UNCONVENTIONAL SECRETION OF THE AUTOPHAGY-RELATED PROTEIN VMP1 VIA EXTRACELLULAR VESICLES AS A POTENTIAL BIOMARKER FOR PANCREATIC CELL INJURY

Mariana S. Tadic[1], Felipe J. Renna[1], Malena Herrera López[1], Tamara Orquera[1], Fabiana López Mingorance[1], Alejandro Ropolo[1], Maria I. Vaccaro[1]*

[1] Universidad de Buenos Aires, CONICET, Instituto de Bioquímica y Medicina Molecular Prof Alberto Boveris (IBIMOL), Buenos Aires, Argentina.

- Correspondence: mvaccaro@ffyb.uba.ar

Abstract

Cellular stress activates various mechanisms, including autophagy and vesicular trafficking, to maintain homeostasis and cope with pathological conditions such as acute pancreatitis. One of these mechanisms involves the unconventional secretion of extracellular vesicles (EVs), lipid bilayer-delimited particles released by almost all cell types into the extracellular medium. Recently, an autophagy-related secretory pathway that releases EVs has been discovered. Our study focuses on Vacuole Membrane Protein 1 (VMP1), an autophagy-related protein implicated in pancreatitis, diabetes, pancreatic cancer, and cellular stress management. VMP1 expression triggers autophagy in mammalian cells through its ubiquitination and its direct binding to BECN1. Here, we demonstrate that VMP1 is secreted to the extracellular medium integrated into the membrane of EVs. Using cell lines expressing different VMP1-tag expression plasmids, we successfully isolated and purified VMP1-containing EVs (VMP1-EVs) from the extracellular medium through ultracentrifugation and immune isolation techniques. The secretion of VMP1-EVs was reduced by mTOR inhibition and in the absence of proteins essential for autophagosome formation, such as ATG5. The secretion of endogenous VMP1 by pancreatic acinar cells was highly induced in experimental models of cell stress, including blocked autophagic flux with Bafilomycin and exposure to supramaximal doses of caerulein. Moreover, immuno-isolation of VMP1-EVs from cell models revealed that VMP1 is a component of EV membranes. Transmission electron microscopy (TEM) and dynamic light scattering (DLS) analysis of immuno-isolated VMP1-EVs indicated a diameter of around 150 nm. Western blot assay demonstrated that the autophagy related protein LC3-II and p62 are co-secreted with VMP1 in cellular stress models. Furthermore, VMP1-EVs are taken up by different host cells, suggesting that VMP1-EVs may be able to mediate remote communication between cells. In conclusion, we have demonstrated for the first time that the pancreatitis-associated transmembrane protein VMP1 is unconventionally secreted as a component of extracellular vesicles via a secretory autophagy pathway.

Keywords: Extracellular Vesicles; Secretory Autophagy; VMP1; Cellular stress; Ubiquitinated Membrane Protein

Introduction

Autophagy is a highly specialized catabolic process that facilitates the selective degradation of aged or damaged cellular components, leading to constant cellular regeneration and maintenance of cellular homeostasis. This fundamental biological process has garnered significant attention due to its critical role in cellular health and function. However, recent discoveries have expanded our understanding of autophagy, revealing new non-canonical functions that have led to the emergence of the term secretory autophagy (SA). SA encompasses conventional and unconventional protein secretion, the production of extracellular vesicles (EVs), and the release of secretory lysosomes (Leidal et al. 2020).

Despite these advancements, the precise mechanisms underlying SA remain poorly defined. The complexity of the underlying cell biology and the novelty of the field contribute to this ambiguity. Canonical autophagy shares significant points of convergence with endosomal processes, given the similarities in the origin of autophagosomes and endosomes. The convergence between autophagic and endosomal pathways involves several shared molecular players and membrane dynamics. For instance, key regulators of the endosomal pathway, including VPS34, ESCRT complex proteins, SNAREs, and various ATG proteins, play fundamental roles in the initiation, maturation, and eventual fusion of autophagosomes with lysosomes (Papandreou and Tavernarakis 2020). This functional interconnection underscores a close link between SA and exocytosis mechanisms, suggesting that autophagy-related pathways might have broader implications in cellular trafficking and secretion beyond degradation processes.

Furthermore, SA has been implicated in various pathophysiological processes by facilitating the secretion of cellular components involved in complex human diseases such as inflammatory diseases, cancer, diabetes, and neurodegenerative disorders (Urano et al. 2018). This autophagic function is particularly triggered during cellular stress conditions such as starvation, endoplasmic reticulum (ER) stress, and the accumulation of misfolded proteins, which activates the unfolded protein response (UPR) (Jahangiri et al. 2022). These stressors can induce a shift towards SA, where instead of merely degrading cellular components, the autophagic machinery aids in the secretion of specific proteins and vesicles.

A pivotal component in the autophagic process is VMP1, an autophagy related transmembrane protein characterized in our laboratory as essential for autophagy (Ropolo et al. 2007; Molejon et al. 2013). VMP1 expression in the pancreas is strongly and rapidly induced in response to acute pancreatitis (Dusetti et al. 2002), leading to the induction of autophagy and localization in the autophagosome membrane (Ropolo et al. 2007). Further studies demonstrated that VMP1 plays a critical role in the cellular response to pancreatic pathology, including diabetes mellitus (Grasso et al. 2009), acute pancreatitis (Grasso et al. 2011; Vanasco et al. 2021), and pancreatic cancer (Lo Ré et al. 2012; Gilabert et al. 2013). In the context of AP, VMP1 is involved in zymophagy, a type of selective autophagy that recognizes and degrades activated zymogen granules(Grasso et al. 2011). Moreover, VMP1 is also integral to mitophagy, which along with zymophagy, promote cell survival and prevent the progression to more severe forms of pancreatic disease(Vanasco et al. 2021). The

significance of VMP1 in autophagy, pancreatic pathology, and cancer-related mechanisms has been widely recognized, with studies demonstrating its strict regulation at both transcriptional and post-transcriptional levels. We characterized the AKT1-GLI3-VMP1 (Lo Ré et al. 2012) and the E2F1-EP300-VMP1 (Ropolo et al. 2020) transcriptional pathways. Also, we identified ubiquitination of VMP1, mediated by the E3 ligase complex CRL4/Cdt2, in pancreatic cancer cells (Renna et al. 2023).

Here we propose that VMP1 expression not only triggers sophisticated selective autophagy processes but also might be involved in secretory pathways triggered by stress and pathological conditions. We have demonstrated for the first time the unconventional secretion of VMP1 in the EV fraction. In our findings, VMP1 emerges as a critical player in secretory pathways known as SA, where this transmembrane protein is integrated into the membrane of EVs. Our observations underscore the dynamic nature of VMP1 secretion and its regulation by autophagy and cellular stress conditions. Given the highlighted role of VMP1 in both disease pathophysiology and membrane trafficking related to autophagy, this novel finding may open new avenues for basic research and clinical applications.

Materials and Methods

Cell Culture, Transfections, and Differentiation

HEK293T, HeLa, and AR42J cell lines were obtained from the American Type Culture Collection (ATCC). MEF WT and ATG5-deficient cells were kindly provided by Dr. María I. Colombo (Universidad Nacional de Cuyo, Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina). Cells were cultured in Dulbecco's modified Eagle medium (Biological Industries) supplemented with 10% fetal bovine serum (Natocor), 200 mM L-alanyl-L-glutamine dipeptide (Gibco™), 100 U/µl penicillin, and 100 µg/µl streptomycin (Biological Industries). Cell lines were maintained at 37ºC with 5% CO_2 in a humidified atmosphere. Mycoplasma contamination was monitored monthly via DAPI staining and biannually with PCR. HEK293T cells were transfected at 60-70% confluence using polyethyleneimine (PEI) as the transfection reagent from Polysciences (cat. no. #24765-1).The cell medium was replaced with conditioned culture medium 5 hours post-transfection. AR42J cells were differentiated using 100 nM dexamethasone for 48 hours.

Treatments

For amino acid deprivation, cells were washed twice with fasting medium (140 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM glucose, 1% BSA, and 20 mM HEPES pH 7.4) and incubated in the same fasting medium for 16 hours to collect VMP1-EVs. PP242 (Santa Cruz) was dissolved in DMSO to prepare a 1 mM stock solution, and cells were treated with 1 µM PP242 for 16 hours. Chloroquine diphosphate (Sigma Aldrich) was dissolved in PBS to create a 10 mM stock solution, and cells were treated with 50 µM chloroquine diphosphate for 16 hours. Bafilomycin A1 (Santa Cruz Biotech, sc-201550A), prepared as a 10 µM stock in DMSO, was used at final concentrations of 20 nM and 100 nM for 16 hours. For

hyperstimulation of CCK receptors, differentiated AR42J cells were treated with 7.4 μM caerulein (Sigma Aldrich) for 16 hours.

Expression Vectors

The plasmids pEGFP-N1–VMP1, pcDNA4-B–hVMP1–V5/His, pLenti–hVMP1–FLAG were performed according to the explained in Renna et a., 2023 (Renna et al. 2023). FLAG–Ub was kindly gifted from Simona Polo (University of Milan).

Generation of Conditioned Culture Medium

For the generation of exosome-depleted conditioned media, Dulbecco's Modified Eagle Medium (Biological Industries) supplemented with 200 mM L-alanyl-L-glutamine dipeptide (Gibco™) and 30% fetal bovine serum (Natocor) was ultracentrifuged using the Beckman XL-90 equipment for 16 hours at 120,000 g. The resulting supernatant was filtered using 0.22 μm filters and placed in sterile containers. Penicillin (100 U/μl) and streptomycin (100 μg/μl), both from Biological Industries, were subsequently added.

Isolation of Extracellular Vesicles

The conditioned cell medium was collected in 15 mL tubes and subjected to low-speed centrifugation (300 g) for 15 min and 2000 g for 20 min. Subsequently, centrifugation was performed at 16,500 g at 4°C for twenty minutes. The resulting supernatant was ultracentrifuged again using the Beckman XL-90 rotor for 2 hours at 4°C at a speed of 120,000 g. The pellet was washed with cold PBS and centrifuged one last time at 120,000 g at 4°C for 1.5 hours. The final pellet was resuspended in PBS for subsequent analysis by western blot, immunofluorescence, or immunoprecipitation.

Western Blot Analysis

After different treatments and transfections, cells were lysed in lysis buffer (50 mM Tris-HCl (pH: 7.4), 250 mM NaCl, 25 mM NaF, 2mM EDTA, 0.1% Triton X-100, and Thermo Scientific™ Pierce Protease Inhibitors). EVs pellets were lysed with Laemmli Buffer. For LC3, Western blot cells were lysed in 50 mM Tris-HCl (pH: 8.0), 150 mM NaCl, 1% Triton X-100, and Thermo Scientific™ Pierce Protease Inhibitors. The protein concentration was determined using the bicinchoninic acid (BCA) protein assay reagent (Pierce). An equal amount of protein was analyzed using SDS-PAGE and transferred to polyvinylidene fluoride PVDF membranes (0.22 μm pore size, Millipore). The membranes were blocked with Odyssey Blocking Buffer (LI-COR) at room temperature for 1 h and incubated with the corresponding primary antibodies overnight at 4 ºC. The primary antibodies used were anti-VMP1 (1:1,000; rabbit mAb #12978, Cell Signaling Technology), anti-actin (1:2,000; rabbit polyAb A2066; Sigma–Aldrich), anti-ubiquitin (1:1000; mouse mAb UBCJ2; Enzo), anti-DYKDDDDK tag (1:6000; mouse mAb #8146, Cell Signaling Technology), anti-α-tubulin (1:4000; mouse mAb T5168; Sigma-Aldrich), anti-SQSTM1/p62 (D5E2) (1:1000; rabbit mAb #8025 Cell Signaling Technology), anti CD63 (1:1000; mouse mAb ab231975) and anti-GFP (1/3000, rabbit pAb TP401, Torrey Pines Biolab). After incubation, the membrane was washed four times with PBS containing 0.1% Tween-20 (PBS-T), washed twice with PBS, and then incubated with the corresponding IRDye secondary antibody (1:15000, IRDye® 680LT goat, anti-rabbit IgG or 1:10000, IRDye® 800CW goat anti-mouse IgG, LI-COR) in

Odyssey Blocking Buffer (LI-COR) for 2 h at room temperature. Finally, the membrane was washed four times with PBST, washed twice with PBS, and scanned with Odyssey® SA (LI-COR).

For the LC3 Western blotting, the membranes were blocked at room temperature for 1 h with 1% bovine serum albumin in Tris-buffered saline (TBS) containing 0.1% Tween-20 (TBS-T). After blocking, the membranes were incubated with the primary antibody anti-LC3B (1:500, rabbit mAb #3868, Cell Signaling Technology) over 24 h at 4ºC. After the incubation, the membranes were washed four times with TBS-T and twice with TBS. Finally, they were incubated with an anti-rabbit HRP-conjugated (1:2500, Amersham NA934, GE Healthcare) secondary antibody in 1% bovine serum albumin in Tris-buffered saline (TBS) containing 0.1% Tween-20 (TBS-T). The membranes were washed four times with TBST, washed twice with TBS, and incubated with PIERCE ECL Plus Western blotting substrate (Cat# 32134, Thermo Scientific) according to the manufacturer's instructions and scanned with cDigit Blot Scanner (LI-COR).

FIJI software was used to determine the density of protein bands. The relative densitometry, normalized to actin, was expressed as the mean ± SEM of three different experiments.

Immunoprecipitation Assays

For the anti-FLAG immunoprecipitations, pellets obtained after ultracentrifugation of conditioned culture media were resuspended in 300 µL of 1X PBS and placed in 2 mL Eppendorf tubes for subsequent incubation with anti-FLAG® M2 Magnetic Beads for 2 hours at 4°C with agitation. The beads were washed five times with PBS containing 0.02% Tween-20. Elution was performed by adding a Laemmli buffer and heating the samples for 5 minutes at 70°C.

For the anti-GFP immunoprecipitations, ChromoTeK GFP-Trap magnetic agarose beads were used. Pellets obtained after ultracentrifugation were resuspended in 300 µL of 1X PBS and incubated with the reagent for 1 hour at 4°C with agitation. The beads were washed five times with PBS containing 0.02% Tween-20, and elution of the VMP1.GFP-EVs was performed by adding Laemmli buffer and heating the samples for 5 minutes at 70°C.

Fluorescence Intensity of VMP1.GFP-EVs

Conditioned culture medium from HEK293T cells previously transfected with the pEGFP-N1-hVMP1 plasmid was ultracentrifuged to obtain the EV fraction. The resulting pellet, rich in VMP1.GFP-EVs, was resuspended in PBS and the fluorescence intensity was subsequently quantified using the SpectraMax i3x microplate reader (Molecular Devices) at a wavelength of 510 nm. The normalized fluorescence intensity (FI) of the VMP1.GFP extracellular vesicles was calculated using the formula: FI EVs / FI WCL, where FI EVs represents the fluorescence intensity of the extracellular vesicles obtained via ultracentrifugation, and FI WCLs represents the fluorescence intensity of the whole cell lysate (WCL) transfected with VMP1.GFP.

Dynamic Light Scattering (DLS)

Dynamic light scattering (DLS) measurements of the pellets obtained from the conditioned culture medium of HEK293T cells transfected with the pLenti-Puro-PGK-hVMP1 plasmid were performed at 25°C using a laser dynamic light scattering system (Zetasizer Nano-ZSP,

Malvern Instruments, UK) equipped with a He-Ne laser (633 nm) and a digital correlator, model ZEN5600.

Transmission Electron Microscopy (TEM)

Following immunoprecipitation of the VMP1.Flag-EVs, the sample was resuspended in PBS. Elution of the EVs was performed using 2% glutaraldehyde prepared in 0.1 M phosphate buffer at 4°C. The sample was then placed on a 200 mesh copper grid with an LR white resin membrane (Sigma Aldrich - L9774) and washed eight times for 2 minutes each with ultrapure water. The sample was contrasted with 2% aqueous uranyl acetate, pH 7, for 3 minutes. Excess liquid was removed with blotting paper, and the sample was air-dried at room temperature for 15 minutes. Observation was performed using a Zeiss 109T transmission electron microscope (80 kV), and images were acquired with a Gatan ES1000w digital camera.

Immunofluorescence

Cells were grown on 15 mm round glass coverslips in 12-well plates. A total of 60,000 HeLa cells and 80,000 AR42J cells were seeded. The following day, cells were transfected with the plasmids of interest if necessary for the experiment and incubated with either control vesicles or VMP1-EVs for 24 hours. After this period, the coverslips were fixed with 3.6% paraformaldehyde in PBS for 20 minutes, followed by three PBS washes. Cells were then permeabilized with 0.1% Triton X-100 in PBS for 5 minutes and blocked for 1 hour in PBS with 1% BSA blocking solution. Coverslips were incubated in a humid chamber at 4°C overnight with the primary antibody prepared in the aforementioned blocking solution. The antibodies used were: anti-FLAG (1:500; DYKDDDDK Tag (9A3) mAb Mouse; Cell Signaling Technology), anti-VMP1 (1:1000 mAb Rabbit #12929; Cell Signaling Technology), and anti-Tubulin (1:1000 mAb T5168; Sigma-Aldrich). The next day, the coverslips were washed three times with PBS and then incubated for 2 hours at room temperature with secondary antibodies prepared in PBS with 1% BSA blocking solution (Alexa Fluor 488, 594, and 647 1:800 from Molecular Probes) and mounted in polyvinyl alcohol (PVA) with DABCO mounting medium.

Observation of the samples and image acquisition were performed using a Carl Zeiss-LSM 880 confocal microscope (objectives 20x Zeiss Plan-Apochromat, NA 0.8, or 63x Zeiss Plan-Apochromat, NA 1.4)

In Vivo Fluorescence of VMP1.GFP-EVs

Conditioned culture medium from HEK293T cells previously transfected with the pEGFP-N1-hVMP1 plasmid was ultracentrifuged to obtain the EV fraction. The resulting pellet, rich in VMP1.GFP-EVs was resuspended in PBS. A drop of this EV suspension was placed on a microscope slide along with a coverslip and sealed with nail polish. The fresh sample was immediately observed using a Carl Zeiss-LSM 880 confocal microscope (objectives 20x Zeiss Plan-Apochromat, NA 0.8, or 63x Zeiss Plan-Apochromat, NA 1.4).

Protease Assay

VMP1.Flag-EVs pellets were generated by differential centrifugation as described previously, resuspended in 60 µL PBS, and divided equally into three fractions for resuspension in PBS, 100 µg/mL trypsin in PBS, or 1% Triton X-100 and 100 µg/mL trypsin in PBS. After incubation and occasional mixing for 30 min at 37°C, protease reactions were stopped with the addition of 10 µL protease inhibitor cocktail and 4× sample buffer before immunoblotting analysis.

Statistical Analysis

The data are expressed as the mean ± SEM. All the shown images are representative of three independent experiments. The statistical analyses of the data were performed using GraphPad Prism 8, as stated in the corresponding figure legends.

Results

VMP1 is secreted to the cellular culture medium using extracellular vesicles

We aimed to investigate whether VMP1 is secreted into the cell culture medium within the extracellular vesicle (EV) fraction. To accomplish this, we collected the conditioned culture media (CM) from HEK293T cells overexpressing VMP1. The media were then subjected to differential ultracentrifugation to obtain a final pellet enriched in EVs.

First, we transfected HEK293T cells with VMP1.GFP or an empty GFP vector as a control. Western blot analysis of the obtained EV fractions revealed that VMP1.GFP is secreted as part of the EVs (Figure 1A).

To verify that VMP1 secretion is independent of the plasmid and tag used, we employed two additional expression plasmids for VMP1 protein expression. We transfected cells with VMP1.V5 and VMP1.FLAG. Western blot analysis of the EV fractions revealed that both VMP1.V5 and VMP1.FLAG were present in the EVs (Figures 1B and 1C), demonstrating the presence of VMP1 in the extracellular vesicle fraction regardless of the tag and promoter used.

These results were corroborated by two additional experimental techniques. First, using confocal microscopy, we visualized the VMP1.GFP-EVs in the fresh pellet obtained after ultracentrifugation of the conditioned culture medium, observing structures compatible with extracellular vesicles smaller than 1 µm (Figure 1D). Second, we measured the fluorescence intensity of the VMP1.GFP-EVs fraction compared to the control. We detected the fluorescent signal only in the EV fraction obtained from the culture medium of VMP1.GFP expressing cells (Figure 1E). These results collectively demonstrated that VMP1 is secreted into the cell culture medium in the EV fraction.

VMP1 is located in the membrane of the VMP1-EV

Knowing that VMP1 is a transmembrane protein of the autophagosome, we proposed that VMP1 could be an integral component of the membrane of EVs. In Figure 2A we show transmission electron microscopy (TEM) images of VMP1.Flag vesicles that were purified and enriched by immunoprecipitation (IP) using magnetic beads conjugated with the anti-Flag antibody. Additionally, measurements of the VMP1 vesicles sizes from the TEM

images revealed a homogeneous distribution, with diameters ranging from 150 to 200 nm (Figure 2B). Using dynamic light scattering (DLS), we estimated their size, confirming the results obtained by TEM and further validating a homogeneous population (Figure 2C).

To confirm the presence of VMP1 in the EV membrane, we conducted a protease protection assay. VMP1.Flag-EV fractions were collected and incubated with trypsin. In the presence of trypsin, the VMP1 protein was partially degraded. This partial degradation was indicated by a decrease in band intensity and the appearance of a lower molecular weight VMP1 band. Specifically, the FLAG tag was almost completely degraded, indicanding that the hydrophilic C-terminal of VMP1 is exposed on the outer surface of the EVs and thus accessible to trypsin (Figure 2D). Moreover, when TX-100 was added, VMP1 was completely degraded by trypsin. This complete degradation indicates that VMP1 is indeed a component of the EV membrane, as it was protected only when the membrane was intact.

This set of results clearly demonstrates that VMP1 is part of the membranous structure of VMP1-EVs. This finding was confirmed by the ability to specifically isolate the population of VMP1-EVs using IP with magnetic beads fused to the anti-tag.

VMP1-EVs are able to be internalized by different host cells

We evaluated whether VMP1-EVs were able to be internalized by different host cells. For this purpose, we incubated HeLa cells overnight with VMP1.GFP-EVs obtained from the conditioned medium of HEK293T cells overexpressing VMP1-GFP. Using confocal microscopy, we visualized VMP1.GFP-EVs within the host cells (Figure 3A).

To confirm that VMP1-EVs were indeed internalized by different cells, we repeated the experiment using AR42J cells incubated with VMP1.Flag-EVs, and performed immunofluorescence against tubulin and Z-stack analysis. The results are presented in Figure 3B, clearly showing that the VMP1-EVs are located within the host cells.

In addition, we incubated Hela cells with VMP1.Flag-EVs, and performed immunofluorescence with anti-Flag and anti-VMP1 antibodies to achieve double labeling of the VMP1-EVs and we detected positive immunolabeling for VMP1.Flag-EVs within the cells (Supplementary Figure 1).

As a whole, these results further confirm that VMP1 is secreted using extracellular vesicles and suggest that VMP1-EVs might have a biological effect participating in intercellular communication.

Effect of mTOR inhibition on the secretion of VMP1.Flag-EVs

Given that VMP1 plays an essential role in the autophagic process, being crucial for autophagosome formation, and that it is ubiquitinated both during the autophagic process and in the vesicles secreted into the extracellular culture medium, we decided to explore the possible involvement of the canonical induction of autophagy pathway by inhibition of mTOR on the secretion of VMP1-EVs into the cell culture medium.

To conduct this analysis, we used the HEK293T cell line expressing VMP1-Flag treated with starvation or PP242 as two different kinds of mTOR inhibitors. Figure 4B presents the

western blot analysis from the EV fraction obtained from VMP1-Flag transfected cells after mTOR inhibition. Figure 4A shows an immunoblot of lysates, where the increase in LC3-II served as a control for autophagy induction by mTOR inhibitors. Quantification of the bands obtained from the EV fraction indicated a significant reduction in the secretion of VMP1.Flag-EVs upon mTOR inhibition (Figure 4C). These findings suggest that the activation of autophagy through the inhibition of mTOR negatively affects the secretion of VMP1.Flag-EVs.

VMP1.Flag-EV secretion is significantly induced by the lysosomal blocking

Chloroquine is recognized for its capacity to induce lysosomal stress and inhibit autophagic flux by preventing lysosomal acidification, acting directly on the proton pump of this organelle. To evaluate the effect of lysosomal blocking on VMP1-EVs secretion, we transfected cells with VMP1-Flag and treated them with chloroquine. We then obtained the EV fraction and performed western blot analysis of cell lysates and EVs. Figure 5A shows the accumulation of LC3-II under chloroquine treatment, which serves as a control of the chloroquine action, since it impedes the degradation of autophagosomes.

The analysis of the EV fraction revealed a significantly enhanced secretion of VMP1.Flag-EVs when autophagic flux is inhibited by chloroquine. Notably, we also found that lysosome blocking induced the secretion of the autophagy cargo p62 in the EV fraction (Figure 5B and 5C). These results demonstrate an association between lysosomal inhibition and the secretion of VMP1-EVs, supporting the involvement of secretory autophagy in the mechanism of VMP1 secretion.

The endogenous VMP1 secretion depends on autophagosome formation

Recently, we discovered that VMP1 is modified by ubiquitination and that this ubiquitination does not initially lead to VMP1 degradation by proteasomes or by autophagy, but regulates autophagy. VMP1 suffers ubiquitination during the early steps of autophagosome formation and remains ubiquitinated during the autophagic flux (Renna et al. 2023). Then, we aimed to investigate whether VMP1 is ubiquitinated within the EV fraction. To address this, in HEK293T cells previously transfected with VMP1, we performed immunoprecipitation using magnetic beads conjugated with a GFP antibody to purify VMP1.GFP vesicles from both cell lysates and the extracellular vesicle fraction. We then conducted immunoblotting (IB) against VMP1 and ubiquitin (Figure 6A). The results revealed that the VMP1 isolated from the EV fraction of cell culture medium is ubiquitinated and suggest that VMP1 secretion is related to autophagy.

We then investigated whether autophagosome formation is required for the secretion of endogenous VMP1 using EVs. We employed the ATG5-deficient MEF cell line to evaluate the role of ATG5 in the endogenous secretion of VMP1 under conditions with or without lysosomal inhibition using Bafilomycin A1 (BafA1). The western blot analysis of the whole cell lysates showed that the inefficient formation of autophagosomes due to ATG5 deficiency resulted in the slight accumulation of VMP1 (Figure 6B). Interestingly, in the western blot of the EV fraction, we observed that ATG5 deficiency significantly impaired the secretion of

VMP1. Specifically, we observed a marked decrease in the secretion of VMP1-EVs in ATG5-deficient cells compared to WT cells (Figure 6C and D). These findings suggest that ATG5 is required for VMP1 secretion and confirm that autophagosome formation is involved in VMP1 secretion.

Notably, lysosomal blocking with BafA1 led to a significant increase in the endogenous VMP1 secretion by MEF WT cells, confirming the results obtained with VMP1.Flag-EV secretion under chloroquine treatment (Figure 5). As a whole, these data indicate that a secretory autophagy mechanism is involved in VMP1 secretion.

The secretion of endogenous VMP1 by pancreatic acinar cells was highly induced in experimental models of cell injury.

We previously demonstrated that experimental pancreatitis induced by hyperstimulation of cholecystokinin receptors (CCK-R) with the analogue caerulein (CAE) at supramaximal dose in the rat pancreatic acinar cells (AR42J), triggers autophagy mediated by transgenic VMP1 expression in mouse pancreas, preventing cell death (Grasso et al. 2011). We then investigated whether the increased VMP1 expression in the context of experimental AP would translate into greater secretion of this protein into the EV fraction.

To address this, we used AR42J cells that, after differentiation to pancreatic acinar cells with 100 nM dexamethasone, were treated overnight with caerulein at supramaximal doses (Figure A). We collected the EV fraction and performed immunoblotting for VMP1 and LC3 proteins (Figure 7B). Upon analyzing the EV fraction obtained after caerulein treatment, we found a notable increase in both VMP1 and LC3-II compared to the control EV secretion (Figure 7C and D).

Knowing that VMP1 is an acute-phase protein overexpressed under vesicular transport disruption caused by pancreatitis, we decided to explore if lysosomal blockage in pancreatic acinar cells might trigger the secretion of endogenous VMP1 in the EV fraction. To address this, we employed BafA1 to induce lysosomal blocking in AR42J cells (Figure 7E). We collected EV fractions for immunoblotting against VMP1, LC3, and p62 proteins. A significant increase in VMP1 secretion was observed upon BafA1 treatment (Figure 7G). Notably, in the EV fraction treated with BafA1, a significant increase in the secretion of LC3 and p62 proteins also occurs (Figure 7F). These findings confirm once again that VMP1 is released using EVs by a secretory autophagy mechanism, evidenced by two different models of cellular stress.

Discussion

Autophagy is a specialized catabolic process that enables the selective degradation of aged or damaged cellular components, thereby contributing to the maintenance of cellular homeostasis. While traditionally recognized for its role in cellular regeneration, recent research has uncovered non-canonical functions of autophagy, collectively referred to as secretory autophagy (SA) (Ponpuak et al. 2015). This process encompasses both conventional and unconventional protein secretion, the production of extracellular vesicles (EVs), and the release of secretory lysosomes (Buratta et al. 2020; Solvik et al. 2021).

Here we have demonstrated that VMP1 is secreted into the EV fraction as a component of the membrane of extracellular vesicles. In our findings, VMP1 emerges as a novel critical player in SA (Ponpuak et al. 2015; Kimura et al. 2017; Zhang et al. 2015), where this transmembrane protein is integrated into the membrane of EVs. Our observations underscore the dynamic nature of VMP1 secretion and its regulation by autophagy and cellular stress conditions.

Interestingly, VMP1, an autophagy related protein, is secreted into the EV fraction independently of the tag or promoter used (Figure 1). Using confocal microscopy, we visualized VMP1.GFP-EVs in the fresh pellet obtained after differential ultracentrifugation of the conditioned culture medium, observing structures consistent with extracellular vesicles smaller than 1 μm (Figure 1D). Furthermore, we detected for the first time the secretion of endogenous VMP1, under conditions of milder plasmid expression (pLenti-hVMP1Flag) (Figure 1B), and under cellular stress (Figures 6 and 7).

Given the intrinsic nature of VMP1 as a transmembrane protein of the autophagosome during the macroautophagy process, we investigated and confirmed its presence as a membrane component of EVs through immunoprecipitation using magnetic beads fused to VMP1-tag antibodies, and transmission electron microscopy (Figure 2A). VMP1-EVs size estimation via TEM and dynamic light scattering (DLS) revealed a homogeneous profile within the range of 150-190 nm, consistent with secretory vesicles (Figure 2), according to the guide MISEV2023 (Welsh et al. 2024).

VMP1 secretion was reduced upon inhibition of mTOR mediated by the pharmacological inducer pp242 or by STV (Figure 4), aligning with studies that have observed decreased EV secretion under similar conditions (Zou et al. 2019; Bhat et al. 2021). Conversely, SA is characterized by the utilization of autophagic machinery components (ATGs) for the secretion of proteins into the extracellular medium. Several authors have demonstrated that ATG5 is necessary for the mechanism of secretory autophagy (Noh et al. 2018; Urano et al. 2018), as well as for the production of EVs (Guo et al. 2017). Our experiments are consistent with these reports, our data have shown that the secretion of endogenous VMP1-EVs, which is markedly induced under lysosomal inhibition in WT cells, is significantly reduced in cells deficient in ATG5 (Figure 6C and D). Furthermore, VMP1 secretion was increased under conditions of cellular stress. Lysosomal blockade facilitating by BafA1 or CQ leads to a substantial increase in VMP1 levels in EV fraction, along with p62 and LC3II (Figure 5B and 7F), resembling the secretory autophagy mechanisms characterized by Debnath and Leidal (Debnath and Leidal 2022). Our findings, as a whole, confirm that the secretion of VMP1 is related to secretory autophagy in response to cellular stress, since it is induced by bafilomycin and depends on autophagosome formation (Solvik et al. 2022).

Given VMP1's critical role in autophagy and disease, its regulation is tightly controlled at both transcriptional and post-translational levels. Recent findings demonstrated that VMP1 is ubiquitinated by the E3 ligase complex CRL4/Cdt2 during autophagic flux in pancreatic cancer cells, and that the inhibition of VMP1 ubiquitination significantly affects VMP1 recruitment and autophagosome biogenesis (Renna et al. 2023). Here we have shown that VMP1 is ubiquitinated in the VMP1.GFP-EVs (Figure 6A), supporting the autophagy dependence of VMP1 secretion. Our finding also suggests a multifunctionality of VMP1

ubiquitination, potentially encompassing its participation in the biogenesis of extracellular vesicles under conditions of cellular stress or injury.

EVs have been widely recognized as critical mediators of intercellular communication, facilitating the transfer of bioactive molecules through mechanisms such as fusion, internalization, and the subsequent release of signaling molecules into target cells (Cocozza et al. 2020; Ginini et al. 2022). This process has been implicated in a variety of physiological and pathological contexts, such as cancer and inflammation, where EV-mediated communication is often enhanced compared to normal conditions (Lai et al. 2014). Using confocal microscopy, we demonstrated that VMP1-EVs generated experimentally can be internalized by different cell types, including HeLa and AR42J cells (Figure 3), further underscoring their potential role in cellular communication. These findings would suggest that VMP1-EVs could be involved in modulating intercellular signaling pathways, an area that has not yet been fully explored. Although significant advances have been made in understanding EV secretion and uptake, the precise mechanisms underlying these processes, particularly in pathological versus physiological conditions, remain largely unclear and warrant further investigation.

SA has emerged as a critical mechanism in various pathophysiological processes, facilitating the secretion of specific cellular components implicated in complex human diseases such as inflammation, cancer, diabetes, and neurodegenerative disorders (Urano et al. 2018). This new autophagic function is triggered under conditions of cellular stress, such as reticular stress, or in response to misfolded proteins (UPR). Additionally, secretory autophagy can be activated when intracellular trafficking is blocked (Jahangiri et al. 2022), which is particularly relevant in the pathophysiology of acute pancreatitis (AP). In the context of AP, secretory autophagy may play a crucial role. AP is characterized by the premature activation of digestive enzymes within the pancreas, leading to tissue damage and inflammation. During AP, cellular stress might induce SA to alleviate endoplasmic reticulum (ER) stress by secreting misfolded proteins, thereby attempting to restore cellular proteostasis. This process should involve the coordinated action of several proteins, including those central to the autophagic and secretory pathways (Zahoor and Farhan 2018).

Several reports have recently studied the composition and role of isolated extracellular vesicles and the relationship between circulating EVs and pancreatitis (Jia et al. 2021). However, due to the complexity and heterogeneity of EVs, multiple mechanisms and source of secretion, the relevance of EVs in the diagnosis and treatment of pancreatitis requires extensive investigation prior to clinical application. Our results have demonstrated a significant increase in the secretion of VMP1-EVs in pancreatic acinar cells suffering from the characteristic features of AP. Interestingly, our findings reveal a novel approach in the study of the relationship between EV secretion and pancreatitis, pointing at VMP1 secretion as a new autophagy-related pathway in response to disease and with potential relevance in the pathophysiology of pancreatic disorders.

Conclusions

Our study demonstrates for the first time the unconventional secretion of an autophagy related transmembrane protein, VMP1. Our data also show that this secretion uses extracellular vesicles and it is closely related to autophagy (Figure 8). Moreover, our findings

emphasize the multifunctional role of VMP1 in responding to cellular stress and disease, not only through the degradation of damaged components via selective autophagy (Grasso et al. 2011; Vanasco et al. 2021) but also in regulating alternative secretory pathways, including the release of EVs. Moreover, VMP1 secretion might contribute to the complex and poorly understood mechanisms of self limitation in most cases of AP. Understanding the mechanisms governing VMP1 secretion could provide new insights into the characterization and functions of secretory autophagy in health and disease.

Author Contributions: For research articles with several authors, a short paragraph specifying their individual contributions must be provided. The following statements should be used "Conceptualization, MIV and MST; methodology, MST, MIV, FJR, MHL, and FLM; validation, MIV; formal analysis, MST MIV, FJR, AR; investigation, MST, FJR, MHL, FLM, AR and TO.; funding acquisition and resources AR and MIV; data curation, FR and AR; writing—original draft preparation, MST, FJR and MIV.; visualization, MST, FRJ and MIV.; supervision, MIV and AR; project administration, MIV. All authors have read and agreed to the published version of the manuscript."

Funding: This work was supported by grants from: Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) [PIP 2021-2023 GI− 11220200101549CO; and PUE 22920170100033]; Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT) [PICT2019- 01664] [PICT-2021- I-A-00328] and Universidad de Buenos Aires [UBACYT 2018-2020 20020170100082BA] [UBACyT 2023-2025 - 20020220300232BA].

Data Availability Statement: All the generated data are compiled and given in MS or Supplementary files. The raw data will be provided upon reasonable request.

Acknowledgments: Authors would like to thank the Vaccaro's lab members for their contributions in the discussion of results and the laboratory's quality assurance system.

Conflicts of Interest: The authors declare no conflict of interest.

Figure legends

Figure 1. VMP1 is secreted to the extracellular medium. (A) Western Blot analysis of VMP1-GFP and empty GFP levels in HEK293T cell lysates (WCL), and EVs lysates from CM separated by ultracentrifugation. Immunoblotted to detect GFP marker (n = 5 biologically independent experiments). (B) Western Blot analysis of VMP1-Flag levels in HEK293T WCL and EVs lysates from CM separated by ultracentrifugation. Immunoblotted to detect the endogenous levels of VMP1 and the indicated marker (n = 5 biologically independent experiments). (C) Western Blot analysis of VMP1-V5 levels in HEK293T WCL and EVs lysates from CM separated by ultracentrifugation. Immunoblotted to detect the presence of the indicated marker (n = 5 biologically independent experiments). (D) Confocal microscopy of VMP1.GFP EVs obtained by ultracentrifugation of the conditioned medium of HEK293T cells transfected with hVMP1-GFP for 48 hours. Scale bar: 15 μM / 1μM. (E) Quantification of fluorescence intensity obtained in the VMP1.GFP extracellular vesicles fraction compared to their control (pEGFP-N1) (n = 3 biologically independent experiments).

Figure 2. VMP1 is part of the membrane of extracellular vesicles (EVs). (A) Representative TEM image of VMP1.Flag EVs. VMP1-Flag EVs were collected from HEK293T cell culture medium and purified through immunoprecipitation using anti-Flag beads. EVs range in size from 150 to 200 nm. Scale bar: 50 nm. (B) The violin plot details the distribution of sizes

(nm) measured in the images of VMP1.Flag-EVs obtained by electron microscopy (n=25). (C) Light scattering intensity of VMP1.Flag-EVs obtained from the conditioned culture medium of HEK293T cells (n=3 biologically independent experiments). (D) Representative blots of indicated proteins from untreated EVs or EVs incubated with 100 µg/mL trypsin and/or 1% Triton X-100 (TX-100) for 30 min at 37°C (n=3 biologically independent experiments)

Figure 3. VMP1-EVs are able to be internalized by different host cells. (A) Representative images of HeLa cells incubated overnight with VMP1.GFP-EVs. Direct immunofluorescence was performed. (B) Z-stack of AR42J cells incubated overnight with VMP1.Flag-EVs. Immunolabeling was performed for Flag, tubulin, and DAPI. Scales: 10 µm and 1 µm.

Figure 4. Effect of mTOR inhibition on the secretion of VMP1.Flag-EVs. (A) Western blot analysis of WCL fractions from HEK293T cells previously transfected with VMP1.Flag, treated with STV or pp242 (1µM) for 16 hours. Immunoblotting for Flag, VMP1, LC3 and actin was performed where actin served as a loading control. (B) Western blot analysis of EV fractions from HEK293T cells previously transfected with VMP1.Flag, treated with STV or pp242 (1µM) for 16 hours. Immunoblotting for anti-Flag was performed. (C) Quantitative analysis of VMP1-Flag EVs secreted in the conditioned culture medium for each treatment. Data are statistically different from each other with ***$p<0.01$ value versus control (n=3 biologically independent experiments).

Figure 5. VMP1-EV secretion is increased under autophagic flux blockade. (A) Western blot analysis of WCL fractions from HEK293T cells previously transfected with VMP1.Flag, treated with CQ (50 µM) for 16 hours. Immunoblotting for Flag, VMP1, LC3 and actin was performed where actin served as a loading control. (B) Western blot analysis of EV fractions from HEK293T cells previously transfected with VMP1.Flag, treated with CQ for 16 hours. Immunoblotting for VMP1 and p62 was performed. (C) Quantitative analysis of VMP1-Flag EVs secreted in the conditioned culture medium for each treatment. Data are statistically different from each other with *$p<0.01$ value versus control (n=3 biologically independent experiments).

Figure 6. The endogenous secretion of VMP1 is induced by Bafilomycin and depends on autophagosome formation. (A) Western blot analysis of cell lysates and extracellular vesicles (EVs) from HEK293T cells previously transfected with the Ub-Flag plasmid and either pEGFP-N1 or VMP1-GFP. Immunoprecipitation was performed using magnetic beads conjugated with anti-GFP, followed by western blot analysis targeting VMP1 and Flag. The specific immunoblot (IB) for VMP1 shows multiple bands with a molecular weight higher than 73 kDa in the eluate obtained from both total lysates and EVs, indicating the ubiquitination of VMP1. The image is representative of three independent experiments.(B) Whole cell lysates (WCL) from MEF WT and ATG5 KO cells treated and untreated with BafA1 (100 µM) for 16 hours. Immunoblotting was performed for the proteins ATG5, VMP1, and LC3. Actin was used as a loading control. (C) Western blot of the EV fraction obtained from the different culture media of MEF WT and ATG5 KO cells treated and untreated with BafA1. Immunoblots were performed against the proteins VMP1 and LC3. (D) Quantitative analysis of VMP1 secreted in the EV fraction for each treatment. Bars represent the mean and SEM of 3 independent experiments. ***$p < 0.01$ by two-way ANOVA.

Figure 7. The secretion of endogenous VMP1 by pancreatic acinar cells was highly induced in experimental models of cell injury. (A) Western blot of WCL from AR42J cells treated and

untreated with CAE (7,4 μM) for 16 hours. Blots were performed against VMP1 and LC3 proteins. Actin was used as a loading control. (B) Western blot of EV fractions collected from control and CAE treatments. An immunoblot was performed against VMP1 and LC3 proteins. (C) Relative density quantification of VMP1 secretion. Bars represent the mean and SEM of 3 independent experiments *$p < 0.05$ by Welch's parametric test. (D) Relative density quantification of LC3 II secretion. Bars represent the mean and SEM of 3 independent experiments *$p < 0.05$ by Welch's parametric test. (E) Western blot of whole cell lysate (WCL) from AR42J cells treated and untreated with BafA1 (20 μM) for 16 hours. Blots were performed against VMP1, p62, and LC3 proteins. Actin was used as a loading control. (F). Western blot of EV fractions collected from control and BafA1 treatments. An immunoblot was performed against VMP1, p62, and LC3 proteins. (G) Relative density quantification of VMP1 secretion. Bars represent the mean and SEM of 3 independent experiments **$p < 0.01$ by Welch's parametric test.

Figure 8. VMP1-EV secretion is modulated by autophagy pathway. Blocking the autophagic pathway with either chloroquine (CQ) or bafilomycin A1 (BafA1) promotes the secretion of VMP1-EVs into the extracellular space. Autophagosome formation is required for the secretion of VMP1-EVs, which is dependent on the autophagy-related protein ATG5.

Supplementary Figure 1. VMP1-EVs are able to be internalized by different host cells. (A) HeLa cells were incubated overnight with VMP1.GFP-EVs obtained from the conditioned medium of HEK293T cells. Direct immunofluorescence was performed to visualize EV internalization. Scale bars: 10 μm and 1 μm. (B) HeLa cells were incubated overnight with VMP1.Flag-EVs, followed by immunolabeling with anti-Flag and anti-VMP1 antibodies. Scale bars: 10 μm and 1 μm. (C) AR42J cells were incubated overnight with VMP1.GFP-EVs and subsequently immunolabeled with anti-VMP1 antibody. Scale bars: 10 μm and 1 μm.

References

Bhat, Owais M., Xinxu Yuan, Rakesh C. Kukreja, and Pin-Lan Li. 2021. “Regulatory Role of Mammalian Target of Rapamycin Signaling in Exosome Secretion and Osteogenic Changes in Smooth Muscle Cells Lacking Acid Ceramidase Gene.” *FASEB Journal: Official Publication of the Federation of American Societies for Experimental Biology* 35 (7): e21732.

Buratta, Sandra, Brunella Tancini, Krizia Sagini, Federica Delo, Elisabetta Chiaradia, Lorena Urbanelli, and Carla Emiliani. 2020. “Lysosomal Exocytosis, Exosome Release and Secretory Autophagy.” *International Journal of Molecular Sciences* 21 (2576): 1–20.

Cocozza, Federico, Eleonora Grisard, Lorena Martin-Jaular, Mathilde Mathieu, and Clotilde Théry. 2020. “SnapShot: Extracellular Vesicles.” *Cell* 182 (1): 262–262.e1.

Debnath, Jayanta, and Andrew M. Leidal. 2022. “Secretory Autophagy during Lysosome Inhibition (SALI).” *Autophagy* 18 (10): 2498–99.

Dusetti, Nelson J., Yongfeng Jiang, Maria I. Vaccaro, Richard Tomasini, Amina Azizi Samir, Ezequiel L. Calvo, Alejandro Ropolo, et al. 2002. “Cloning and Expression of the Rat Vacuole Membrane Protein 1 (VMP1), a New Gene Activated in Pancreas with Acute Pancreatitis, Which Promotes Vacuole Formation.” *Biochemical and Biophysical Research Communications* 290 (2): 641–49.

Gilabert, Marine, Maria Inés Vaccaro, Martin E. Fernandez-Zapico, Ezequiel L. Calvo, Olivier Turrini, Véronique Secq, Stéphane Garcia, et al. 2013. “Novel Role of VMP1 as Modifier of the Pancreatic Tumor Cell Response to Chemotherapeutic Drugs.” *Journal of Cellular Physiology* 228 (9): 1834–43.

Ginini, Lana, Salem Billan, Eran Fridman, and Ziv Gil. 2022. “Insight into Extracellular Vesicle-Cell Communication: From Cell Recognition to Intracellular Fate.” *Cells* 11 (9). https://doi.org/10.3390/cells11091375.

Grasso, Daniel, Alejandro Ropolo, Andrea Lo Ré, Verónica Boggio, María I. Molejón, Juan L. Iovanna, Claudio D. Gonzalez, Raúl Urrutia, and María I. Vaccaro. 2011. “Zymophagy, a Novel Selective Autophagy Pathway Mediated by VMP1-USP9x-p62, Prevents Pancreatic Cell Death.” *The Journal of Biological Chemistry* 286 (10): 8308–24.

Grasso, Daniel, Maria L. Sacchetti, Lidia Bruno, Andrea Lo Ré, Juan L. Iovanna, Claudio D. Gonzalez, and Maria I. Vaccaro. 2009. “Autophagy and VMP1 Expression Are Early Cellular Events in Experimental Diabetes.” *Pancreatology: Official Journal of the International Association of Pancreatology ... [et Al.]* 9 (1-2): 81–88.

Guo, Huishan, Maneka Chitiprolu, Luc Roncevic, Charlotte Javalet, Fiona J. Hemming, My Tran Trung, Lingrui Meng, et al. 2017. “Atg5 Disassociates the V1V0-ATPase to Promote Exosome Production and Tumor Metastasis Independent of Canonical Macroautophagy.” *Developmental Cell* 43 (6): 716–30.e7.

Jahangiri, Babak, Ali Kian Saei, Patience O. Obi, Narjes Asghari, Shahrokh Lorzadeh, Shirin Hekmatirad, Marveh Rahmati, et al. 2022. “Exosomes, Autophagy and ER Stress Pathways in Human Diseases: Cross-Regulation and Therapeutic Approaches.” *Biochimica et Biophysica Acta, Molecular Basis of Disease* 1868 (10): 166484.

Jia, Yu-Chen, Yi-Xuan Ding, Wen-Tong Mei, Yu-Ting Wang, Zhi Zheng, Yuan-Xu Qu, Kuo Liang, Jia Li, Feng Cao, and Fei Li. 2021. “Extracellular Vesicles and Pancreatitis: Mechanisms, Status and Perspectives.” *International Journal of Biological Sciences* 17 (2): 549–61.

Kimura, Tomonori, Jingyue Jia, Suresh Kumar, Seong Won Choi, Yuexi Gu, Michal Mudd, Nicolas Dupont, et al. 2017. “Cellular and Molecular Mechanism for Secretory Autophagy.” *The EMBO Journal* 36 (1): 42–60.

Lai, Charles P., Osama Mardini, Maria Ericsson, Shilpa Prabhakar, Casey Maguire, John W. Chen, Bakhos A. Tannous, and Xandra O. Breakefield. 2014. “Dynamic Biodistribution of Extracellular Vesicles in Vivo Using a Multimodal Imaging Reporter.” *ACS Nano* 8 (1): 483–94.

Leidal, Andrew M., Hector H. Huang, Timothy Marsh, Tina Solvik, Dachuan Zhang, Jordan Ye, Fui Boon Kai, et al. 2020. “The LC3-Conjugation Machinery Specifies the Loading of RNA-Binding Proteins into Extracellular Vesicles.” *Nature Cell Biology* 22 (2): 187–99.

Lo Ré, Andrea E., Maite G. Fernández-Barrena, Luciana L. Almada, Lisa D. Mills, Sherine F.

Elsawa, George Lund, Alejandro Ropolo, Maria I. Molejon, Maria I. Vaccaro, and Martin E. Fernandez-Zapico. 2012. “Novel AKT1-GLI3-VMP1 Pathway Mediates KRAS Oncogene-Induced Autophagy in Cancer Cells.” *The Journal of Biological Chemistry* 287 (30): 25325–34.

Molejon, Maria I., Alejandro Ropolo, Andrea Lo Re, Veronica Boggio, and Maria I. Vaccaro. 2013. “The VMP1-Beclin 1 Interaction Regulates Autophagy Induction.” *Scientific Reports* 3. https://doi.org/10.1038/srep01055.

Noh, Shin Hye, Heon Yung Gee, Yonjung Kim, He Piao, Jiyoon Kim, Chung Min Kang, Gahyung Lee, et al. 2018. “Specific Autophagy and ESCRT Components Participate in the Unconventional Secretion of CFTR.” *Autophagy* 14 (10): 1761–78.

Papandreou, Margarita-Elena, and Nektarios Tavernarakis. 2020. “Crosstalk between Endo/Exocytosis and Autophagy in Health and Disease.” *Biotechnology Journal* 15 (8): e1900267.

Ponpuak, Marisa, Michael A. Mandell, Tomonori Kimura, Santosh Chauhan, Cédric Cleyrat, and Vojo Deretic. 2015. “Secretory Autophagy.” *Current Opinion in Cell Biology*. Elsevier Ltd. https://doi.org/10.1016/j.ceb.2015.04.016.

Renna, Felipe J., Juliana H. Enriqué Steinberg, Claudio D. Gonzalez, Maria Manifava, Mariana S. Tadic, Tamara Orquera, Carolina V. Vecino, et al. 2023. “Ubiquitination Is a Novel Post-Translational Modification of VMP1 in Autophagy of Human Tumor Cells.” *International Journal of Molecular Sciences* 24 (16). https://doi.org/10.3390/ijms241612981.

Ropolo, Alejandro, Cintia Catrinacio, Felipe Javier Renna, Veronica Boggio, Tamara Orquera, Claudio D. Gonzalez, and Maria I. Vaccaro. 2020. “A Novel E2F1-EP300-VMP1 Pathway Mediates Gemcitabine-Induced Autophagy in Pancreatic Cancer Cells Carrying Oncogenic KRAS.” *Frontiers in Endocrinology* 11 (June): 1–14.

Ropolo, Alejandro, Daniel Grasso, Romina Pardo, Maria L. Sacchetti, Cendrine Archange, Andrea Lo Re, Mylene Seux, et al. 2007. “The Pancreatitis-Induced Vacuole Membrane Protein 1 Triggers Autophagy in Mammalian Cells.” *The Journal of Biological Chemistry* 282 (51): 37124–33.

Solvik, Tina A., Tan A. Nguyen, Yu-Hsiu Tony Lin, Timothy Marsh, Eric J. Huang, Arun P. Wiita, Jayanta Debnath, and Andrew M. Leidal. 2021. “Autophagy Cargo Receptors Are Secreted via Extracellular Vesicles and Particles in Response to Endolysosomal Inhibition or Impaired Autophagosome Maturation.” *bioRxiv*. bioRxiv. https://doi.org/10.1101/2021.08.12.456045.

Solvik, Tina A., Tan A. Nguyen, Yu Hsiu Tony Lin, Timothy Marsh, Eric J. Huang, Arun P. Wiita, Jayanta Debnath, and Andrew M. Leidal. 2022. “Secretory Autophagy Maintains Proteostasis upon Lysosome Inhibition.” *The Journal of Cell Biology* 221 (6). https://doi.org/10.1083/jcb.202110151.

Urano, Yasuomi, Chinatsu Mori, Ayano Fuji, Keito Konno, Takayuki Yamamoto, Shohei Yashirogi, Mayu Ando, Yoshiro Saito, and Noriko Noguchi. 2018. “6-Hydroxydopamine Induces Secretion of PARK7/DJ-1 via Autophagy-Based Unconventional Secretory Pathway.” *Autophagy* 14 (11): 1943–58.

Vanasco, Virginia, Alejandro Ropolo, Daniel Grasso, Diego S. Ojeda, María Noé García, Tamara A. Vico, Tamara Orquera, Jorge Quarleri, Silvia Alvarez, and María I. Vaccaro. 2021. “Mitochondrial Dynamics and VMP1-Related Selective Mitophagy in Experimental Acute Pancreatitis” 9 (March): 1–16.

Welsh, Joshua A., Deborah C. I. Goberdhan, Lorraine O’Driscoll, Edit I. Buzas, Cherie Blenkiron, Benedetta Bussolati, Houjian Cai, et al. 2024. “Minimal Information for Studies of Extracellular Vesicles (MISEV2023): From Basic to Advanced Approaches.” *Journal of Extracellular Vesicles* 13 (2): e12404.

Zahoor, Muhammad, and Hesso Farhan. 2018. “Crosstalk of Autophagy and the Secretory Pathway and Its Role in Diseases.” *International Review of Cell and Molecular Biology* 337 (January):153–84.

Zhang, Min, Samuel J. Kenny, Liang Ge, Ke Xu, and Randy Schekman. 2015. “Author Response: Translocation of Interleukin-1β into a Vesicle Intermediate in

Autophagy-Mediated Secretion." eLife Sciences Publications, Ltd. https://doi.org/10.7554/elife.11205.023.

Zou, Wenchong, Mingqiang Lai, Yue Zhang, Lei Zheng, Zhe Xing, Ting Li, Zhipeng Zou, et al. 2019. "Exosome Release Is Regulated by mTORC1." *Advancement of Science* 6 (3): 1801313.

Figure 1.

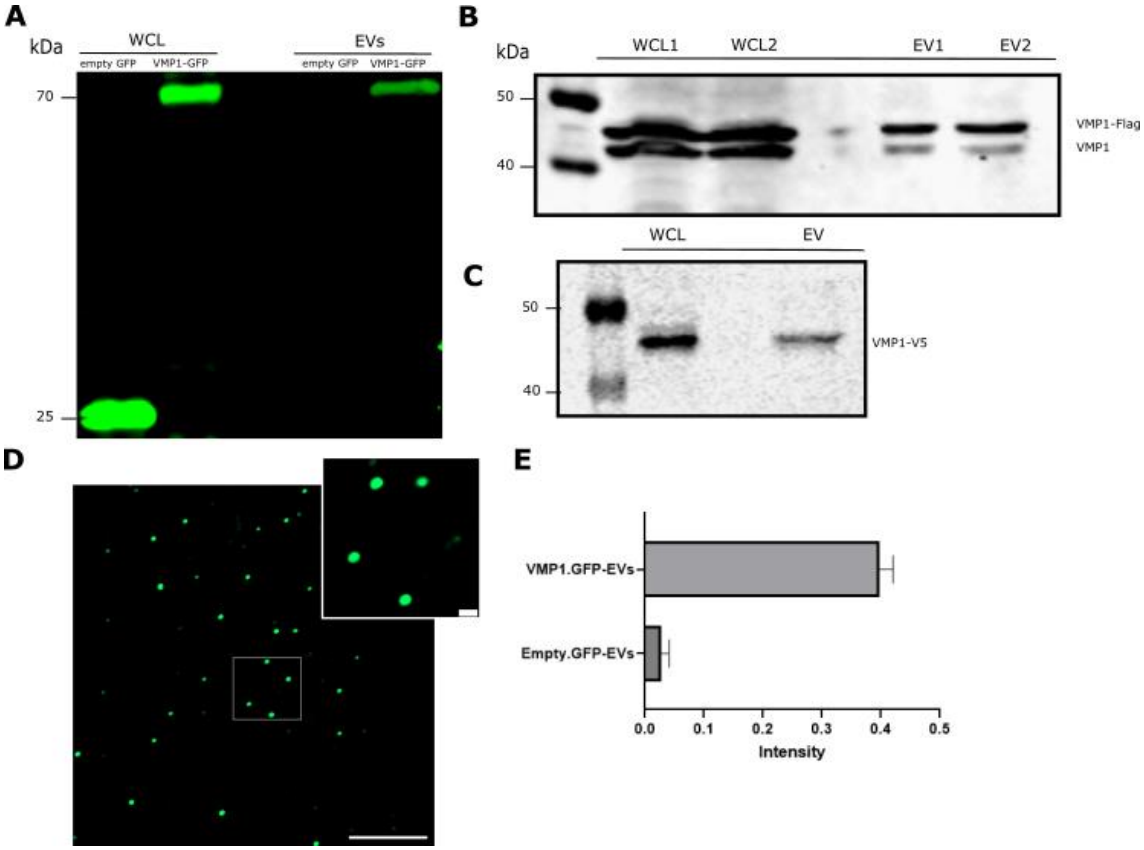

A
kDa
WCL
EVs
empty GFP
VMP1-GFP
empty GFP
VMP1-GFP
70
25
B
kDa
WCL1
WCL2
EV1
EV2
50
40
VMP1-Flag
VMP1
C
WCL
EV
50
40
VMP1-V5
D
E
VMP1.GFP-EVs
Empty.GFP-EVs
0.0
0.1
0.2
0.3
0.4
0.5
Intensity

Figure 2.

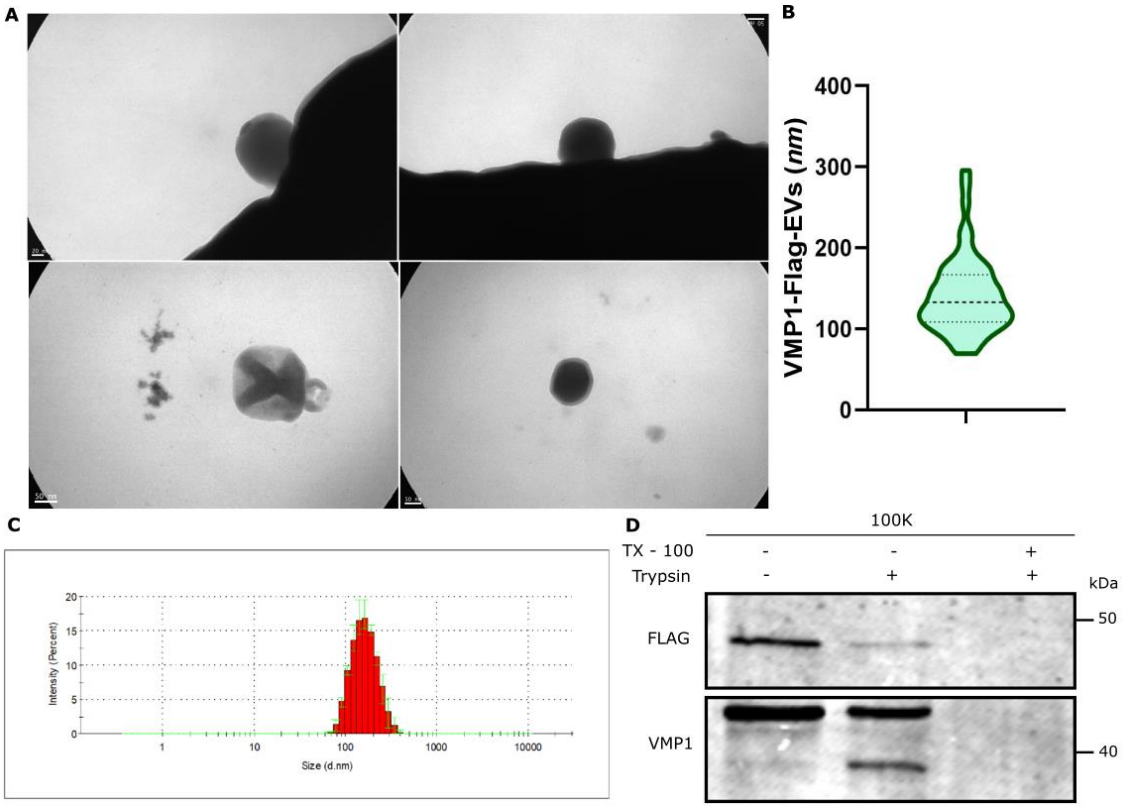

A
B
VMP1-Flag-EVs (nm)
400
300
200
100
0
C
Intensity (Percent)
Size (d.nm)
D
100K
TX - 100
Trypsin
kDa
FLAG
VMP1
50
40

Figure 3.

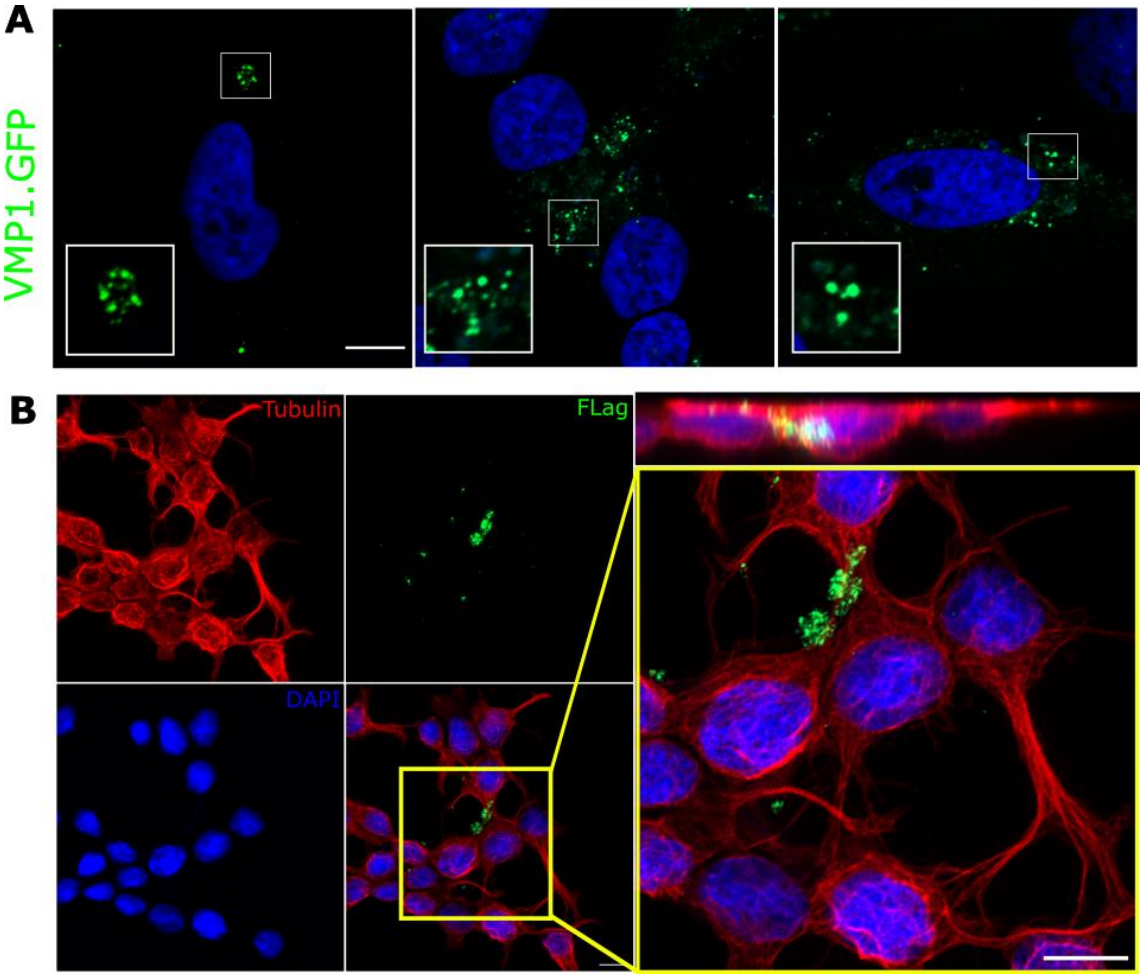


Figure 4.

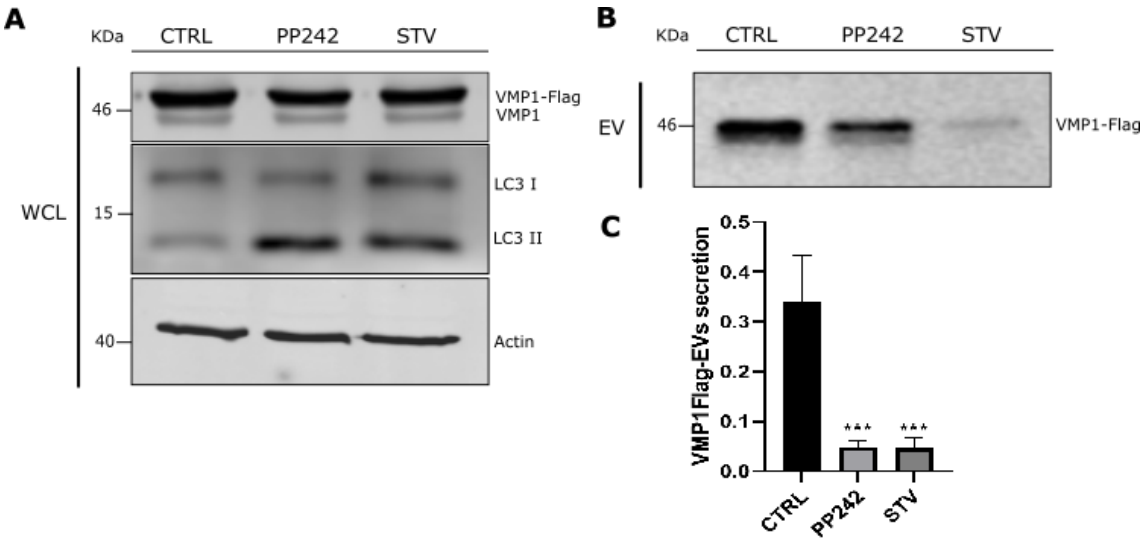


Figure 5.

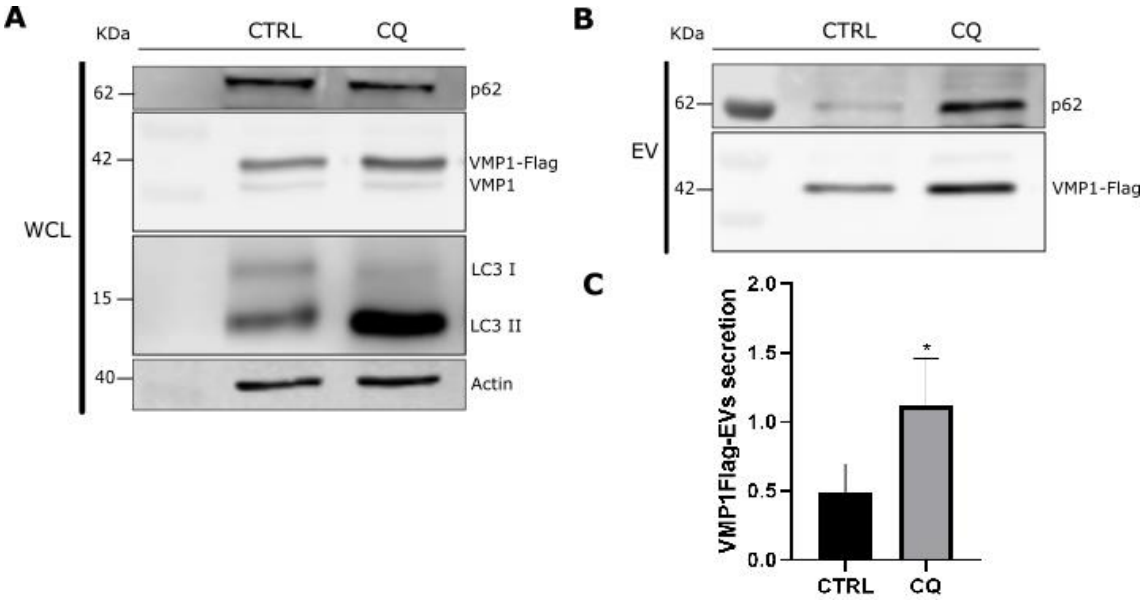


Figure 6.

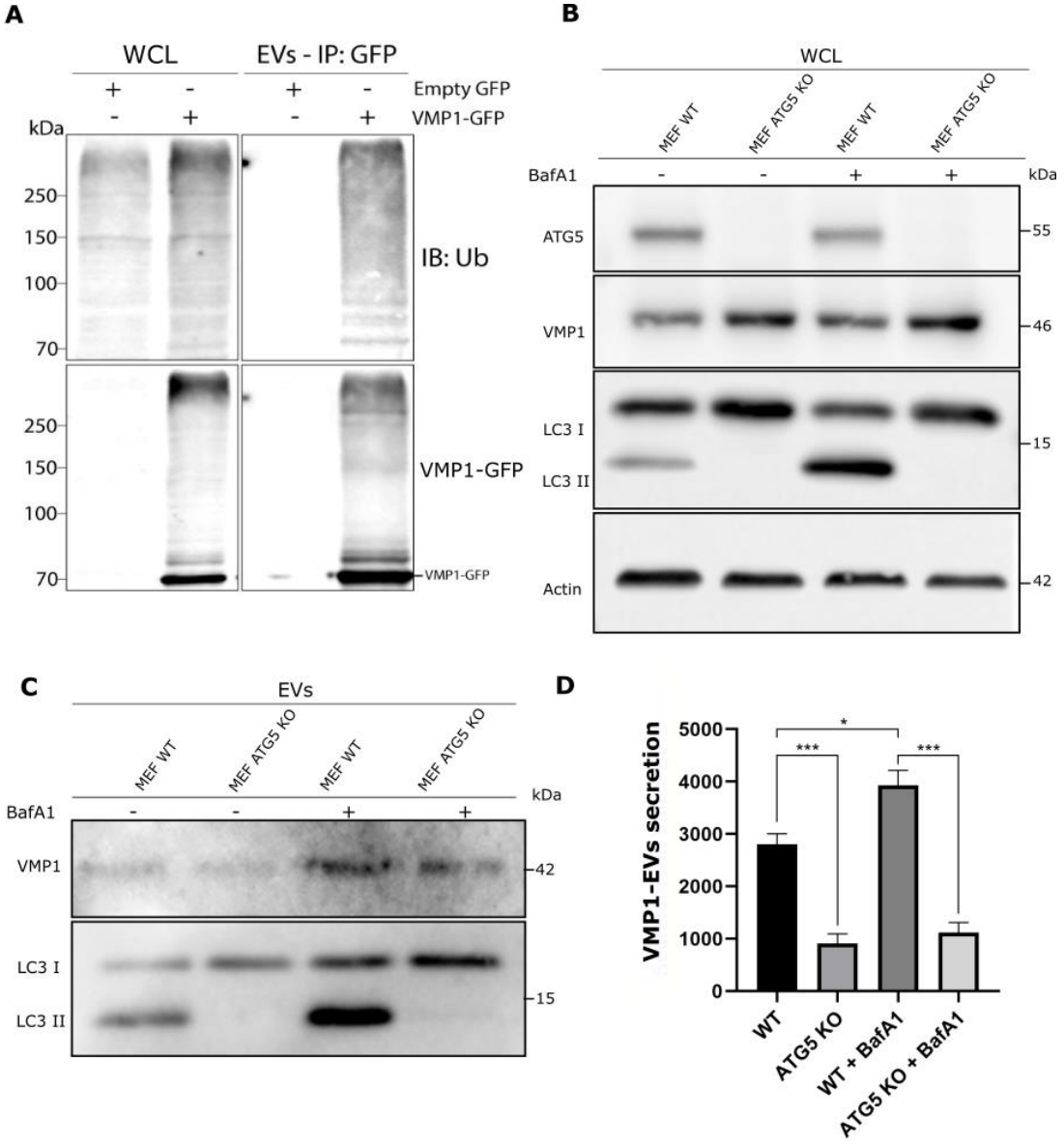

A
WCL
EVs - IP: GFP
Empty GFP
VMP1-GFP
kDa
250
150
100
70
IB: Ub
VMP1-GFP
B
WCL
MEF WT
MEF ATG5 KO
BafA1
ATG5
VMP1
LC3 I
LC3 II
Actin
55
46
15
42
C
EVs
D
VMP1-EVs secretion
5000
4000
3000
2000
1000
0
WT
ATG5 KO
WT + BafA1
ATG5 KO + BafA1

Figure 7.

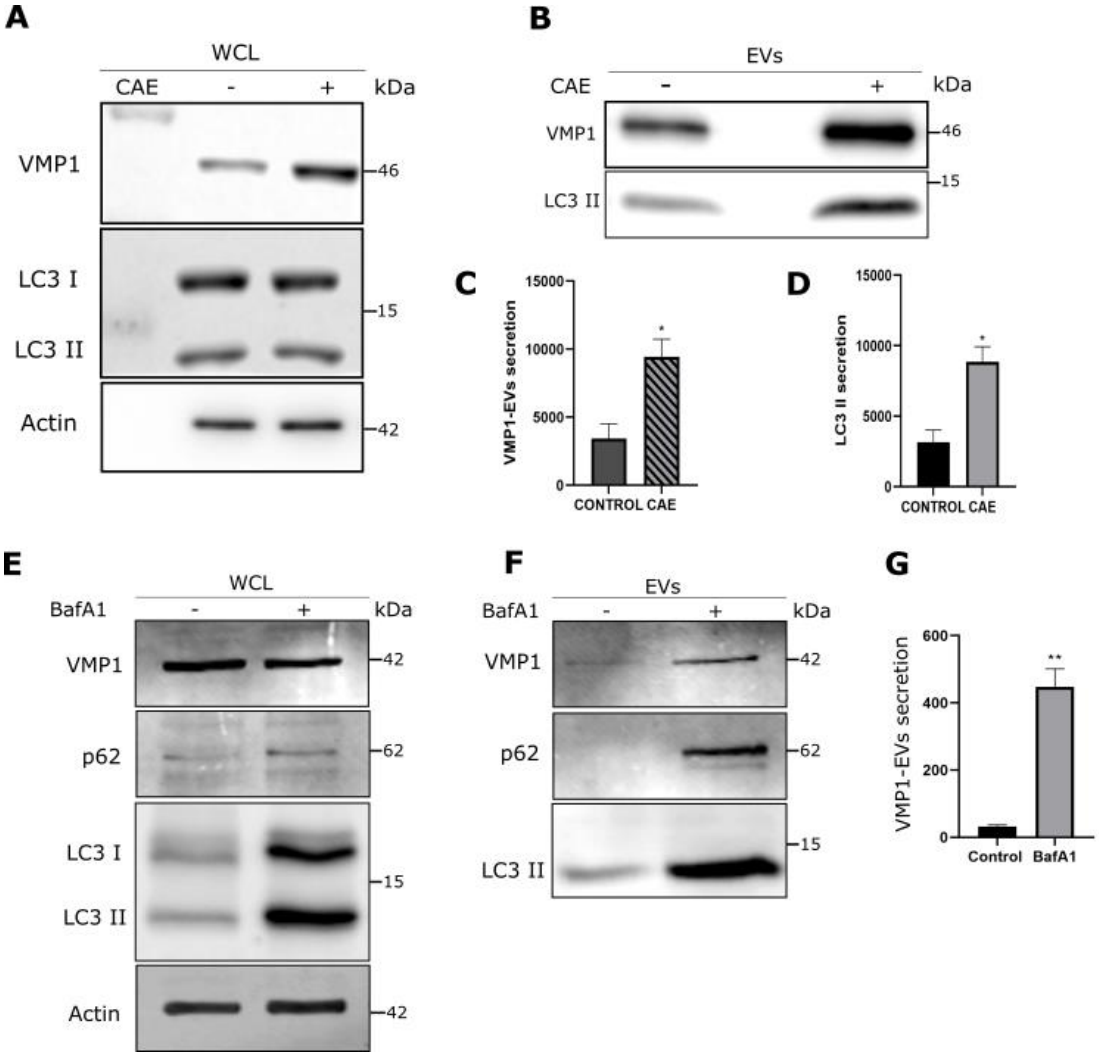
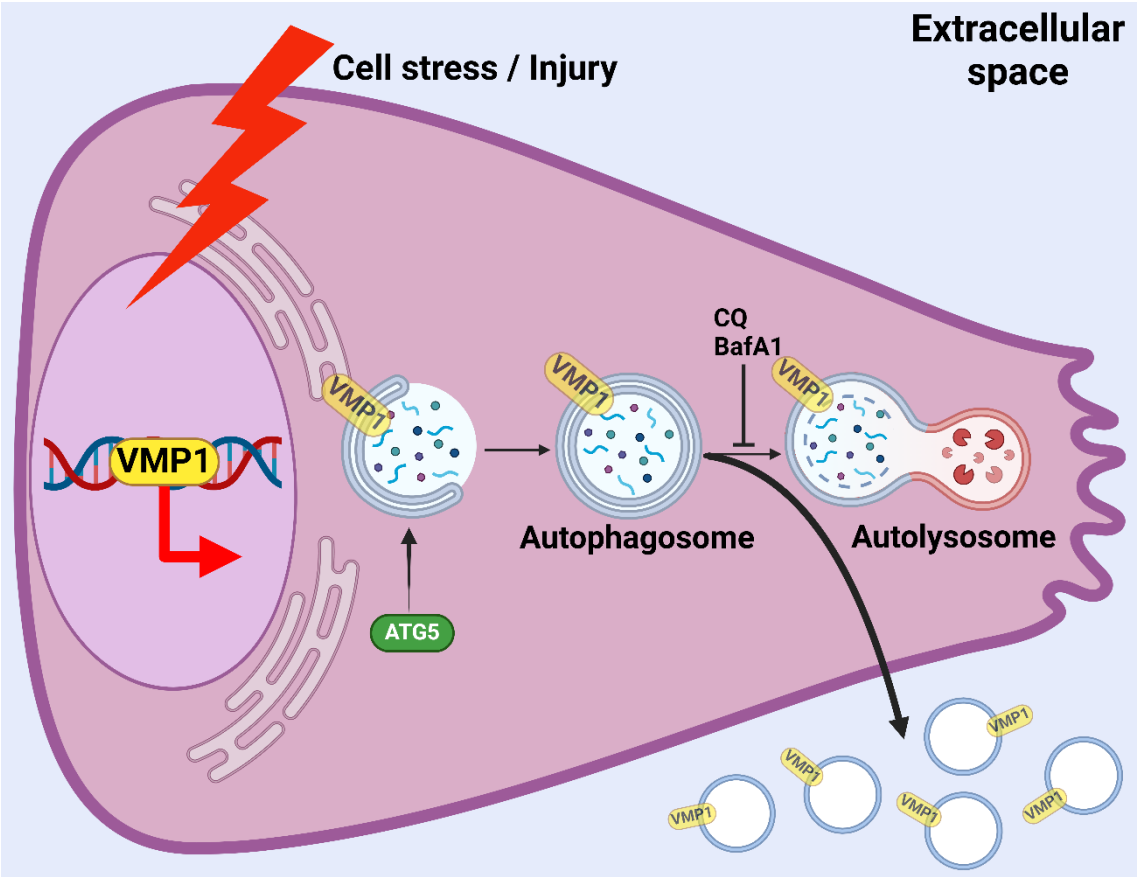

A
WCL
CAE
-
+
kDa
VMP1
46
LC3 I
LC3 II
15
Actin
42
B
EVs
CAE
-
+
kDa
VMP1
46
LC3 II
15
C
VMP1-EVs secretion
15000
10000
5000
0
CONTROL CAE
D
LC3 II secretion
15000
10000
5000
0
CONTROL CAE
E
WCL
BafA1
-
+
kDa
VMP1
42
p62
62
LC3 I
LC3 II
15
Actin
42
F
EVs
BafA1
-
+
kDa
VMP1
42
p62
62
LC3 II
15
G
VMP1-EVs secretion
600
400
200
0
Control
BafA1

Figure 8.


Extracellular space
Cell stress / Injury
VMP1
VMP1
VMP1
CQ
BafA1
VMP1
Autophagosome
Autolysosome
ATG5
VMP1
VMP1
VMP1
VMP1
VMP1