Role of caveolae mechanics in the dynamics of extracellular vesicles involved in tumor progression

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Role of caveolae mechanics in the dynamics of extracellular vesicles involved in tumor progression

Rôle de la mécanique des cavéoles dans la dynamique des vésicules extracellulaires impliquées dans la progression tumorale

Soutenue par
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Le 31 Octobre 2023

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Sigue abriendo los caminos
el surco de tu destino.
La alegría de sembrar
no te la pueden quitar,
la alegría de sembrar
es tuya, de nadie más.

-La pala, Víctor Jara
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"...An office is for not dying. An office is a place to live life to the fullest. To the max. To... an office is a place where dreams come true." – Michael Scott.

This dream could not have come true without all the people that I met throughout this journey.

First I would like to thank my supervisor Christophe, who trusted me to come from Chile and work in this exciting project. Every project comes with complications and roadblocks, and I feel privileged to have had the support of Christophe in these times, whether it was by trusting in my proposals to overcome the issues or by his willingness to arrange meetings with different experts that could help me. Of all that I have learned under his direction, I believe what stood the most for me was the importance of finding joy in what one does. I am tremendously grateful for the invaluable knowledge and guidance I have received from him during these past four years.

"The people that you work with are, when you get down to it, your very best friends." – M.S.

By being more than 11,000 kilometers away from home, across the equator and the Atlantic Ocean, one realizes that your colleagues are in reality more than just colleagues, they are the people that you see every single day, who you talk with, take a coffee together after lunch, or get together after work. For this I would like to thank all my colleagues, in no particular order, my office mates Changting and Satish, for the daily conversations about everything including life, science, cultural exchanges, and so on. It’s good to have people that you get along with sitting right next to you.

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and motivation to get involved in my project, even when you’ve learned that working with EVs comes with more fails than successes.

“I live by one rule: No office romances, no way. Very messy, inappropriate… no. But, I live by another rule: Just do it… Nike.” -MS

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“And I knew exactly what to do, but in a much more real sense I had no idea what to do.” –MS

Whenever I felt stuck in the project I’d meet with experts from different places so in this part I’d like to take the time to thank all the people that have helped me as advisors, contributors or collaborators.

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“The only time I set the bar low is for limbo. Always keep the bar raised no matter what.” –MS

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RÉSUMÉ DE LA THÈSE

Les vésicules extracellulaires (VE) sont des vésicules lipidiques qui sont libérées par toutes les cellules étudiées à ce jour et présentes dans tous les fluides corporels humains. Les VE contiennent du matériel génétique et des protéines qui peuvent être transférés à d'autres cellules et produire un effet sur celles-ci. La cavéoline-1 (Cav1) est un composant clé des petites invagination de la membrane plasmique appelées cavéoles, où elles fonctionnent comme mécano-capteurs et régulateur de la tension membranaire. La Cav1 facilite la migration et l'invasion des cellules tumorales et son expression est souvent augmentée dans les stades avancés du cancer. Des niveaux élevés de Cav1 ont ainsi été rapportés dans les VE de patients atteints d'un cancer avancé. Étant donné l'importance des forces mécaniques dans le micro-environnement des cellules cancéreuses, nous avons émis l'hypothèse que les cavéoles et/ou Cav1 pourraient être des acteurs clés dans la régulation de la biologie des VE et la progression du cancer sous contraintes mécaniques. Pour tester cette hypothèse, nous avons soumis différentes lignées cancéreuses exprimant (WT) ou délétées pour l'expression de Cav1 (KO) à des systèmes de stress mécanique en 2D et 3D. Les VE ont été purifiées et analysées par suivi des nanoparticules (NTA). Nous avons trouvé une augmentation conséquente de la libération des VE après un stress mécanique à la fois dans les modèles 2D et 3D. Cette augmentation était strictement dépendante de la présence de Cav1 et corrélée à une fusion accrue des corps multivésiculaires avec la membrane plasmique, indiquant que la population de VE augmentée lors d'un stress...
mécanique est constituée d'exosomes. Nous avons observé que Cav1 était enrichie dans les VE après une contrainte mécanique. L'augmentation des VE suite à un stress mécanique est considérablement réduite suite à la régulation négative du complexe de tri endosomal ESCRT-0, confirmant la nature exosomale de ces VE. L'analyse lipidomique a révélé des différences dans la composition lipidique des VE provenant de cellules Cav1KO et après un stress mécanique, suggérant une différence dans les propriétés de ces VE. Enfin, il a été démontré que les EVs provenant de cellules soumises à un stress mécanique favorisent un phénotype de migration et d'invasion accru, dépendant de Cav1, dans les cellules cancéreuses. Ces résultats nous permettent de conclure que le stress mécanique est associé à la libération accrue de VE et à l'acquisition de traits métastatiques dans les cellules réceptrices in vitro, Cav1 étant un acteur clé de ce processus. En parallèle, nous avons également étudié l'implication de Cav1 dans l'endocytose des EVs, un processus clé dans le contexte de la communication cellulaire médiée par les EVs. Nous avons utilisé différents marqueurs protéiques pour suivre et visualiser la capture des VE dans les cellules réceptrices. La capture des VE était fortement diminuée dans les cellules réceptrices Cav1 KO par rapport aux cellules WT. Nous avons également fait l'observation que Cav1, et non les cavéoles, était nécessaire à la capture des VE par les cellules, et que l'efficacité de capture était corrélée au niveau d'expression de Cav1 dans les cellules réceptrices. Dans l'ensemble, nos travaux ont révélé un rôle nouveau de Cav1 dans la communication intercellulaire médiée par les VE et la propagation des phénotypes métastatiques par le contrôle mécanique de la sécrétion et la dynamique des VE.
Extracellular vesicles (EVs) are lipid-enclosed vesicles that are released by all cells studied to date and present in all human bodily fluids. EVs contain genetic material and proteins that are able to be transferred to and generate an effect in other cells. Caveolin-1 (Cav1) is a key component of the small invagination of the plasma membrane called caveolae, where it functions as mechano-sensors and membrane tension buffering device. Cav1 facilitates the migration and invasion of tumor cells and its expression is often increased in the late stages of cancer. Interestingly, high levels of Cav1 have been found in EVs of patients with advanced cancer. Given the importance of mechanical forces in the microenvironment of cancer cells, we hypothesized that caveolae and/or Cav1 may represent key players in the regulation of EV biology and cancer progression under mechanical strain. To test this hypothesis, we subjected different cancer cell lines, having (WT) or deleted for Cav1 expression (KO) to 2D or 3D systems of mechanical stress. EVs were purified from these cells and analyzed by nanoparticle tracking analysis. We found a striking increase in the release of EVs after mechanical stress both in 2D and 3D models. This increase was strictly dependent on the presence of Cav1 and correlated with enhanced fusion of multivesicular bodies to the plasma membrane, indicating that the population of EVs increased upon mechanical stress corresponding to exosomes. We observed that Cav1 was enriched in EVs after mechanical strain. The increase of EVs observed after mechanical stress was drastically reduced upon downregulation of the endosomal sorting complex ESCRT-0, further
confirming their exosomal nature. Lipidomic analysis revealed differences in the lipid composition of EVs in Cav1−/− cells and after mechanical stress, suggesting a difference in the properties of these EVs. Finally, EVs from mechanically stressed cells were shown to promote enhanced migration and invasion phenotype in triple negative breast cancer cells, whereas EVs from Cav1 depleted cells did not. These data allow us to conclude that mechanical stress is associated with increased release of EVs and the acquisition of metastatic traits in receiving cells in vitro, with Cav1 being a key player of this process. In parallel, we also studied the involvement of Cav1 in the uptake of EVs, a key process in the EV mediated cellular communication context. We used different protein markers to follow and visualize the uptake of EVs. EV uptake was strongly decreased in Cav1−/− cells as compared to WT receiving cells. We also made the observation that Cav1 but not caveolae was required for this uptake of EVs by cells and that the uptake efficiency was correlated with the level of Cav1 expression in receiving cells. Overall, our work has revealed a new role for Cav1 in cell-to-cell communication and the propagation of metastatic phenotypes through the mechanical control of EV production and dynamics.
Abbreviations

α-SMA, Alpha-smooth muscle actin
CAF, Cancer associated fibroblast
Cav1, Caveolin-1
Cav2, Caveolin-2
Cav3, Caveolin-3
CLIC, Clathrin-independent carriers
CTC, Circulating tumor cells
DMD, Duchenne muscular dystrophy
ECM, Extracellular matrix
EGFR, Epidermal growth factor receptor
EMT, Epithelial-mesenchymal transition
EM, Electron microscopy
eNOS, Endothelial nitric oxide synthase
ER, Endoplasmic reticulum
ESCRT, Endosomal sorting complex required for transport
EV, Extracellular vesicles
FAP, Fibroblast activation protein
FGF, Fibroblast growth factor
GEEC, GPI-AP enriched early endosomal compartment
GUV, Giant unilamellar vesicle
HCK, Hyperckemia
HGF, Hepatocyte growth factor
IMD, Intramembrane domain
IL1β, Interleukin 1 beta
ILV, Intraluminal vesicles
IGF, Insulin growth factor
ISC, In situ carcinomas
JNK, Jun N-terminal Kinase
KO, Knockout
LacCer, Lactosylceramide
LGMD, Limb-girdle muscular dystrophy
IncRNA, Long non-coding RNA
MAPK, Mitogen-activated protein kinase
MLC, p-Myosin Light Chain
MMP, Matrix metallo-proteinases
miRNA, MicroRNA
mRNA, Messenger RNA
MVB/MVE, Multivesicular body
NTA, Nanoparticle tracking analysis
OM, Oligomerization domain
PDGF, Platelet-derived growth factor
PIP2, Phosphatidylinositol bisphosphate
PM, Pin motif
RMD, Rippling muscle disease
ROCK, Rho-associated protein kinase
SM, Signature motif
SD, Scaffolding domain
TGF-β, Transforming growth factor beta
TNFα, Tumor necrosis factor alpha
TME, Tumor microenvironment
Tregs, CD4+ CD25+ FOXP3+ regulatory T cells
UPR, Unfolded protein response
VEGF, Vascular endothelial growth factor
WT, Wild type
1. INTRODUCTION

Chapter 1: Extracellular Vesicles

Extracellular vesicles (EVs) are small membranous particles present in all bodily fluids and released to the extracellular space by all cell types studied thus far. EVs can range in size from 30 to 1000 nanometers and are classified based on their biogenesis, size, and content (Théry et al., 2018). They are known to play important roles in intercellular communication, immune regulation, and disease pathogenesis (Kugeratski & Kalluri, 2021).

1.1.1 Historical perspective

What could be considered as the beginning of the exploration of the biology of EVs is a series of observations in two articles published one by Erwin Chargaff in 1945 and another by Erwin Chargaff together with Randolph West in 1946, where they describe a very particulate sediment that is obtained after centrifugation at 31,000 g of blood samples with high clotting potential (Chargaff, 1945; Chargaff & West, 1946). Two decades later, Wolf observed small particles in human plasma that were derived from platelets and proposed that they were involved in blood clotting, calling them "platelet dust" (Wolf, 1967). H. Clarke Anderson, on the other hand, identified small vesicles in the matrix of epiphyseal cartilage that were associated with calcification, which were called "matrix vesicles" (Anderson, 1969). These early observations laid the foundation for further studies on the role of EVs in
physiological and pathological processes. Later on, Aaronson and colleagues were able to visualize and isolate a heterogeneous population of extracellular particles from *Ochromonas danica*, a flagellated alga, using centrifugation; it is Aaronson who first used the term “extracellular vesicles” (Aaronson et al., 1971). The first insight about one of the biogenesis pathways of EVs comes from the work of Eladio Nunez and collaborators. They were the first to observe the presence of vesicle-containing endosomal structures in the bat thyroid gland that they called them multivesicular bodies (MVBs). They suggested that the fusion of the membrane of the MVBs with the plasma membrane of the cell leads to the release of the small vesicles that now we know as intraluminal vesicles (ILVs) (Nunez et al., 1974). The 1980s were a prolific decade for EV research, Clifford Harding and colleagues used electron microscopy to study the process of transferrin receptor trafficking in reticulocytes and observed the release ILVs from MVBs fused with the plasma membrane (Harding et al., 1983). In parallel, Bin-Tao Pan and Rose Johnstone also observed the release of small vesicles from sheep reticulocytes and proposed that these vesicles were involved in the shedding of membrane proteins (Pan & Johnstone, 1983). Later and for the first time, Johnstone coined the term "exosome" to specifically describe these small vesicles derived from the endocytic pathway (Johnstone et al., 1987) (Figure 1).
Figure 1. Timeline of major milestones in EV research

From the very first observations made by E. Chargaff and R. West to the functional relevance breakthrough and the formation of national and international societies, this timeline depicts the growing interest in the EV field throughout time (Couch et al., 2021).

In 1996, EVs from immune cells were shown to contain various proteins, including MHC class II molecules, therefore demonstrating that EVs are capable of antigen presentation. This report was a ground-breaking moment, as it revealed that EVs have the potential to serve functional purposes in various biological processes, including in disease (Raposo et al., 1996). Since then, the growing interest in EV research has led to an in-depth exploration of the composition and content of the vesicles as well as their physiological role. Early studies showed that EVs carry a diverse range of proteins, including receptors, enzymes, and cytoskeletal proteins (Théry et al., 2001).

The presence of specific proteins in EVs can provide insights into the cellular origin of the vesicles and their potential functions. In addition to proteins, EVs carry a specific subset of lipids, including phosphatidylserine, sphingomyelin, and cholesterol (Subra et al., 2007). The lipid composition of EVs can also vary depending on the cell type and physiological or pathological state of the parental cell (Figure 2). For example, cancer-derived EVs have been shown
to have a distinct lipid profile compared to EVs derived from normal cells (Skotland et al., 2017). Interestingly, when comparing the lipid profile of small EVs to the lipid composition of the parental cells, there is a notable enrichment of certain lipid species, such as sphingolipids, phosphatidylserine and cholesterol in the EV population, on the other hand, phosphatidylcholine and phosphatidylinositol are found to be much more abundant in the total lipid composition of the parental cells compared to the EVs (Skotland et al., 2023) (Figure 2).

**Figure 2. Enrichment of lipid species in cells and EVs**
Enrichment of lipid classes in cells or exosomes calculated as mol% of lipids in these samples. (LPE) lysoPE, (LPI) lysoPI, (CHOL) cholesterol. GM2, Gb3, GD1, GM1, GM3, LacCer, and HexCer are all glycosphingolipids (GSLs) (Llorente et al., 2013).

EVs were found to carry various types of nucleic acids with functional capabilities, including messenger RNA (mRNA), microRNA (miRNA), and long non-coding RNA (lncRNA) (Ratajczak et al., 2006; Skog et al., 2008; Valadi et al., 2007) (Figure 3).
Figure 3. Overall composition and cargo of EVs
Schematic representation of the different components of EVs, including families of proteins, lipids, and nucleic acids. Among the proteins we can find tetraspanins, including CD63, CD81, Lamp1 and CD9. Typical species of lipids found in EVs include sphingomyelin, phosphatidylserine, cholesterol and ceramide, among others. And for nucleic acids, we can typically find miRNAs, mRNAs, and other noncoding RNAs (Colombo et al., 2014).

The presence of specific nucleic acids in EVs can provide insights into the gene expression profile of the parental cell and may have implications for disease diagnosis and monitoring. Overall, the specific cargo carried by EVs is highly dependent not only on the cell type that secretes the EVs but also on the physiological or pathological state of the parental cell.
1.1.2 EV subtypes and biogenesis

Based on their biogenesis, EVs can be classified into several types, including exomeres, exosomes, microvesicles, and apoptotic bodies (Figure 4).

Exomeres are the newest members of the EV family and the smallest type of EVs, with a diameter of less than 50 nm. They lack an external membrane structure yet are still able to carry lipids, proteins and nucleic acids. The biogenesis of exomeres has not been described to date and it is hypothesized that exomeres are rather a heterogeneous group of complexes that are purified together because of their biophysical properties rather than a single type of entity (Tosar et al., 2022; Zhang et al., 2018).

Figure 4. Extracellular vesicle subtypes
Schematic representation of the classification of EVs according to their biogenesis and size. Depicted we can see exomeres, the newly discovered EV subtype, exosomes, that are released after the fusion of MVBs to the plasma membrane, and large EVs which are mainly released by shedding of the plasma membrane, which include microvesicles, ectosomes, microparticles, oncosomes and apoptotic bodies (Cocozza et al., 2020).

Microvesicles are formed by the outward budding of the plasma membrane and are released into the extracellular space; they are larger than exosomes, with a diameter of 100-1000 nm (van Niel et al., 2018). Given their
heterogeneity, microvesicles can be formed by multiple mechanisms including the endosomal sorting complex required for transport (ESCRT) machinery, ceramide-dependent production, bleb scission, among others (Bianco et al., 2009; Sedgwick et al., 2015; Wang & Lu, 2017).

Apoptotic bodies are large EVs with a diameter of over 1000 nm that are released by cells undergoing programmed cell death (apoptosis). They are formed by the fragmentation of the apoptotic cell and contain cellular debris, including organelles and nucleic acids (Hristov et al., 2004).

Exosomes are another type of EVs with a diameter of roughly 50-150 nm that are formed by the inward budding of the endosomal membrane, resulting in the formation of MVBs. The MVBs can then fuse with the plasma membrane, releasing the ILVs into the extracellular space as exosomes (Théry et al., 2002). The biogenesis of MVBs can be divided into ESCRT-dependent and ESCRT-independent pathways, the former being the most extensively described. The ESCRT-dependent pathway is a complex multi-step process that begins with the recognition and recruitment of ubiquitinated cargo proteins by the ESCRT-0 complex, composed of a heterodimer of HRS and STAM subunits which contain ubiquitin-binding domains, to the early endosomal membrane (Raiborg & Stenmark, 2009). In humans, there are two isoforms of STAM, STAM1 and STAM2. Downregulation of ESCRT-0, either by HRS or STAM depletion, shows impairment in the recruitment of other ESCRT members that results in defects in the endosomal membrane invagination, ILV formation, and ultimately impaired exosome secretion.
Next, the ESCRT-I complex, which is a heterotetramer of TSG101, MVB12, VPS28, and VPS37, is recruited to the endosomal membrane by ESCRT-0 (Hurley & Hanson, 2010). ESCRT-I contributes to the clustering of ubiquitinated cargo to the early endosome and acts as a bridge between ESCRT-0 and ESCRT-II (Katzmann et al., 2001). The ESCRT-I complex recruits the ESCRT-II complex, which is a heterotetramer of two VPS22 subunits, one VPS25, and one VPS36 subunit (Carlton & Martin-Serrano, 2007). The ESCRT-II complex also has ubiquitin binding domains but more importantly will stabilize the ESCRT-III complex involved in membrane scission (Alam et al., 2004). ESCRT-III is composed of CHMP2A/B, CHMP3, CHMP4A/B/C, and CHMP6. The main role of this complex is to constrict the inward budding membrane and recruit the ESCRT-associated AAA-ATPase VPS4 that catalyzes the disassembly of the ESCRT-III complex and drives membrane scission and ILV formation (Hurley & Hanson, 2010; Wollert et al., 2009). The ESCRT pathway has additional but mechanistically similar roles in membrane abscission during cytokinesis, viral budding, membrane repair, and in autophagy (Carlton & Martin-Serrano, 2007; Fujii et al., 2009; Jimenez et al., 2014; Rusten & Stenmark, 2009).

Independently of ESCRT proteins, cells can generate MVBs and secrete exosomes in different ways involving other protein species or lipids, although the composition, shape, and size may differ in many cases.

Several reports have shown that tetraspanins, which are protein scaffolds embedded in the membranes through four transmembrane domains,
contribute to ILV cargo sorting. Cells depleted of key subunits of all ESCRT complexes were still able to form MVBs (Stuffers et al., 2009). Furthermore, in melanocytes, it has been shown that PMEL, a protein essential for the formation of melanosome amyloid fibrils, is sorted into ILVs independently of the ESCRT machinery through a process that relies on cholesterol- and sphingolipid-rich microdomains in a CD63 dependent manner (van Niel et al., 2011). Tetraspanin-6 has been suggested to interact with syntenin, a PDZ-domain-containing protein, and induce an ESCRT-independent formation of ILVs that leads to release of a specific subpopulation of exosomes containing amyloid precursor protein (Guix et al., 2017).

Ceramide, a lipid produced by hydrolysis of sphingomyelin by nSMase2 sphingomyelinase has been shown to play a role in exosome biogenesis, through the promotion of ceramide-rich microdomains with a spherical curvature of the endosomal membrane, leading to the inward budding of ILVs into the lumen of MVBs in oligodendroglial cells without the need for ESCRT function (Trajkovic et al., 2008). Moreover, the nSMase2-ceramide pathway promotes the secretion of exosomes with a specific packaging of miRNAs with a functional significance in tumor invasion (Singh et al., 2014). The detailed mechanism of how ceramide is able to induce the formation of ILVs and release of exosomes is not fully understood but it is proposed that ceramide can recruit tetraspanins that help in the budding of the plasma membrane or induce curvatures and scission of the MVB membrane (Trajkovic et al., 2008) (Figure 5).
Figure 5. Biogenesis of extracellular vesicles
Schematic representation of the different pathways involved in the biogenesis of exosomes and microvesicles. 1. Membrane-bound proteins are recruited in microdomains of the MVB (here called MVE) membrane (bottom) or at the plasma membrane (top). 2. Other molecules such as proteins and nucleic acids are recruited to be sorted into ILVs (bottom) or microvesicles (top). 3. These clustered microdomains together with other proteins induce the budding of the plasma membrane towards the inside of the MVB (bottom) or outside of the cell (top), and posterior scission to form ILVs (bottom) or microvesicles, accordingly (top) (van Niel et al., 2018).

The ESCRT accessory protein ALIX has been demonstrated to interact with syntenin and play a role in exosome biogenesis and exosomal sorting of syndecans, transmembrane proteoglycans thought to act as coreceptors, independently of ubiquitination (Baietti et al., 2012). The membrane scaffolding protein flotillins have been shown to control specific exosomal cargo sorting, whereby activation of the small GTPase Rab31 leads to its binding to flotillins and epidermal growth factor receptor (EGFR) sorting into
ILVs via lipid microdomains (Wei et al., 2021). Caveolin-1 (Cav1), a key protein in the formation of the characteristic small invaginations, called caveolae, in the plasma membrane has been described to be sorted into ILVs and released in exosomes (Hayer et al., 2010). Ubiquitinated Cav1 regulates sorting of extracellular matrix (ECM) cargoes into exosomes such as tenascin-C and fibronectin (Albacete-Albacete et al., 2020). Additionally, Cav1 can regulate the specific loading of miRNAs into ILVs through its interaction with the RNA binding protein hnRNPK (Robinson et al., 2021). The intricate relationship between Cav1 and EVs as well as their physiological implications of Cav1 bearing EVs will be further explored in detail below.

1.1.3 EVs in pathology, focus on cancer

EVs have gained significant attention in recent years due to their involvement in various physiological and pathological processes, including the development and progression of neurologic, cardiovascular diseases, inflammation, and cancer (Yáñez-Mó et al., 2015) (Figure 6).
Figure 6. Schematic representation of different roles for circulating EVs in diseases

Circulating EVs have the potential to facilitate intercellular communication by transferring bioactive molecules like proteins and RNA. This process can have significant impacts on various systemic processes, including immune function, inflammation, and disease- and organ-specific functions (Shah et al., 2018).

EVs have been found to be involved in the pathogenesis of various neurological disorders, including Alzheimer's disease, Parkinson's disease, and multiple sclerosis. EVs can carry misfolded proteins, such as beta-amyloid and alpha-synuclein, which are involved in Alzheimer's and Parkinson's diseases, respectively (Dinkins et al., 2014; Herman et al., 2023).

In addition, EVs can modulate neuroinflammatory responses by carrying pro-inflammatory molecules, such as interleukin-1β (IL-1β) and tumor necrosis factor alpha (TNFα) (Spiers et al., 2022).
The role of EVs in cardiovascular diseases, such as atherosclerosis, myocardial infarction, and heart failure, has been extensively studied. EVs derived from endothelial cells, platelets, and immune cells can modulate vascular inflammation and thrombosis, contributing to the progression of cardiovascular diseases (Oggero et al., 2019; Zarà et al., 2019).

Extracellular vesicles are actively involved in promoting and regulating inflammatory processes in various conditions, including rheumatoid arthritis, systemic lupus erythematosus, and inflammatory bowel disease. EVs can transfer bioactive molecules, such as cytokines, chemokines, and microRNAs, between cells, thereby modulating immune responses and contributing to the perpetuation of chronic inflammatory diseases (Buzas et al., 2014). For example, EVs can induce the differentiation of monocytes into pro-inflammatory macrophages, promoting the production of pro-inflammatory cytokines and the destruction of tissues (Vasina et al., 2011).

In the context of cancer, EVs have been implicated in multiple aspects of tumor progression and metastasis, such as promoting cell proliferation, invasion, angiogenesis, and immune evasion. Interestingly, tumor cells have been shown to release an increased amount of EVs compared to healthy cells (Silva et al., 2012; Vasconcelos et al., 2019) (Figure 7).
Figure 7. EV mediated pro-metastatic effects

Tumor-derived EVs affect the local tumor microenvironment by inducing enhanced migration, invasiveness potential, ECM remodeling and motility among other phenotypes (left). Tumor-derived EVs can also affect distal sites by establishing pre-metastatic niches through the induction of increased vascular permeability, ECM remodeling or recruitment of bone marrow-derived cells (Tkach & Théry, 2016).

Cancer progression is often characterized by alterations in the ECM, which can be modulated by EVs. EVs can enhance directional cell movement within the ECM by interacting with components like fibronectin. These components bind to integrins found on the surface of EVs, creating a substrate that facilitates cell-EV interactions, encourages cell attachment and increases cell movement speed (Sung et al., 2015). In rat pancreatic adenocarcinoma, it was demonstrated that CD151 and tetraspanin-8, two tetraspanins found in EVs, contribute to ECM degradation, driving cells to a motile phenotype (Yue et al., 2015). Matrix metalloproteinases (MMPs) are membrane-associated...
proteins that are secreted and are required for ECM remodeling. MMPs have been observed to be associated to EVs, specifically in the case of skin cancer cells that are able to promote invasion by secreting exosomes containing MMP-MT1 and MMP2 that mediate invadopodia biogenesis, stability and activity in matrix degradation (Hoshino et al., 2013).

Tumor-derived EVs can travel to distant secondary sites to play a crucial role in initiating pre-metastatic niche formation, the process of preparing a microenvironment in specific organs for the colonization of circulating tumor cells (Figure 7). The pre-metastatic niche remodeling is mediated by EVs by establishing a supportive and receptive niche that promotes tumor cell colonization and metastasis. EVs derived from melanoma cells can instruct bone marrow progenitors to adopt a pro-metastatic by reprogramming them to exhibit a pro-vasculogenic phenotype and inducing increased vascular permeability at pre-metastatic locations (Peinado et al., 2012). Pancreatic ductal adenocarcinoma-derived EVs are uptaken by Kupffer cells, which leads to the secretion of transforming growth factor β (TGF-β) and increased fibronectin production by hepatic stellate cells, creating a fibrotic microenvironment (Costa-Silva et al., 2015). In a breast cancer model, miR-105 is specifically expressed and secreted by highly metastatic cells and can be transferred to endothelial cells via exosomes. Overexpressed miR-105 in tumors is transferred to endothelial cells where it destabilizes the tight junctions and alters the integrity of endothelial barriers, thereby facilitating distant metastases in the lung and brain (Zhou et al., 2014). EVs, specifically microvesicles, released by CD105+ cancer stem cells trigger angiogenesis
and promote lung premetastatic niche formation on normal human endothelial cells, resulting in a significant increase in lung metastasis (Grange et al., 2011). Ral GTPases A and B in breast cancer cells are responsible for the organotropic targeting of pro-metastatic EVs towards lungs, where the presence of the adhesion molecules MCAM/CD146 in EVs where found to be crucial for this tropism (Ghoroghi et al., 2021). Furthermore, integrin patterns in EVs from lung-tropic models were distinct; integrins α6β4 and α6β1 were associated with lung metastasis, whereas integrin αvβ5 was linked to liver metastasis (Hoshino et al., 2015).

EVs can also serve as means of communication between both cancerous and non-cancerous cells, such as epithelial cells, cancer-associated fibroblasts (CAFs), endothelial cells, and immune cells, where they can play a crucial role in reprogramming cells and tissues to promote cancer progression. In melanoma cells, exosomes containing the immune checkpoint inhibitor PD-L1 have been found to contribute to immunosuppression by inhibiting the function of immune T cells (Chen et al., 2018). In another study, exosomes from prostate cancer cells were shown to induce an adenosine-mediated immunosuppressive effect, which led to the suppression of dendritic cell function by inhibiting TNFα- and IL-12 production through the induction of the expression of CD73 (Salimu et al., 2017). Tumor-secreted EVs have been described to promote the activation of CAFs in in vivo experiments where the population of fibroblasts expressing CD140a, an activated fibroblast marker, were increased after EV injection in a miRNA-125b dependent manner (Vu et al., 2019). Similarly, it has been shown that exosomes from various types of
cancer cells can trigger the differentiation of fibroblasts into myofibroblasts (Webber et al., 2010). This transformation is known to further contribute to the formation of an environment that supports tumor growth and metastasis by increasing contractile activity, vascularization and angiogenesis (Hinz et al., 2001; Orimo et al., 2005).

Interestingly, the caveolae protein Cav1 is significantly abundant in tumor-derived EVs and has been proposed to serve as a biomarker for cancer progression (Campos et al., 2019; Logozzi et al., 2009; Tahir et al., 2006). The presence of Cav1 in tumor-derived extracellular vesicles not only serves as a potential biomarker for the progression of various cancers but also promotes malignancy. Cav1 was found to be highly expressed in various melanoma cell lines and it was observed that Cav1 silencing led to reduced tumor growth and angiogenesis (Felicetti et al., 2009). Cav1 bearing EVs were shown to stimulate in vitro anchorage independence, migration, and invasion through paracrine and autocrine mechanisms. Notably, they are capable of transferring metastatic properties from the donor melanoma cell to the less aggressive recipient cell line. In another study, the proteome of the EVs from various melanoma cell lines revealed that their specific protein compositions are dependent on the aggressive nature of the parental cells, with Cav1 being one of the varying proteins (Lazar et al., 2015). In metastatic breast cancer, cells were observed to release Cav1-loaded EVs, which in turn, upon uptake by non-metastatic receiving cells, prompted the acquisition of pro-metastatic traits. Such traits included a marked increase in invasion and migration capabilities, in a Cav1 dependent manner. This effect was
suggested to be conferred, at least partially, by the presence of specific cell adhesion-related proteins unique to EVs released from Cav1 expressing cells (Campos et al., 2018). Indeed, Cav1 was found to be required for exosomal sorting of specific ECM proteins such as tenascin-c, promoting local tumor microenvironment remodeling for enhanced tumor invasion and the generation of distant ECM-enriched stromal niches in vivo (Albacete-Albacete et al., 2020).

In summary, the identification of the caveolar protein Cav1 within the protein composition of EVs originating from cancer cells and its role in various cancer models implicates it as a significant factor involved in cancer progression. Further study of its role in EV dynamics can therefore offer valuable insights into the mechanisms of cancer progression. This is the main topic of the present work.

**Chapter 2: Caveolae**

**1.2.1 Structure and formation**

Caveolae were first observed in 1953 in endothelial cells and two years later the term caveola intracellularis or “intracellular cave” was suggested (Palade, 1953; Yamada, 1955). Caveolae are specialized, cup-shaped invaginations of the plasma membrane with a diameter of 60-80 nm that are present in most cell types with a few exceptions such as red blood cells, platelets, immune cells, and some neuronal cell types. Caveolae research traversed from the
early years of electron microscopy to the discovery of the essential protein components (Echarri & Del Pozo, 2012). The structural and functional integrity of caveolae is attributed to two groups of proteins known as caveolins and cavins. The caveolin family of proteins is composed of the homologues Cav1, caveolin-2 (Cav2), and the muscle specific caveolin-3 (Cav3) (Kurzchalia et al., 1992; Rothberg et al., 1992; Scherer et al., 1996; Tang et al., 1996; Way & Parton, 1995). The cavin protein family is composed of four members, cavin-1, cavin-2, cavin-3 and the muscle-specific cavin-4 (Kovtun et al., 2015). Particularly, Cav1 together with cavin-1 (initially called polymerase I and transcript-release factor, PTRF) in non-muscle cells and Cav3 together with cavin-4 in muscle cells are strictly required for caveolae formation. The interaction between caveolins and cavins is crucial because the absence of either protein leads to a loss in the formation of caveolae (Drab et al., 2001; Hill et al., 2008) (Figure 8).
Figure 8. Caveolar components
Schematic representation of the structural components of caveolae. A. Caveolin monomer and oligomer structure (See figure 9 for more details). B. Cavin monomer and trimer structure (See figure 10 for more details). C. Interaction between caveolins and cavins triggers the assembly of the budded caveolae (Lamaze et al., 2017).

Cav1 is a 21 kDa protein that has distinct domains, in the N-terminal region (residues 1-101) there is the signature motif (SM), an oligomerization domain (OM), a pin motif (PM) and a scaffolding domain (SD), in the central region (residues 102-134) there is the intramembrane domain (IMD), and in the C-terminal region (residues 135-178) there are three palmitoylation sites. Together, these regions control Cav1 trafficking and are overall key elements in the biogenesis and functions of caveolae (Machleidt et al., 2000; Ohi & Kenworthy, 2022; Porta et al., 2022). Recent cryo–electron microscopy data revealed that the Cav1 complex is preferably composed of 11 protomers.
forming a flat disk stabilized by numerous interactions between the protomers that are embedded in the plasma membrane. Furthermore, structural analysis revealed that a single caveola is composed of 12 faces, each formed by one Cav1 8S disc complex, meaning that in total, a single caveolae contains roughly 132 copies of Cav1 protomers (Porta et al., 2022) (Figure 9). Importantly, phosphorylation and ubiquitination sites can be found on the cytoplasmic side of Cav1 (Hayer et al., 2010a; Schlegel et al., 2001). For the formation of caveolae, first, it is necessary for Cav1 to oligomerize into what are called 8S complexes; this first step occurs at endoplasmic reticulum exit sites shortly after synthesis (Hayer et al., 2010b). The formation of the 8S complexes is dependent on the oligomerization domain, scaffolding domain and signature motif (Hayer et al., 2010b; Porta et al., 2022). Later, Cav1 is transported from the Golgi apparatus to the plasma membrane in caveolin-enriched vesicles, where different species of lipids, including cholesterol play a crucial role in the oligomerization of Cav1 (Hayer et al., 2010b; Pol et al., 2005). At the plasma membrane, Cav1 is known to recruit specific species of lipids such as sphingolipids and phospholipids, therefore, generating particular lipid nanodomains within the plasma membrane (Krishna & Sengupta, 2019; Prakash et al., 2021; Sonnino & Prinetti, 2009).
Figure 9. Cav1 structure
A. Top view and B. side view of the 11-unit oligomer of Cav1 that forms the 8S complex. C. Top view and D. side view of the Cav1 monomer with its structural features, including the signature motif (SM), oligomerization domain (OM), pin motif (PM), scaffolding domain (SD), and intramembrane domain (IMD) (Modified from Porta et al., 2022).

Once Cav1 oligomers reach the plasma membrane, cavins are recruited to form an outer filamentous coat that assists in the transition from a Cav1 oligomer flat disk to a curved structure characteristic of caveolae (Hill et al., 2008; Ludwig et al., 2016). All four cavin proteins share sequence homology and possess conserved secondary helical structure elements (HR1 and HR2), which have been found to be important in the recruitment of phosphatidylserine and PIP2 and subsequent caveolae formation (Burgener et al., 1990; Hill et al., 2008; Kovtun et al., 2014). Similarly, to Cav1, cavins form oligomers composed of 3 cavin subunits that will further polymerize to form the caveolar coat (Gambin et al., 2014) (Figure 10).
Figure 10. Cavins protein family structure
Schematic representation of cavins structure and complex formation. A-B. Cavins can form a trimeric homo-oligomer complex of cavin1 or hetero-oligomers complexes where two Cavin1 subunits can bind either a single Cavin2 or Cavin3 subunit. Cavin2 and Cavin3 cannot interact with each other. C. Cavin oligomeric particles can assemble; D. and these can associate with PS and PIP-containing membranes. E. Cavins are only stably associated with caveolae in conjunction with caveolin (Modified from Kovtun et al., 2014).

In conjunction with these two primary components of caveolae, there exists other accessory proteins working synergistically, including the GTPase dynamin2 which associates with caveolae to stabilize it at the plasma membrane, the caveolar “neck” protein EHD2 whose role is mainly attributed to the stabilization of caveolae through their interaction with the actin cytoskeleton, pacsin2/syndapin II, and pacsin3/syndapin III (Lamaze et al.,
Caveolae have been shown to have distinct and multifunctional roles in various cellular processes including endocytosis, cholesterol transport, signal transduction, and mechanosensing, among others (Cheng & Nichols, 2016). Additionally, they have been shown to play roles in the pathogenesis of several diseases, such as cancer, heart disease, and neurological disorders. These roles will be reviewed in the following paragraphs.

1.2.2 Caveolae and lipid regulation

Caveolae play a pivotal role in regulating lipid homeostasis. These distinct regions of the plasma membrane are rich in cholesterol and sphingolipids and compartmentalize cellular processes by serving as organizing centers for the assembly of signaling molecules (Thomas & Smart, 2008).

Cav1, 2, and 3 have a binding domain for cholesterol, suggesting an intricate involvement in cholesterol dynamics. As already mentioned, the association of caveolin proteins with cholesterol is critical for the formation and stability of caveolae structures (Pol et al., 2005). Moreover, Cav1 is an important regulator of cholesterol levels within various subcellular compartments. For example, in fibroblasts taken from mice lacking Cav1, the build-up of cholesterol within mitochondria is linked to impaired functioning of this organelle (Graf et al., 1999). In humans, a stop codon mutation in Cav1, that
leads to the absence of Cav1 expression and no caveolae, leads to severe depletion of adipose tissue accompanied with insulin resistance and dyslipidemia (Kim et al., 2008).

Caveolae and caveolin proteins are also directly involved in lipid transport and storage. For example, Cav1 has been shown to bind fatty acids and is suggested to be part of the machinery for intracellular transport of lipids, especially long-chain fatty acids (Pohl et al., 2002; Trigatti et al., 1999). The expression of Cav1 in human embryonic kidney (HEK) cells was found to aid in the absorption of fatty acids by cells, with cholesterol playing a role as well (Meshulam et al., 2006). In adipocytes, where caveolae are abundantly found, they are essential sites for the regulation of lipolysis, a process whereby triglycerides stored inside fat cells are broken down to produce glycerol and free fatty acids (Cohen et al., 2004). In adipocytes Cav1 is required to maintain the cholesterol content of lipid droplets, conversely, cholesterol induces the translocation of caveolins to lipid droplets where they bind together. Moreover, adipocytes from Cav1 knockout mice show decreased levels of free cholesterol, modified lipid and protein composition in lipid droplets (Blouin et al., 2010; Le Lay et al., 2006). Cavin1 deficiency has also been related to lipid-related defects. Cavin1 depleted mice show increased levels of insulin and triglycerides in their bloodstream, have significantly reduced amounts of fat tissue, along with a lower tolerance for glucose, therefore displaying a clear lipodystrophic phenotype (Liu et al., 2008).
Recent evidence has revealed the nanoscale lipid profile of caveolae. Using de novo assembly of caveolae on a caveolae deficient cellular model it was observed that Cav1 alone is able to cluster phosphatidylserine, PIP₃, and cholesterol, cavin1 alone recruits mostly PIP₂, PIP₃, and phosphatidic acid, revealing that Cav1 and cavin1 have the ability to cluster different lipids at the plasma membrane. Finally, the expression of both proteins led to a unique lipid profile composed mainly of PIP₂, phosphatidylserine and cholesterol (Zhou et al., 2021) (Figure 11).

**Figure 11. Lipid profile of caveolae**  
A. Schematic representation of the lipid species enriched in caveolae. Caveolae are highly enriched in cholesterol among other species, including sphingolipids and
gangliosides (Sonnino & Prinetti, 2009). B. Schematic comparison of the lipid profiles associated with Cav1 (left), cavin1 (middle), or caveolae (right). Colors of square boxes indicate lipid species; the size of the box indicates the level of co-clustering with the indicated proteins (faded boxes indicate statistically insignificant association) (Zhou et al., 2021).

Given the crucial roles of caveolae in regulating lipid metabolism and trafficking, alterations in caveolae structure or function can have profound effects on cellular lipid homeostasis and contribute thereby to several pathophysiological conditions like lipid disorders, cardiovascular disease, and cancer.

1.2.3 Caveolae in cell trafficking

Initially, it was believed that caveolae functioned as carriers for endocytosis. This assumption was based on their prominent presence in the plasma membrane and their morphological resemblance to clathrin-coated pits. Some cargoes that have been characterized to be endocytosed by caveolae include simian virus 40, Shiga and cholera toxins and albumin. These cargoes however can be endocytosed by alternative means (Ghitescu et al., 1986; Norkin & Kuksin, 2005; Richards et al., 2002; Schubert et al., 2001). Once caveolae undergo endocytosis, they can be observed colocalized with early endosomes and other endosomal structures (Shvets et al., 2015). Recently, researchers in the field have come to a consensus that the primary function of caveolae is not related to endocytosis (Parton et al., 2020).

Interestingly, increasing evidence related EV uptake to Cav1-mediated endocytosis. Exosomes derived from Epstein-Barr virus infected cells were
found to be internalized by epithelial acceptor cells in a Cav1 dependent pathway, that lead to the colocalization of the EVs with Rab5 and Rab7 positive endosomes (Nanbo et al., 2013). Pharmacological inhibition of Cav1 with nystatin or filipin sterol binding agents, has been shown to lead to a reduction in EV uptake in lung cancer and pancreatic cells (Javeed et al., 2015; Wei et al., 2017). Contrary to this, there is also evidence showing that Cav1 is a negative regulator of exosome uptake, where knockout of Cav1 in fibroblasts induces an increased uptake of EVs (Svensson et al., 2013). It appears that the role of Cav1 in the uptake of EVs is still under debate. Further research is needed to fully understand the mechanisms and factors that determine the involvement of Cav1 in EV uptake.

1.2.4 Caveolae as mechanosensing and mechanotransducing domains

Cells have to face different stresses in both normal and abnormal conditions, such as changes in volume, stretching, compression or shear stresses, which have the potential to increase tension at the plasma membrane. A lipid bilayer is incapable of supporting major increases in membrane area, however, considerable stretching in cell length or significant swelling are frequently observed and reported without compromise to cell viability. Therefore, a rapid underlying mechanosensitive mechanism regulating membrane tension must be in place.

The first suggestions that caveolae could be mechanosensitive were postulated in 1975, where caveolae from frog skeletal muscle fibers were
observed to “open” after stretching, an effect also documented in stretched smooth muscle from the sea slug *Aplysia californica*, this suggested that caveolae could serve as "safety valves" that would open and flatten to protect the plasma membrane from rupturing (Dulhunty & Franzini-Armstrong, 1975; Prescott & Brightman, 1976). Nevertheless, this potential role of caveolae was not pursued at the time given that further research generated contradictory results, showing that caveolae were rather semi-permanent structures in the plasma membrane, with no differences in quantity between mechanical stress and resting conditions (Gabella & Blundell, 1978; Poulos et al., 1986).

It was not until 2011, where it was established for the first time the role of caveolae as mechanosensors that flatten upon increase of PM tension, and thereby buffer membrane tension increase. This rapid flattening of caveolae happens in an ATP and actin-independent manner. Furthermore, the interaction between Cav1 and cavin is lost after mechanical stress. On the other hand, when stress is relieved, caveolae undergo a complete reassembly process that is dependent on actin and ATP (Sinha et al., 2011) (Figure 12).
Figure 1.2. Caveolae flattening after exposure to mechanical stress
Schematic representation of the process of flattening of caveolae after mechanical stress. At steady state, Cav1 and cavin together form budded caveolae. Upon tension enhancing cues such as swelling or stretching of the plasma membrane, budded caveolae immediately flatten out (Sinha et al., 2011).

The mechano-protective role of caveolae has subsequently been observed in vivo. Loss of cavin-1 in zebrafish revealed a severe disorganization of the cellular membrane that protects the skeletal muscle, notochord lesions and severe locomotor defects. Furthermore, strenuous muscle activity led to sarcolemmal damage and notochord cell collapse in the absence of caveolae (Garcia et al., 2017; Lim et al., 2017; Lo et al., 2015).

Recent evidence has proven that indeed this flattening of caveolae can regulate intracellular signaling through its components (Torrino et al., 2018) (Figure 13). Myotubes derived from patients with mutations in the CAV3 gene demonstrate a notable reduction in the quantity of caveolae at the plasma membrane. As a consequence, these mutant myotubes exhibit an abnormal
reaction to mechanical stress, as they are unable to effectively mitigate the heightened membrane tension caused by such stress. This deficiency subsequently triggers persistent over activation of the IL6/STAT3 signaling pathway. Reconstituting functional caveolae through the reexpression of Cav3 reversed these defects (Dewulf et al., 2019). Upon caveolae flattening, Cav3 can also interact with BRCA1, a protein that help repair damaged DNA, regulating its expression levels, subcellular location, and function, there playing a key role in the cellular protection against stress induced DNA damage (McMahon et al., 2021).
Figure 13. Consequences of caveolae mechanical response on signaling
Schematic representation of possible signaling events triggered by mechanical stress-dependent caveolae flattening. At steady state (left), Cav1 and cavin1 are at the plasma membrane in the caveolar compartment, after mechanical stress (right), Cav1 is released from the caveolae and free cavin1 can translocate to the nucleus to promote gene transcription (Nassoy & Lamaze, 2012).

On the other hand, Cav1 has been shown to interact with various signaling molecules, such as the endothelial nitric oxide synthase (eNOS), EGF, and TFG-β receptors among others (Bucci et al., 2000; Engelman et al., 1998; Strippoli et al., 2015). In a study in mice, the effect of blood flow-induced shear stress was evaluated, when comparing WT with Cav1−/− mice. It was
found that the absence of Cav1 lead to an impaired shear stress regulation of vessel diameter both in vivo and in vitro. Furthermore, they found that Cav1 is necessary for flow-induced eNOS activation. However, reconstitution of Cav1 in the endothelium of the Cav1−/− mice rescued this impairment (Yu et al., 2006).

In smooth muscle cells, it was shown that mechanical strain induced the colocalization of PI3-K with Cav1 at focal adhesion sites. In addition, the presence of Cav1 is necessary for integrin-mediated PI3-K/Akt activation during cyclic stretch of cells in vitro and in a model of elevated tensile force in vivo. On the contrary, this activation of Akt was not present in Cav1 knockout mice (Sedding et al., 2005).

The disassembly of caveolae has been shown to play a critical role in mechanoprotection, as evidenced by the abnormal response to mechanical stress observed in cells with absent or reduced caveolae quantity. Understanding the mechanosensing and mechanotransducing roles of caveolae and Cav1 may have implications for developing therapies targeting disorders and conditions associated with altered response to mechanical stress.

1.2.5 Caveolae in disease

Caveolae related diseases are inherited diseases that arise from mutations in the caveolin genes, primarily CAV1 and CAV3. Gene mutations or mislocalization or deficiency of the encoded proteins, lead to a broad range of
disorders affecting multiple organs and systems including lipodystrophy, musculopathies and cancer (Yin et al., 2016).

Lipodystrophy is a rare and heterogeneous genetic disorder that is characterized by a loss of adipose tissue or its abnormal accumulation. Several clinical studies have identified lipodystrophy-associated mutations in the CAV1 gene. For example, a mutation in patients that results in a heterozygous frameshift mutation in CAV1 has been associated with partial atypical lipodystrophy and hypertriglyceridemia (Cao et al., 2008). A stop codon mutation in CAV1 in human patients leads to depletion of adipose tissue, hypercholesterolemia, insulin resistance, dyslipidemia, and hypercalcemia (Kim et al., 2008). Similarly, loss of Cavin1 in mice induces elevated levels of triglycerides in the bloodstream, a significant decrease in adipose tissue mass, impaired glucose tolerance, and high levels of insulin in the blood (Liu et al., 2008).

Several studies have reported an association between caveolar dysfunction and musculopathies (Gazzerro et al., 2010). A loss of Cav3 in mice induces an exclusion of the dystrophin-glycoprotein complex, a critical regulator of muscle function, from lipid raft domains, and the development of T-tubules mainly in a longitudinal orientation, which is typical of immature muscle cells (Galbiati et al., 2000). The numerous Cav3 mutations lead to the expression of non-functional Cav3 protein, unable to oligomerize and form caveolae in striated muscle cells. Thus, patients bearing these mutations suffer from a broad spectrum of muscle dystrophies including limb-girdle muscular
dystrophy (LGMD), hyperckemia (HCK) and rippling muscle disease (RMD) (Minetti et al., 1998). On the other hand, overexpression of Cav3 was shown to induce a phenotype similar to Duchenne muscular dystrophy (DMD). It induces a significant increase in the abundance of caveolae in skeletal muscle, hypertrophic, immature, and necrotic muscle fibers, and a decrease in the expression of skeletal muscle β-dystroglycan, an important protein involved in muscle stability (Pradhan & Prószyński, 2020).

The role of caveolae in cancer has remained a controversial topic for decades, with the function of Cav1 being the most studied to date in this complex pathology. Cav1 has been described both as a tumor suppressor gene as well as an oncogene. This contradictory role has been suggested to be dependent on the type and stage of cancer (Williams & Lisanti, 2005) (Figure 14).

**Figure 14. The dual role of caveolae in cancer**
In breast cancer, Cav1 has been described with a dual role. On one hand, Cav1 acts as a tumor suppressor by inhibiting breast cancer cell proliferation, autophagy, invasion, and migration while promoting apoptosis (Left). On the other hand, Cav1 can also act as a tumor promoter in breast cancer by stimulating breast cancer cell
proliferation, autophagy, invasion, migration, and metastasis, while inhibiting apoptosis and anoikis (Right) (Qian et al., 2019).

Several studies have demonstrated the tumor suppressor role of Cav1. The genes encoding for Cav1 and Cav2 are localized in a locus that is normally deleted in various human cancers, including but not restricted to squamous cell carcinomas of the head and neck, prostate cancers, renal cell carcinomas, ovarian adenocarcinomas, colon carcinomas, and breast cancers, suggesting that the genes contained in this area, including the caveolins isoforms are important for tumor suppression (Engelman et al., 1998). The presence of a specific mutation in the transmembrane domain of Cav1, where proline 132 is changed to leucine (P132L), has adverse consequences on the formation of caveolae within the body (Han et al., 2023). This mutation has been associated with various disease conditions, with a particular emphasis on aggressive forms of breast cancer, where 9-16% of patient cases present this mutation (Bonuccelli et al., 2009; Shatz et al., 2010). In fibroblasts, the downregulation of Cav1 displays unrestricted growth. They have the ability to develop tumors in mice with weakened immune systems, and they exhibit excessive activation of the Ras-p42/44 mitogen-activated protein (MAP) kinase cascade (Galbiati et al., 1998). A recent study has discovered a new mechanism in which Cav1 plays a role in inhibiting tumor progression. This mechanism involves the suppression of the unfolded protein response (UPR) signaling pathway, a key pathway needed to adapt and respond to endoplasmic reticulum stress conditions, promoting cell survival. The study found that Cav1’s ability to suppress the UPR signaling is dependent on the presence of serine-80, an amino acid that is involved in
Cav1 secretion and its localization to the endoplasmic reticulum (Díaz et al., 2020). Alternatively, the presence of Cav1 could lead to an enhanced cell migration and invasion phenotype. In fact, an earlier study from the same group demonstrated that increased expression of Cav1 is associated with increased migration, invasion and anchorage-independent growth, therefore enhanced malignancy in a model of endometrial cancer cells (Diaz-Valdivia et al., 2015). Accordingly, multiple reports have characterized the pro-metastatic effect of Cav1 in different models of cancer. In metastatic cancer cells, Cav1 stimulates the activation of Rab5, leading to increased Rac1 activity, which ultimately promotes enhanced migration and invasion of tumor cells (Díaz et al., 2014). The tyrosine (Tyr14) phosphorylation of Cav1 has been found to be important for the stimulation of Rho activation which leads to enhanced invasion and migration in various breast cancer cells lines (Joshi et al., 2008). Furthermore, tyr14 was identified as a substrate for Src kinase, a known oncoprotein associated with enhanced tumor cell migration, invasion, survival and focal adhesion signalling (Wong et al., 2020). In Hs578T cells, Cav1 overexpression induces an increased formation of colonies in soft agar and a decreased apoptosis (Wu et al., 2007). Similarly, MCF7 cells that have been transfected with human CAV1 plasmid show improved cell survival by the suppression of anoikis, a programmed cell death that happens when the connection between a cell and the ECM and neighboring cells, which is facilitated by integrins, is disturbed (Ravid et al., 2005).

Finally, enhanced expression of Cav1 has been reported in metastatic cancers. Cav1 upregulates the expression of the fucosyltransferase pofut1,
resulting in the activation of the Notch pathway, leading to the generation of a more aggressive glycosylation phenotype in hepatocellular carcinoma, which promotes invasion and metastasis (Zhang et al., 2019). When MDA-MB-231 cells are submitted to a fluid-induced low shear stress environment, similar to the shear stress metastatic cells have to undergo while migrating in the circulatory system, the expression of Cav1 mRNA and protein is increased. This enhanced expression of Cav1 provides these cells with resistance to anoikis. The mechanism by which Cav1 impairs anoikis resistance is through the inactivation of caspase-8, a key enzyme involved in programmed cell death (Chen et al., 2022).

The specific molecular mechanisms underlying this contradictory reported behavior are still not fully understood and require further investigation. To date, the consensus is that the role of Cav1 in cancer may depend on the stage of the cancer, with clear differences between early and late phenotypes (Campos et al., 2019). The specific context of each cancer type is another important aspect to consider as well as the intricate interactions between caveolin-1 and other proteins in order to fully comprehend its dual role in the progression of such a complex pathology as cancer.

**Chapter 3: Mechanics of cancer progression**

One important aspect of cancer research is understanding the mechanics of cancer. These particular terms refer to the physical properties and behaviors of cancer cells and their surrounding environment that facilitate cancer
growth, invasion, and metastasis. The research in this field has revealed how alterations in cellular mechanics, such as changes in cell-cell interactions, adhesion to extracellular matrix, and mechanical properties, contribute to the development and progression of cancer.

1.3.1 ECM remodeling

Cancer cells reside within a complex ecosystem known as the tumor microenvironment (TME), comprising various components such as neighboring cells, blood vessels, and ECM (Northcott et al., 2018). The ECM is often altered in cancer, with increased stiffness or cross-linking that can provide rigidity and anchorage for invading cancer cells (Paszek et al., 2005; Yu et al., 2011).

ECM remodeling involves two main processes: degradation and synthesis. Cancer cells can secrete enzymes called MMPs, which include collagenases, gelatinases, stromelysins, among others, that degrade ECM components, such as collagen and fibronectin (Table 1). Their activity is tightly regulated in healthy tissues but becomes dysregulated in cancer, leading to excessive ECM degradation (Niland et al., 2022). These enzymes can break down the ECM barriers, allowing cancer cells to invade surrounding tissues and migrate to distant sites. The increased production and activity of MMPs by cancer cells contribute to the invasive and metastatic potential of tumors (Westermarck & Kähäri, 1999). The dysregulated expression of MMPs in cancer is influenced by various factors. Several signaling pathways, such as
the TGF-β pathway and the vascular endothelial growth factor (VEGF) pathway, can upregulate the production of MMPs in cancer cells leading to increased metastatic phenotypes (Gomes et al., 2012; Hiratsuka et al., 2002) (Figure 15).

**Table 1. Classification and function of matrix metalloproteinases (MMPs)** (Grobulewska et al., 2012).

<table>
<thead>
<tr>
<th>Classification</th>
<th>Metalloproteinase</th>
<th>Substrates</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Matrilysins</strong></td>
<td>MMP-7 (matrilisin, metalloendopeptidase)</td>
<td>Collagen type IV, glycoprotein, gelatine</td>
</tr>
<tr>
<td><strong>Collagenases</strong></td>
<td>MMP-1 (interstitial collagenase; collagenase 1)</td>
<td>Collagen type I, II, III, V, VII, VIII, X, gelatine, HL-1/P, MMP-2, MMP-9, fibronectin</td>
</tr>
<tr>
<td></td>
<td>MMP-2, MMP-9, fibronectin</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MMP-8 (neutrophil collagenase; collagenase 2)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MMP-13 (collagenase 3)</td>
<td></td>
</tr>
<tr>
<td><strong>Stromelysins</strong></td>
<td>MMP-3 (stromelysin 1)</td>
<td>Proteoglycans, fibronectin, laminin, elastin, gelatine, vitronectin, plasminogen, fibrinogen, fibrin, collagen type III, IV, V, utihrombin III, MMP-1, MMP-2, MMP-8, MMP-9, MMP-13</td>
</tr>
<tr>
<td></td>
<td>MMP-10 (stromelysin 2)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MMP-11 (stromelysin 3)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MMP-18 (collagenase 4)</td>
<td></td>
</tr>
<tr>
<td><strong>Gelatinases</strong></td>
<td>MMP-2 (gelatinase A; 72 KDa metalloproteinase)</td>
<td>Collagen type I, IV, V, VII, X, gelatine, elastin, laminin</td>
</tr>
<tr>
<td></td>
<td>MMP-9 (gelatinase B; 92 KDa metalloproteinase)</td>
<td></td>
</tr>
<tr>
<td><strong>Membrane-type MMPs</strong></td>
<td>MMP-14 (MT1-MMP)</td>
<td>Collagen type I, II, III, gelatine, elastin, laminin, fibronectin, fibrin, proMMP-2, 13</td>
</tr>
<tr>
<td></td>
<td>MMP-15 (MT2-MMP)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MMP-16 (MT3-MMP)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MMP-24 (MT5-MMP)</td>
<td></td>
</tr>
<tr>
<td><strong>(B) GPI-anchored MMPs</strong></td>
<td>MMP-17 (MT4-MMP)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MMP-25 (MT6-MMP)</td>
<td></td>
</tr>
<tr>
<td><strong>Others MMPs</strong></td>
<td>MMP-11 (stromelysin)</td>
<td>Acmelagenine, agrecanes, elastine</td>
</tr>
<tr>
<td></td>
<td>MMP-12 (macrophage metalloelastase)</td>
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<tr>
<td></td>
<td>MMP-19</td>
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<td></td>
<td>MMP-20 (enamelysin)</td>
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<td></td>
<td>MMP-23</td>
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<td></td>
<td>MMP-27</td>
<td></td>
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<tr>
<td></td>
<td>MMP-28 (epilysin)</td>
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In addition to degradation, cancer cells can also actively synthesize ECM components. They can produce and deposit molecules, such as fibronectin, collagen, and proteoglycans, which can influence the mechanical properties of the ECM (Huibbers et al., 2010; Kaplan et al., 2005). These newly synthesized
ECM molecules can further modify the structure and composition of the ECM, again, promoting tumor growth and metastasis. Indeed, the presence of a higher concentration of proteoglycans in the ECM has been associated with a more aggressive tumor phenotype and an increased stiffness of the TME (Varga et al., 2012). Fibronectin is a glycoprotein that plays a role in cell adhesion, migration, and tissue organization. Cancer cells can upregulate the production of fibronectin in surrounding CAFs, leading to the deposition of fibronectin-rich ECM around tumors. This deposition can facilitate cell adhesion, support angiogenesis, and promote interactions between cancer cells and the ECM that drive tumor growth and metastasis (Wang & Hielscher, 2017). Collagen fibers form a scaffold-like structure that gives strength and stability to tissues. In cancer, the remodeling of collagen fibers is essential for tumor invasion and metastasis (Lu et al., 2012) (Figure 15).

**Figure 15. Mechanisms of ECM remodeling**

A. Secreted factors activate stromal cells, which differentiate into CAFs leading to the secretion and deposition of ECM components along with the secretion of ECM components from cancer cells. B. Cross-linking and alignment of collagen fibers
increases matrix stiffness around the tumor. Increased matrix stiffness promotes the interaction between ECM components and cell-surface receptors on tumor cells that triggers mechanosignaling. C. CAFs and tumor cells secrete ECM-degrading proteases, including MMPs. ECM degradation generates bioactive molecules that induce pro-tumorigenic ECM signaling that promotes tumor proliferation, migration, invasion and angiogenesis (Modified from Winkler et al., 2020).

1.3.2 Metastasis

As tumors progress and metastasize, the changes that occur are not limited to biochemical signaling or genetic expression. The physical environment undergoes significant alterations at each stage of tumor progression; hyperplasia, invasion, dissemination and colonization at secondary sites (Gensbittel et al., 2021; Northcott et al., 2018) (Figure 16).

Increase of cell contractility, uncontrolled growth of cells, and abnormal ECM results in an increase of stiffness in in situ carcinomas (ISC) that has already been observed in various tumors, including breast, pancreatic, and colorectal cancers (Brauchle et al., 2018; Butcher et al., 2009; Levental et al., 2009). Cancer cells thrive in these conditions in different ways, for example, through the assembly of filopodia, lamellipodia and invadosomes that enhance migration and invasion (Wolf et al., 2013). Stiff substrates have the ability to trigger a mesenchymal/basal-like invasive phenotype in tumor cells through increased contractile force mediated by integrins and the formation of mature focal adhesions (Mekhdjian et al., 2017).

It is important to acknowledge that many mechanotransduction pathways are regulated by oncogenes or tumor suppressor genes (Broders-Bondon et al.,
2018). Therefore, the profound changes that cells undergo in these modified mechanical environments can induce cellular transformations comparable to genetic mutations (Yu et al., 2011). Disturbances in this system can impact many cellular processes like cell differentiation, cell viability, proliferation, adhesion, migration, among others, all of which contribute to the promotion of cellular invasion.

**Figure 16. Mechanics of cancer**
Schematic representation of the mechanical stimuli on tumor cells throughout cancer progression. **A.** Normal tissue environment presenting balanced forces between cells and ECM. **B.** The tumor microenvironment consists of cancer cells exhibiting increased tension, enveloped by an ECM that becomes progressively stiffer, and
various stromal cell types such as fibroblasts, immune cells, and vascular cell types. 

**C.** The growth of the tumor within the surrounding stromal tissue leads to compression of both the tumor and the neighboring tissue. The increased stiffness of the ECM intensifies the resistance of the stromal tissue to compression and further amplifies the solid stress. **D.** A gradient of pressure within the surrounding tissue leads to the movement of fluid from the central region of the tumor to its outer edges, facilitating the spread of cancer cells to distant locations (metastasis). After escaping from the primary tumor, cancer cells migrate, guiding their movement towards blood vessels. During this process, tumor cells experience significant shear stresses as they pass between endothelial cells during intravasation and extravasation, as well as while traveling through the bloodstream to potential secondary tumor sites (Northcott et al., 2018).

When ISC becomes invasive and cancerous cells start migrating, moving through the circulatory system, and infiltrating other tissues and organs, they will experience other types of mechanical strains, including solid forces and fluid forces that generate shear stress. It can be shear stress generated by the blood flow along the surface of cells, known as hemodynamic shear stress, but also the stress induced by solid forces encountered when tumor cells come into contact with endothelial cells during the process of intravasation and extravasation from the blood vessels (Wirtz et al., 2011). Hemodynamic flow forces can favor the arrest, adhesion, and extravasation of circulating tumor cells (CTCs) *in vivo*. In a model of zebrafish, blood flow-induced endothelial cell remodeling is a crucial mechanism facilitating the extravasation of CTCs to promote metastasis (Follain et al., 2018).

Interestingly, tumor cells are more resistant to shear stress than normal cells (Mitchell et al., 2015). Normal levels of shear stress in the body, ranging from approximately 5 to 30 dynes/cm² (0.5-3 Pa), have been found to stimulate the movement and attachment of tumor cells (Ma et al., 2017; Xiong et al., 2017). Nevertheless, shear stress levels similar to those experienced during
exercise, approximately 60 dynes/cm² (6 Pa), have been shown to induce cell death in tumor cells (Regmi et al., 2017).

The activation of mechano-signaling pathways can promote tumor progression by enhancing cancer cell survival. Low shear stress can induce the activation of mechanical signaling pathways that promote tumor growth and migration. Studies have demonstrated that low shear stress conditions activate multiple signaling pathways in a Cav1-dependent manner, including AKT/Src and Rho-associated protein kinase (ROCK)/p-Myosin Light Chain (MLC) pathways, leading to enhanced cell motility and adhesion (Xiong et al., 2017). Furthermore, shear stress was found able to induce an epithelial-mesenchymal transition in circulating tumor cells by activating Jun N-terminal Kinase (JNK) signaling, therefore increasing their survival rate during hematogenous dissemination (Xin et al., 2020).

Therefore, for a tumor cell to survive and thrive during its journey from the primary ISC to a secondary site, it must constantly adjust and adapt to the different mechanical stresses it encounters.

### 1.3.3 EV dynamics

The ECM has been demonstrated to impact the secretion of EVs from surrounding cells through its stiffness and mechanical properties. Several studies have highlighted the significance of ECM-induced mechanical force on EV biogenesis, with evidence suggesting that these mechanical forces can
influence regulatory pathways (Liu et al., 2022; Patwardhan et al., 2021; Schwager et al., 2019; Xiao et al., 2022).

For example, it was discovered that a stiff ECM induced the release of a specific population of EVs with particular miRNAs. These EVs were found capable of regulating prostate cancer cell motility and migration, a finding that implies that mechanical force can modulate cancer metastasis (Liu et al., 2022). Moreover, in a model of breast cancer it was shown that stiff ECMs promote exosome secretion in a YAP/TAZ pathway-dependent manner. These exosomes are able to induce alterations in cell shape, attachment, and protrusion kinetics that can enhance cellular motility and invasiveness (Patwardhan et al., 2021).

Another type of mechanical stress that has been well established as a regulator of EV dynamics is shear stress. This type of mechanical stress has been found to be crucial for platelets to release microvesicles where integrins serve as mechanical sensors and downstream signal transducers (Pang et al., 2018). Endothelial cell-derived EVs have been shown to present a very distinct miRNA cargo in shear stressed cells. Interestingly, these EVs are able to modulate the function of endothelial cells, including their angiogenesis, migration, and inflammatory response (Chung et al., 2022). Shear stress can also affect other aspects of EV dynamics, endothelial cells were subjected to low or high shear stress and their ability to uptake EVs was analyzed. Interestingly, low and oscillating shear stress promoted the uptake of EVs in vitro and in vivo, respectively (Qin et al., 2022). Shear as well as cyclic stretch
has been shown to induce an increased release of EVs in different cell models. The amount of EVs released after these types of mechanical stress have been described to range from 10 to 100 times the amount of EVs released in resting conditions (Guo et al., 2021), this is of particular interest, as it reveals that it may not only be the nature of the EVs that is affected upon mechanical stress, but also the amount of EVs secreted, suggesting a potential combined effect exerted on EV receiving cells.

Cyclic stretch is another type of mechanical strain that cells have to endure in normal conditions, in tissues that are cyclically stretched due to breathing, heart beating or peristalsis of the gut. It has been described that cyclic stretch is able to induce the release of EVs with the ability to suppress IL-1β production and pyroptosis, an inflammatory cell death, through the blockade of NF-κB signaling in human monocytes and macrophages (Wang et al., 2019).

When cells are stretched due to changes in osmotic pressure or when mice are experiencing increased pressure in their hearts, it triggers the release of exosomes that are rich in the angiotensin II type 1 receptor. These exosomes are then released from the heart and can affect how blood vessels respond to neurohormonal stimulation (Pironti et al., 2015).
2. HYPOTHESIS AND OBJECTIVES

So far, we have discussed topics related to EVs, caveolae, cancer, and the role of cell mechanics in these processes. We have explored the different mechanisms by which EVs are secreted and their diverse functions in carrying various molecular cargoes such as nucleic acids, proteins, and lipids, and delivering them to their target cells. One of the key conclusions is that EVs play a crucial role in intercellular communication and cell-to-cell signaling across different physiological contexts.

Additionally, we have also discussed the importance of caveolae in cellular and tissue homeostasis, and in disease development, including cancer, where we explored the contradictory role of Cav1 in cancer progression. Moreover, we have examined the mechanical properties of caveolae in mechano-protection and signaling.

Finally, we delved into the field of cell mechanics, including mechanics of cancer and EV dynamics. Even though there is an increasing interest in the topics listed above, there are still enormous gaps in knowledge in certain areas. Understanding the role of mechanical stress as a regulator of these processes is critical for identifying new therapeutic targets and developing interventions for cancer. Therefore, the overall aim of my thesis was to investigate the potential role of caveolae in EV biology in a context of cancer and enhanced mechanical strains.
For this I have pursued three main objectives:

1) To investigate the effect of different types of mechanical stress on EV biology, including secretion, uptake, composition, and physiological relevance from a cancer perspective,

2) To explore the role of caveolae and its components in the mechanically-induced changes in EV biology,

3) To shed light into the molecular mechanism by which caveolae may regulate these effects.
3. RESULTS
Caveolin-1 and mechanical stress control the release of a pro-metastatic subpopulation of small extracellular vesicles

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Abstract

Extracellular vesicles are lipid-enclosed vesicles that are released by most cells and present in all human bodily fluids. EVs contain genetic material and proteins that can be transferred to other cells to generate various effects. Caveolin-1 (Cav1) is a key structural component of the small invaginations of the plasma membrane called caveolae, where it functions as mechano-sensors and membrane tension buffering device. High levels of Cav1 have been found in EVs of patients with advanced cancer. Given the importance of mechanical forces in the microenvironment of cancer cells, we investigated the possible role of caveolae and/or Cav1 in the regulation of EV dynamics and cancer progression under mechanical strain. We found that different types of mechanical stress induced a striking increase in the release of EVs purified from several cancer cell lines. We further characterize these EVs as exosomes that were enriched in Cav1 and found that functional Cav1 was required for the increase if EVs induced by mechanical stress. Lipidomic analysis revealed differences in the composition of EVs isolated from Cav1−/− cells and cells under mechanical stress. Finally, we could show that EVs from mechanically stressed cells were able to promote enhanced migration and invasion of cancer cells in a Cav1-dependent manner. Altogether, these data reveal a new role for Cav1 in cell-to-cell communication and the acquisition of metastatic traits through the mechanical control of EV production and dynamics.
Introduction

Extracellular vesicles (EVs) are small membranous particles present in all bodily fluids and released to the extracellular space by all cell types studied thus far (Théry et al., 2018). EVs have gained significant attention in recent years due to their involvement in various physiological and pathological processes, including cancer (Yáñez-Mó et al., 2015). In the context of cancer, EVs have been implicated in multiple aspects of tumor progression and metastasis, such as promoting cell proliferation, invasion, angiogenesis, and immune evasion. Interestingly, tumor cells have been shown to release higher amounts of EVs compared to healthy cells. Tumor-derived EVs can affect the local tumor microenvironment, travel to distant secondary sites or serve as means of communication between both cancerous and non-cancerous cells, promoting cancer progression (Chen et al., 2018; Peinado et al., 2012; Sung et al., 2015).

Caveolae are specialized, 60-80 nm cup-shaped invaginations of the plasma membrane that are present in most cell types (Lamaze et al., 2017; Palade, 1953). Caveolins together with cavins are required for the structural and functional integrity of caveolae (Kovtun et al., 2015). Mutations or abnormal expression of caveolae components have been associated with various disorders, including cancer (Le Lay & Kurzchalia, 2005). The caveolae protein caveolin-1 (Cav1) is more abundant in tumor-derived EVs and has been proposed as a biomarker for cancer progression (Campos et al., 2019; Lamaze & Torrino, 2015). In 2011, a new function of caveolae was established as mechano-sensors and mechano-transducers that play an essential role in cell mechano-protection (Dewulf et al., 2019; Sinha et al., 2011; Torrino et al., 2018). As tumors progress and metastasize, their physical microenvironment undergoes
significant alterations. During its metastatic journey from a primary site to a secondary site, tumor cells must constantly adapt and respond to the different mechanical stresses they encounter.

In this study, we explored the role of caveolae in EV dynamics in cancer cells subjected to mechanical strains. We observed a drastic increase in the amount of EVs released after mechanical stress. The increase of EV release required Cav1 and the endosomal sorting complex endosomal sorting complex required for transport (ESCRT)-0 proteins, indicating their exosomal origin. This specific subpopulation of Cav1-positive EVs contained a particular lipid signature and was able to induce the acquisition of metastatic traits in receiving cells, including increased migration and invasion in vitro. We have therefore unveiled a novel mechanism by which cancerous cells take advantage of the mechanical properties of caveolae to adapt to the mechanical environment of tumors and to promote the secretion of pro-metastatic EVs.
Results

**Mechanical stress enhances the release of small EVs.**

In order to investigate the effect of mechanical stress on the release of small EVs, we first applied a hypo-osmotic shock, where cells are subjected to a short and acute hypo-osmotic medium that induces the flattening of caveolae (Sinha et al., 2011) (**Figure S1A and S2B**). EVs were purified by ultracentrifugation from WT HeLa cells under resting or osmotic shock conditions. The size distribution measured by nanoparticle tracking analysis (NTA) shows that most secreted EVs are ranging from 50-200 nm (**Figure 1A**). We observed a drastic increase in EV release in cells subjected to hypo-osmotic shock with a 3.5-fold increase in the number of secreted EVs detected by NTA (**Figure 1B**). We used electron microscopy (EM) to assess the purity and integrity of released EVs. EM images revealed the presence of EVs with a characteristic “donut” shape albeit with a smaller diameter (~80 nm mean size) than the values obtained through NTA measurement (~128 nm mean size). The substantial reduction in observed EV size is a known artifact of the preparation method used to visualize EVs in TEM (Wu et al., 2015) (**Figure 1C and 2D**). We confirmed by western blot the presence of *bona fide* EV markers, including CD63 and CD9 (Mathieu et al., 2021). Also, the endoplasmic reticulum (ER) protein, calnexin commonly used as a negative marker was detected in cell extracts but not in EVs as expected. The two main components of caveolae, Cav1 and cavin1 were detected in WT HeLa EVs, with an enrichment of both proteins (~2.5-fold increase) in EVs from HeLa cells subjected to hypo-osmotic shock compared to EVs from resting cells (**Figure 1E**).
Cav1 is required for the enhanced release of small EVs induced by mechanical stress.

Considering the role of caveolae in mechano-sensing, we examined their potential involvement in the increase of EV release induced by mechanical stress. Remarkably, in HeLa cells stably knocked out for Cav1 expression, we no longer observed the increase in the number of secreted EVs induced by hypo-osmotic (Figure 2A). To rule out that this effect was specific of the two-dimensional hypo-osmotic shock model, we tested a three-dimensional osmotically induced mechanical compression in multicellular spheroids grown in an agarose bed with high molecular weight dextran (100 kDa), a high molecular weight sugar that is neutral for mammalian cell metabolism (Figure S1D and S1E) (Dolega et al., 2017). We also found that this different type of mechanical strain caused the disassembly of caveolae without compromising cell viability (Figure S1E-G). Dextran compression of spheroids induced a 2-fold significant increase in the number of secreted EVs detected by NTA as compared to resting spheroids. Similarly to the results observed with hypo-osmotic shock, the absence of Cav1 prevented the increase in secreted EVs (Figure 2B). Cavin1 is required for caveolae morphogenesis and stability at the plasma membrane. In the absence of cavin1, caveolae are not formed and only Cav1 is present at the plasma membrane albeit at a lower expression level (Drab et al., 2001; Hill et al., 2008). In HeLa cells stably knocked out for cavin1, we could still measure an increased number of secreted EVs after mechanical stress, yet the increase was significantly less than in WT HeLa cells (Figure 2A and 2B). Because of the co-transcriptional regulation of cavin1 and Cav1 expression, cavin1 depleted cells are known to express lower amounts of Cav1 (Hill et al., 2008). We therefore re-expressed Cav1 in Cavin1−/− HeLa cells to restore basal levels of Cav1. Under this condition, we could measure
a level of EVs similar to the one induced by mechanical stress in WT HeLa cells (Figure S2D). These data indicate that Cav1 but not caveolae are required for the increase of EV release by mechanical stress.

We could extend this mechanism to other cell types, including Hs578t and MDA-MB-231 triple negative breast cancer cell lines. In these cells, hypo-osmotic shock or dextran compression induced an increase in the number EVs detected through NTA as compared to control cells, and this increase was also dependent on the presence of Cav1 (Figure S2A-C).

Different mechanisms could lead to an increase of EV production, including enhanced secretion of EVs by producing cells or impaired reuptake. We addressed the latter possibility by selectively measuring the release of EVs, using a CD63-pHluorin construct previously described (Verweij et al., 2018). CD63-pHluorin is a pH sensitive plasmid that becomes fluorescent once the pH becomes neutral inside endosomal acidic compartments such as multivesicular bodies (MVB) that contain intraluminal vesicles (ILVs). This construct allows therefore to visualize the fusion of MVBs at the plasma membrane and the release of ILVs as exosomes to the extracellular medium in live cells. In line with the EV secretion results measured by NTA, the exosomal release activity of HeLa cells was increased after osmotic shock (Figure 2C). The increase in fusion activity after mechanical stress was absent in HeLa Cav1−/− cells, confirming that the increased EV detection is due to an increase in EV release from producing cells. Altogether, this data reveals a novel Cav1-dependent mechanism by which hypo-osmotic shock and 3D mechanical compression induce a drastic increase in the release of small EVs i.e. exosomes from cells.

The mechanical release of small EVs requires the endosomal ESCRT-0 complex.
We next explored the mechanism by which the MVB pathway is affected by mechanical stress or Cav1 depletion. First, we analyzed by EM the characteristics of MVBs in Hs578t cell spheroids in resting conditions, and after 5 min, 1 day or 5 days of dextran compression (Figure 3A). While the diameter of MVBs observed after mechanical compression did not significantly change over time (Figure 3B), the overall number of MVB-like structures in compressed Hs578t spheroids was significantly reduced as compared to resting spheroids for all tested compression times (Figure 3C). In parallel, in HeLa cells subjected to osmotic shock, we observed the presence of enlarged CD63-positive endosomal-like structures that were decorated with Cav1 (Figure S1C, inserts).

We next evaluated the protein levels of the components of the ESCRT pathway, one of the key machineries controlling MVB biogenesis and exosome production. Signal transducing adaptor molecule (STAM) binds to hepatocyte growth factor-regulated tyrosine kinase substrate (HRS) to form the ESCRT-0 at the early endosome (Migliano et al., 2022). We found by western blot that HRS and STAM were significantly downregulated to approximately 45% expression in the absence of Cav1. We also found that Lamp1, a lysosomal marker, and LC3b a marker of autophagy, were both upregulated in Cav1 depleted cells, further confirming the alteration in the endosomal pathways (Figure 4A and 4B). Next, we tested the effect of ESCRT-0 downregulation on the mechanical increase of EV release. The treatment of WT HeLa cells with siRNA targeting HRS resulted in a strong downregulation of HRS (>90%) and a milder downregulation of STAM (~50%) (Figure 4C). We next purified EVs from HRS downregulated cells subjected to hypo-osmotic shock. We observed a significant reduction in the secreted EVs detection through NTA in HeLa cells treated with siRNA against HRS, confirming the established role of HRS in EV biogenesis (Colombo et
al., 2013, Colombo et al., 2014). Interestingly, we observed that mechanical stress did not increase the number of secreted EVs by HeLa cells downregulated for HRS, indicating that the increased EV release after mechanical stress is also dependent on ESCRT-0, pointing towards a co-regulation of this effect by both Cav1 and ESCRT-0 (Figure 4D).

Small EVs from mechanically stressed cells present a distinct lipid signature.

Caveolae organize lipid nanodomains at the plasma membrane that are enriched in cholesterol and sphingolipids, and have been described to play a central role in regulating lipid homeostasis (Krishna & Sengupta, 2019; Prakash et al., 2021; Sonnino & Prinetti, 2009). We evaluated whether mechanical stress and Cav1 depletion would affect the lipid composition of EVs. For this we performed lipidomic analysis of EVs purified from WT or Cav1−/− HeLa cells under resting conditions or after exposure to mechanical stress. Indeed, mass spectrometry analysis revealed differences in the lipid composition between EVs from WT and Cav1 depleted HeLa cells, as well as differences between EVs from resting or mechanically stressed HeLa cells.

Mechanical stress led to the specific enrichment of lipid species in EVs including phosphatidylcholine, O-phosphatidylcholine, phosphatidylethanolamine, O-phosphatidylethanolamine, and phosphatidylinositol. In contrast, mechanical stress led to a decrease in the cholesterol and ceramide content of EVs. Depletion of Cav1 in cells induced an increase in the presence of lactosylceramide (LacCer), and a decrease in ceramide, and the gangliosides Gb4 and GM2 in EVs (Figure 5A and 5B). These differences in lipid composition of EVs induced by mechanical stress led us to hypothesized that EVs released by mechanical stress could convey distinct specific functions on receiving cells.
Mechanically stressed cells release small EVs that promote enhanced migration and invasion of cancer cells.

We examined the potential impact of EV uptake in cancer biology through cell proliferation and invasion assays. Cell proliferation was investigated using a wound-healing assay (Ilina & Friedl, 2009). We found that EVs from mechanically stressed cells promoted faster wound closure in WT Hs578t cells compared to EVs from resting cells (Figure 6A-C). Importantly, we observed that Cav1 was required, as EVs secreted from Cav1-deleted cells failed to enhance wound closure in both resting and mechanical stress conditions. We also analyzed the invasive potential of these EVs by pre-incubating recipient cells with the EVs for 24 hours and then analyzing their invasiveness using a thin layer of matrigel, which primarily consists of collagen type IV, entactin, heparan sulfate proteoglycan, and laminin. We observed a similar effect, where EVs from mechanically stressed cells enhanced the invasion capabilities of Hs578t cells through the ECM, while EVs from resting cells also increased the invasion, albeit to a lesser extent. The absence of Cav1 in secreting cells completely prevented the enhanced invasion ability conferred by the EVs (Figure 6D).

In summary, these data indicate that EVs secreted by HeLa cells in response to mechanical stress enhanced the migratory and invasion capabilities of Hs578t cells through the ECM in a Cav1 dependent manner, thereby increasing their tumorigenic potential. In contrast, EVs from resting HeLa cells do not confer these properties to Hs578t cells.

Discussion
In this study, we characterized EVs whose release is specifically increased after mechanical stress, and identified Cav1, the primary constituent of caveolae, as a key player in this process. EVs released from mechanically stressed cancer cells displayed distinct lipid composition differences and promoted enhanced *in vitro* migration and invasion. These effects were dependent on the presence of Cav1.

The average size distribution of EVs purified from HeLa WT, Cav1−/−, and cavin1−/− ranged up to ~350 nm, with over 80% comprising vesicles smaller than 200 nm, falling within the typical exosome size range. The exosomal nature of these vesicles was confirmed through monitoring MVB fusion to the plasma membrane using the pHluorin-CD63 construct and by the dependence on a functional ESCRT-0 complex.

In addition to universally accepted EV markers such as CD63 and CD9 (Keerthikumar et al., 2016; Mathieu et al., 2021), we also detected the presence of the two main caveolar proteins, Cav1 and cavin1, in the EV samples from WT HeLa cells. This finding is consistent with prior studies reporting the presence of Cav1 in EVs from cancerous cells (Campos et al., 2018; Logozzi et al., 2009).

The molecular mechanisms responsible for the increase in EV release during mechanical stress remain unclear. We discovered that both Cav1 and the ESCRT-0 complex were essential for the mechanically induced increase in EV release. Previous studies have suggested a potential interaction between Cav1 and the ESCRT machinery. Thus, in HeLa cells, depleting HRS reduced the ubiquitination-dependent targeting of Cav1 to ILVs within MVBs. In this study, it was also proposed that overexpressing Cav1 leads to the accumulation of Cav1 in endosomal structures (Hayer et al., 2010a).

Given the critical role of caveolae in mechano-sensing and mechano-protection, we decided to test our hypothesis in cancer cell lines with either Cav1 expression or
depletion, considering the mechanical challenges that cancer cells encounter during their progression from a growing tumor to metastasis (Northcott et al., 2018). It is known that Cav1 acts as an enhancer of metastatic traits, promoting cell migration, invasion and anoikis resistance (Díaz et al., 2014; Urra et al., 2012; Wang et al., 2018). Yet the exact mechanism by which it exerts these effects is still poorly understood. Recently, the presence of Cav1 in EVs from breast cancer cells was associated with increased migration and invasion of T47-D epithelial and MDA-MB-231 recipient cells (Campos et al., 2018). Considering this, we assessed the metastatic potential of EVs released from mechanically stressed or resting WT and Cav1-depleted cells. We observed increased migration and invasion of Hs578t cells after incubation with EVs from HeLa cells in resting conditions, and this effect was further enhanced when incubated with EVs secreted under mechanical stress. Again, this depended on the presence of Cav1 in donor cells. Since Hs578t cells contain endogenous Cav1, it suggests that the enhanced migration and invasion effects of EVs may not be solely due to the presence of Cav1 within the EVs themselves. Instead, it may be linked to changes in the overall composition of the EV subpopulation induced by mechanical stress, where Cav1 could play a role.

We assessed the potential components of the EVs responsible for mediating these effects, taking into account the established role of Cav1 in lipid clustering at the plasma membrane and lipid nanodomain assembly (Pohl et al., 2002; Pol et al., 2005). Mechanical forces can alter lipid distribution in reconstituted membranes, as seen in giant unilamellar vesicles (GUV), where increased tension triggers lipid phase separation (Colom et al., 2018). We performed lipidomics to investigate whether Cav1 and mechanical stress could recruit distinct lipids to the membrane of MVBs and, consequently, EVs. Our lipidomic assays revealed significant differences in the lipid
proportion and composition of EVs, both between WT and Cav1 depleted cells, as well as between resting and mechanically stressed donor cells. In WT cells, mechanical stress led to a slight increase in the content of globoside Gb4 and ganglioside GM2 in EVs. Notably, Cav1 depletion in donor cells prevented the incorporation of these lipids in EVs. Gb4 has been demonstrated to stimulate the Raf-MEK-ERK MAPK signaling pathway, leading to increased cancer cell proliferation (Park et al., 2012). GM2 role in cancer progression is well-documented, as it is highly expressed in various human tumors and functions as a pro-cancer signaling regulator (Sasaki et al., 2021). In this context, we cannot rule out potential changes in the protein composition of EVs. Differential EV protein composition can influence various aspects of EV biology, including their impact on receiving cells. This can occur through a protein cargo carrying molecules that activate pro-metastatic signaling pathways or by inducing differential EV uptake by receiving cells, as seen with the clustering of integrins in EVs that facilitate surface docking and internalization (Hoshino et al., 2015). The potential changes in the protein cargo of EVs remain to be defined.

Our data establish a connection between the response of caveolae to mechanical stress and EV production. We and others have shown that upon mechanical stress, caveolae immediately flatten out to counteract membrane tension increase and prevent rupture (Sinha et al., 2011). This flattening is immediately followed by caveolae disassembly, releasing caveolae coat components into the cytoplasm and non-caveolar Cav1 at the plasma membrane. This process is linked to mechano-transduction (Dewulf et al., 2019; Nassoy & Lamaze, 2012; Torrino et al., 2018). Our discovery that Cav1, rather than caveolae, is responsible for EV production under mechanical stress aligns with Cav1’s presence in both MVBs and EVs. Under mechanical stress, caveolae flatten out and release non-caveolar Cav1, which is
subsequently endocytosed into the endosomal network, engulfed in ILVs, and eventually secreted in EVs. Cells lacking Cav1 fail to respond to mechanical stress and do not enhance EV production, whereas cells lacking cavin1 can produce EVs, albeit at a reduced level. These data indicate a correlation between the level of EV production under mechanical stress and the quantity of Cav1 present in MVBs. This was further validated by the increase of EV production when Cav1 was overexpressed in HeLa cells. In support of these findings, EM observation of MVBs revealed a significant enrichment of Cav1 in mechanically stressed cells (Figure S3).

In conclusion, our findings emphasize the importance of Cav1 in regulating the dynamics of EVs in the context of the constant mechanical strains that cancer cells must endure. This mechanism provides an explanation for how cancer cells can utilize their mechanical environment to further promote cancer progression.

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Materials and Methods

Cell lines
HeLa, Hs578t, MDA-MB-231 cells along with the Cav1−/− and cavin1−/− sub lines were cultured in Dulbecco's modified Eagle's medium (DMEM-Glutamax, Gibco), with 10% FBS (FBS, Gibco), 100U/ml penicillin and 100 μg/ml streptomycin (Gibco). Cell lines were grown at 37°C, under 5% CO2. Cell spheroids were prepared following the classical agarose cushion protocol. First, 50 µl of agarose 1.5% w/v in PBS (ultrapure agarose, Invitrogen) and 50 µl per well were dispensed in 96-well plate and incubated for 10-15 min for polymerization at room temperature. Then, cells were seeded on agarose cushion at 105 cells per well. Spheroid formation usually takes between 24 and 48 h. Cav1−/− and cavin1−/− cells were obtained by CRISPR-Cas9 gene editing.

Antibodies and plasmids
Primary antibodies used include rabbit anti-cav1 (BD Transduction Laboratories, cat. no. 610059; 1:2,000 for WB and 1:200 for IF), mouse anti-Hrs (Abcam, cat. no. ab56468; 1:2,000 for WB), rabbit anti-STAM1/2 (Abcam, cat. no. ab76061; 1:2,500 for WB), mouse anti-CD9 (clone MM2/57; Sigma, cat. no. CBL162; 1:1,000 for WB), mouse anti-α-tubulin (clone B512; Sigma, cat. no. T5168; 1:5,000 for WB), rabbit anti-LC3B (Abcam, cat. no. ab51520; 1:3,000 for WB), mouse anti-CD63 (Santa Cruz, cat. no. sc-5275; 1:2,000 for WB and 1:100 for IF), mouse anti-calnexin (BD transduction laboratories. cat. no. 610523; 1:2,000 for WB), rabbit anti-PTRF (Proteintech. cat. no. 18892-1-ap. 1:1,000 for WB). Secondary antibodies used include mouse-HRP.
(Jackson ImmunoResearch, cat. no. 715-035-151; 1:5,000 for WB) and rabbit-HRP (Jackson ImmunoResearch, cat. no. 711-035-152; 1:5,000 for WB), mouse-Alexa 488 (Invitrogen, cat. no. A21202; 1:200 for IF), mouse-Cy3 (Jackson ImmunoResearch, cat. no. 715-166-150; 1:200 for IF), rabbit-Alexa 488 (Invitrogen, cat. no. A21206; 1:200 for IF), rabbit-Cy3 (Jackson ImmunoResearch, cat. no. 111-166-045; 1:200 for IF).

The pCMV-CD63-pHluorin construct (Addgene, #130901) was transfected in HeLa cells using the lipofectamine LTX reagent (Invitrogen) protocol, with 1 µg of plasmid DNA. Cells were imaged 48 hours after transfection.

**RNA interference**

For hrs knockdown we used the following: Control siRNA (Dharmacon, Thermo Fisher, cat. no. SI03650325; 5′-AATTCTCCGAACGTGTCACGT-3′), the hrs pool of four siRNA SMARTpool ON-TARGETplus HGS siRNA (Thermo Fisher, L-016835-00-0005; 5′-GAGGUAACGUCGUAAACA-3′, 5′-GCACGUCUUUCCAGAAUUC-3′, 5′-AAAGAACUGUGGCCAGACA-3′ and 5′-GAACCCACACGUCGCCUG-3′). Transfection was done using Lipofectamine RNAiMAX according to the manufacturer’s protocol (Thermo Fisher), siRNA was used at 20 nM. Depletion efficiency was assessed by immunoblotting.

**Mechanical stress models**

To induce swelling of cells in 2D cultures we used hypo-osmotic shock. Hypo-osmotic shock was performed on cells by using the corresponding growth medium diluted appropriately in deionized water (1:9 dilution for 30 mOsm hypo-osmotic shock).
To induce compression of multicellular spheroids, we used osmotic stress as described by Cappello’s team (Dolega et al., 2017). Shortly, we prepared hyperosmotic medium by adding high molecular weight dextran to reach a final concentration of 160 g/ml (2X solution). We used 2 MDa dextran (Sigma Aldrich, 95771) to avoid the penetration of the polymer in the cellular spheroid. Dextran containing medium was added on spheroid at 80 g/ml final concentration. For long-term compression experiments, dextran containing culture medium is renewed after 3 days.

EV isolation

For the hypo-osmotic shock experiments, $6 \times 10^6$ of cells were seeded per T75 culture flask. After 24 h, cells were washed 1 X with PBS (Gibco) and 12 ml of serum free iso or hypo-osmotic culture medium. Medium was changed after 5 min to iso-osmotic, serum free, culture medium and incubated for the corresponding time periods. For the osmotic induced compression experiments, spheroids were washed with 1X PBS (Gibco) and 100 µl of serum free medium containing or not dextran. After treatments with mechanical stimulations (Hypo-osmotic shock or osmotic induced compression) culture supernatant was collected and was subjected to serial centrifugations (2,000 g for 10 min, 11,000 g for 30 min at 4°C), followed by ultracentrifugation at 100,000 g for 90 min at 4°C (45Ti or TLA110 rotors, Beckman Coulter). The pellet containing EVs was washed in cold 1x PBS (Gibco) and ultracentrifuged again at 100,000 g for 90 min at 4°C.
EV characterization by Nanoparticle Tracking Analysis

NTA was performed using ZetaView PMX-120 (Particle Metrix) equipped with a 488 nm laser, with software version 8.05.02 to measure the concentration and distribution size of particles by evaluating the Brownian motion in a light scattering system. The samples were diluted in 1X PBS (Gibco) to obtain a concentration of $10^6$ to $10^9$ particles/ml per field.

Western blotting

Cell and EV extracts were separated by SDS-PAGE, transferred to a nitrocellulose, blocked in 1x PBS containing 3% BSA or 5% nonfat milk according to the antibody used. Membranes were probed overnight at 4°C. Bound antibodies were developed using SuperSignal™ West Femto Maximum Sensitivity Substrate (Thermo Scientific) and the ChemiDoc Touch Imaging System (BioRad). Protein bands were quantified by densitometric analysis using the ImageJ.

Electron microscopy

For imaging cell spheroids, epon embedding was used to preserve the integrity of cell structures for electron microscopy (EM). Spheroids were fixed sequentially for 1 h at room temperature with 1.25% glutaraldehyde in 0.1 M Cacodylate and then overnight at 4 °C. Cells were washed extensively with 0.1 M Cacodylate, pH 7.2. Post-fixation was performed for 1 h at room temperature with 1% OsO4 in 0.1 M Cacodylate, pH 7.2. Spheroids were dehydrated through a graded-concentration series of ethanol (50, 70, 90, then 100%, each for 10 min at RT). Embedding was finally performed in LX112 resin. Cells were infiltrated with an increasing ratio of LX112: ethanol solution (1:2, 1:1 and 2:1) and finally with pure LX112. Samples in resin were polymerized overnight at
60°C. Semi-thin 500 nm sections were sliced using a Leica UCT ultra microtome and mounted onto microscopic glass slides and dried on a hot plate. Semithin sections were stained for 30 s on a hot plate with a mix of Azure B and basic fuchs in sodium tetraborate (Morikawa et al., 2018). Sections were then mounted in DPX for microscopy and covered with coverslips. Micrographs were acquired on an Upright Wide field Leica DM6000b Microscope equipped with a color CoolSNAP HQ2 camera. Ultrathin 65 nm sections were sliced using a Leica UCT ultra microtome and mounted on nickel formvar/carbon-coated grids for observations. Contrast was obtained by incubation of the sections for 10 min in 4% uranyl acetate followed by 1 min in lead citrate. Caveolae were identified based on their ultrastructural features. The length of plasma membranes observed were measured using ImageJ software and the number of the structures observed was reported to µm of membrane.

For imaging EVs: EVs pellets were resuspended in 50-100 µl of 1x PBS, 5 µl were deposited on a coated-side of a formvar-carbon grid and left for 20 minutes to adhere. Grids were then transferred on 2% PFA droplets for 20 minutes fixation and then washed 10 times on filtered distilled water droplets. Grids were incubated for 10 minutes in 0.4 % uranyl acetate in 2 % methylcellulose droplets on ice and in the dark. Grids were removed from the droplet with a stainless steel loop and excess liquid was removed by pushing the loop on a Whatman filter. Grids are then air dried for 30 minutes on the loops.

Electron micrographs were acquired on a Tecnai Spirit electron microscope (FEI, Eindhoven, The Netherlands) equipped with a 4k CCD camera (EMSIS GmbH, Münster, Germany).
Immunofluorescence

After the treatments, cells were fixed with 4% PFA (EMS) for 15 min at room temperature. Cells were incubated for 1 h in a blocking solution: 1x PBS with 0.1% triton and 0.3% BSA. Then primary and secondary antibodies were successively incubated during 1 h each at RT in 1x PBS containing 0.1% triton and 0.1% BSA. Coverslips were then mounted on microscope slides with Fluoromount G (Invitrogen). Images were acquired on a Zeiss LSM 780 confocal microscope with a 63x/1.46 Oil objective. At least 10 cells per replicate were imaged.

Live TIRF microscopy

Cells were grown in 35-mm imaging plates (FluoroDish WPI; IBIDI). An inverted Eclipse Ti-E (Nikon) full motorized with PFS 2 (Perfect Focus System) to reduce the drift was used with a 100 × CFI PlanApoTIRF, oil, 1.49/0.12-mm objective (Nikon), images were acquired with Metamorph (Molecular Devices). A cage incubator (Life Imaging Services) was used to keep the imaging chamber at 37°C and 5% CO₂. To visualize fusion events, 1-3 min videos were acquired in different areas to analyze at least 20 cells per condition.

Sample preparation for lipidomics

For lipidomics analysis, 20 ug of protein corresponding to around 109 EV particles were spiked with 1.65 μL of internal standard lipid mixture containing 500 pmol of Chol-d6, 100 pmol of Chol-16:0-d7, 100 pmol of DAG 17:0-17:0, 50 pmol of triglycerides 17:0-17:0-17:0, 100 pmol of SM 18:1;2-12:0, 30 pmol of Cer 18:1;2-12:0, 30 pmol of GalCer 18:1;2-12:0, 50 pmol of LacCer 18:1;2-12:0, 300 pmol of PC 17:0-17:0, 50 pmol of PE 17:0-17:0, 50 pmol of PI 16:0-16:0, 50 pmol of PS 17:0-17:0, 30 pmol of
PG 17:0-17:0, 30 pmol of PA 17:0-17:0, 40 pmol of Gb3 18:1;2-16:0(d9), 25 pmol of GM3 18:1;2-18:0-d5, 25 pmol of GM2 18:1;2-18:0-d9, 25 pmol of GM1 18:1;2-18:0-d5, 30 pmol of lysophosphatidic acid (LPA) 17:0, 30 pmol of lysophosphatidylcholine (LPC) 12:0, 30 pmol of lysophosphatidylethanolamine (LPE) 17:1, and 30 pmol of LPS 17:1 and subjected to lipid extraction at 4°C, as described elsewhere (78). Briefly, the sample was dissolved in 200 μL of 155 mM ammonium bicarbonate and then extracted with 1 mL of chloroform-methanol (10:1) for 2 hours. The lower organic phase was collected, and the aqueous phase was re-extracted with 1 mL of chloroform-methanol (2:1) for 1 hour. The lower organic phase was collected and evaporated in a SpeedVac vacuum concentrator (Thermo Fisher Scientific). Lipid extracts were dissolved in 100 μL of infusion mixture consisting of 7.5 mM ammonium acetate dissolved in propanol/chloroform/methanol (4:1:2 vol/vol). Samples were analyzed by direct infusion in a Q Exactive mass spectrometer (Thermo Fisher Scientific) equipped with a TriVersa NanoMate ion source (Advion Biosciences). A total of 5 μL of sample was infused with gas pressure and voltage set to 1.25 psi and 0.95 kV, respectively.

DAG, TAG and CE were detected as ammonium adducts and PC, PC O- and SM were detected as protonated ions in the 10:1 extract, by positive ion mode fourier transform mass spectrometry (FTMS) by scanning m/z = 580–1,000 Da, Rm/z =200= 280,000 with lock mass activated at a common background (m/z = 680.4802) for 30 s. Every scan is the average of two microscans, automatic gain control (AGC) was set to 1E6 and maximum ion injection time (IT) was set to 200 ms. Cer and hexosylceramide (HexCer) were detected as acetate adducts and PE, PE O-, PG, LPC and LPE were detected as deprotonated ions in the 10:1 extract, after polarity switch by negative ion mode FTMS by scanning m/z = 520–1,050 Da, at Rm/z = 200 = 280,000 with lock mass activated at a common background (m/z = 529.4626) for 30 s. Every scan is the
average of two microscans, AGC was set to 1E6 and IT was set to 50 ms. Hex2Cer, Gb3 and Gb4 were detected as protonated ions in the 2:1 extract in positive ion mode FTMS by scanning m/z = 800–1,600 Da, at Rm/z=200= 280,000 with lock mass activated at a common background (m/z = 1,194.8179) for 30 s. GM1, GM2, and GM3 were detected as deprotonated ions in the 2:1 extract in negative ion mode after polarity switch in FTMS by scanning m/z = 1,100–1,650 Da, at Rm/z=200= 280,000 with lock mass activated at a common background (m/z = 1,175.7768) for 30 s. Every scan is the average of two micro-scans, AGC was set to 1E6 and IT was set to 50 ms. PA, PI, PS, LPA and LPS were detected as deprotonated ions in the 2:1 extract in negative ion mode in FTMS by scanning m/z = 520–1,100 Da, at Rm/z=200 = 280,000 with lock mass activated at a common background (m/z = 529.4626) for 30 s. Every scan is the average of two micro-scans, AGC was set to 1E6 and IT was set to 50 ms.

All data were acquired in centroid mode.

All data were analysed with the lipid identification software, LipidXplorer (Herzog et al. Genome Biology 2011, 12:R8). Tolerance for MS identification was set to 2 ppm. Data post-processing and normalization to internal standards were done manually.

**Migration and invasion assays**

Migration assays of Hs578t cells were performed by seeding 300,000 cells in each well of a 24-well plate 24 h before the experiment. The cells were incubated with 1 μg/ml of EVs 37 °C for 4 h. After incubation, a scratch was done in the middle of each well with a pipette tip, cells were washed 1 time with PBS and incubated with FBS-free culture medium. Cell migration was monitored using time-lapse microscopy (IncuCyte Live Cell Analysis Systems, 4x objective lens, Sartorius) with an interval of
3 h during 21 h. Image analysis was performed with ImageJ using a wound healing
size tool (Suarez-Arnedo et al., 2020).

Invasion assays were performed in 8 μm pore size 24-well inserts (Greiner bio-one)
pretreated with Matrigel (BD Biosciences) according to the manufacturer’s
instructions. Briefly, the inserts were coated with Matrigel and were left in a 37°C
incubator for 30 minutes before seeding. Then, 300,000 Hs578t cells previously
treated with 1 μg/ml of EVs during 24 h were resuspended in serum-free medium and
added to the top of each chamber insert, and medium with FBS as a chemo-attractant
was added to the bottom chamber. After 24 h, inserts were removed, washed and cells
that migrated to the lower side of the inserts were stained with 0.1% toluidine blue,
washed, and counted in an inverted microscope.

**Statistical analysis**

All analyses were performed using GraphPad Prism version 6.0 to 8.0, (GraphPad
Software). Two-tailed (paired or unpaired) Student’s t-tests were used when
comparing only two conditions. For more than two conditions, the Kruskal–Wallis test,
ordinary one-way ANOVA or two-way ANOVA were used. Significantly different
comparisons of means are marked on the graphs with asterisks. Error bars denote
SEM or SD.

**Figures Legends**

**Figure 1: Characterization of EVs from mechanically stressed cells.**

A. Concentration (particles/ml) and size (nm) of EVs from WT HeLa cells in response
to hypo-osmotic shock analyzed by NTA. B. Quantification of EVs released from WT
HeLa cells in response to hypo-osmotic shock. Graph shows number of total secreted EVs. **C.** Vesicle concentration and size of EVs from WT HeLa cells in response to hypo-osmotic shock analyzed by EM. **D.** Representative EM images showing EVs isolated from WT HeLa cells in resting conditions or after hypo-osmotic shock. Scale bar 150 nm. **E.** Western Blot analysis of CD63, CD9, calnexin, Cav1 and cavin1 of WT, Cav1\(^{-/-}\) or cavin1\(^{-/-}\) HeLa cell lysates and their corresponding purified EVs. Red arrow indicates cavin1. Gels were loaded with EVs from the same number of secreting cells. ****: p<0.0001.

**Figure 2: Mechanical stress effects on EV release.**

**A.** Quantification of EVs released by WT, Cav1\(^{-/-}\) and cavin1\(^{-/-}\) HeLa cells in response to hypo-osmotic shock. Graph shows the number of total secreted EVs. **B.** Quantification of EVs released by WT, Cav1\(^{-/-}\) and cavin1\(^{-/-}\) HeLa cells in response to dextran compression. Graph shows the number of total secreted EVs. **C.** Quantification of MVB fusion activity (events per cell and time) of WT or Cav1\(^{-/-}\) HeLa cells in response to hypo-osmotic shock. **** p < 0.0001, *** p < 0.001, ** p < 0.01.

**Figure 3: Impact of mechanical stress on MVBs.**

**A.** Upper panel, representative image of an Hs578t cell spheroid. Scale bar 50 µm. Lower panel, representative EM image of an MVB in an Hs578t cell spheroid. Scale bar 10 nm. **B.** Quantification of MVB diameter in Hs578t cell spheroids after 5 min, 1 day or 5 days of dextran compression. **C.** Quantification of the number of MVBs in Hs578t cell spheroid after 5 min, 1 day or 5 days of dextran compression, represented by area. **** p < 0.0001.
Figure 4: Analysis of the ESCRT pathway in EV release.
A. Western Blot analysis of HRS, STAM, Lamp1 and LC3B in WT or Cav1−/− HeLa cells. B. Protein signal quantification graphs normalized to the stain free gel. C. Western Blot analysis of HRS, STAM, Cav1 and tubulin in HeLa cells treated with siRNA control or siRNA against HRS. D. Quantification of EVs released by HeLa cells treated with siRNA control or siRNA against HRS in resting conditions or after mechanical stress. Graph shows the number of total secreted EVs. *** p < 0.001, ** p < 0.01.

Figure 5: Lipid signature of EVs from mechanically stressed cells.
A. Mol percentile of each lipid class in EVs from WT or Cav1−/− HeLa cells in resting conditions or after osmotic shock. B. Comparison of the fold change in lipid classes between EVs from WT or Cav1−/− HeLa cells in resting conditions or after hypo-osmotic shock. PC, phosphatidylcholine; PE, phosphatidylethanolamines; PI, phosphatidylinositol; PS, phosphatidylserines; Chol, cholesterol; CE, cholesteryl ester; DAG, diacylglycerols; TAG, triacylglycerols; PG, phosphatidylglycerols; PA, phosphatidic acids; LPC, lysophosphatidylcholines; LPE, lysophosphatidylethanolamine; LPA, lysophosphatic acid; Cer, ceramides; GlcCer, glucosylceramide; LacCer, lactosylceramide; Gb3, globoside 3; Gb4, globoside 4; GM1, ganglioside 1; GM2, ganglioside 2; GM3, ganglioside 3; SM, sphingomyelins. ** p < 0.01, * p < 0.05.

Figure 6: Migration and invasion of Hs578t cells treated with EVs.
A. Representative images of wound healing assays on Hs578t cells pre-treated with HeLa EVs from WT or Cav1−/− cells in resting conditions or after exposure to
mechanical stress. B. Wound closure quantification represented in percentage over time. C. Quantification of the slope of the wound closure curves. D. Quantification of invasive Hs578t cells pre-treated with HeLa EVs from WT or Cav1−/− cells in resting conditions or after hypo-osmotic shock. ** p < 0.01.

Supplementary figure 1: Validation of mechanical stress models.

A. Schematic representation of the effect of hypo-osmotic shock on caveolae. In resting conditions, caveolae are fully budded invaginations where Cav1 and cavin1 can interact. Upon hypo-osmotic shock, caveolae flatten out, resulting in the diffusion of non-caveolar Cav1 at the plasma membrane and loss of interaction between Cav1 and Cavin1. This process is reverted upon return to resting conditions

B. Immunofluorescence of Cav1 and CD63 in HeLa cells in resting conditions and after exposure to hypo-osmotic shock.

C. Magnified inserts from B showing enlarged endosome-like structures in the cell after osmotic shock.

D. Schematic representation of the dextran compression model on spheroids. Cells are seeded on an agarose bed and left to spontaneously form spheroids. High molecular weight dextran is used to induce osmotic induced mechanical compression of the spheroids.

E. Representative images of Hs578t spheroids at resting conditions and after 5 min, 1 day and 5 days of mechanical compression.

F. Representative EM images of caveolae structures (*) observed on spheroids.

G. Quantification of caveolar structures in Hs578t spheroids at resting conditions and after 5 min, 1 day and 5 days of mechanical compression. Graph represents the data in caveolae per area. * p < 0.05.
Supplementary figure 2: Effect of mechanical stress on EV secretion in additional cell lines.

A. Quantification of EVs released by WT, Cav1<sup>−/−</sup> and cavin1<sup>−/−</sup> Hs578t cells after exposure to hypo-osmotic shock. Graph shows total secreted EVs. B. Quantification of EVs released by WT, Cav1<sup>−/−</sup> and cavin1<sup>−/−</sup> Hs578t cells after dextran compression. Graph shows total secreted EVs. C. Quantification of EVs released by WT, Cav1<sup>−/−</sup> and cavin1<sup>−/−</sup> MDA-MB-231 cells after exposure to hypo-osmotic shock. Graph shows total secreted EVs. D. Quantification of EVs released by WT, cavin1<sup>−/−</sup>, and cavin1<sup>−/−</sup> overexpressing Cav1 HeLa cells after exposure to hypo-osmotic shock. Graph shows total secreted EVs. ** p < 0.01, * p < 0.05.

Supplementary figure 3: Cav1 localization at MVBs after mechanical stress.

A. Representative EM image of an MVB in MLEC cell in resting conditions. B. Representative EM image of an MVB in MLEC cell during hypo-osmotic shock. Immunogold labelling against Cav1. Arrows indicate MVB structures. Scale bar 100 nm.
Figure 1: Characterization of EVs from mechanically stressed cells.

A

NTA

particles/mL

Size (nm)

ctrl hypo

B

EM

particles/mL

Size (nm)

ctrl hypo

C

D

E

cells EVs

MW (KDa) WT Cav1−/− PTRF−/− Protein ladder ctrl ctrl hyp hypo hyp hyp hyp hyp

CD63

CD9

calinexin

Cav1

cavin1
Figure 2: Mechanical stress effects on EV release.

A  WT  Cav1⁻⁻  cavin1⁻⁻
B  WT  Cav1⁻⁻  cavin1⁻⁻
C  WT  Cav1⁻⁻

particles / mL

0  2x10⁹  4x10⁹  6x10⁹  8x10⁹

ctl  hypo  ctl  hypo  ctl  hypo

particles / mL

0  1x10⁹  2x10⁹  3x10⁹

ctl  dextran  ctl  dextran  ctl  dextran

fusion activity (fold change)

0  1  2  3  4  5  6  7  8

ctl  hypo  ctl  hypo

WT  Cav1⁻⁻
Figure 3: Impact of mechanical stress on MVBs.

A

B

C

MVB diameter (µm)

MVB per 100 µm²

ctrl 5 min 1 day 5 days

ctrl 5 min 1 day 5 days

**** ****
Figure 4: Analysis of the ESCRT pathway in EV release.

A

![Western blot images showing MW (kDa) and protein bands for HRS, STAM1, Lamp1, and LC3b.]

B

**HRS**

**STAM1**

**Lamp1**

**LC3b**

Fold change to control

C

![Western blot images showing MW (kDa) and protein bands for HRS, STAM1, Cav1, and Tubulin.]

D

**siCTRL**

**siHRS**

Particles/mL

siCTRL vs. siHRS: **p < 0.01**
Figure 5: Lipid signature of EVs from mechanically stressed cells.

A

B

- **A**
  - WT control
  - Cav1Δ control
  - WT hypo
  - Cav1Δ hypo

- **B**
  - PC
  - PCO
  - PE
  - PEO
  - PI
  - PS
  - Chol
  - TAG
  - PI
  - PS
  - Chol
  - TAG
  - PI
  - PS
  - Chol
  - GM2
  - GM3
  - SM

- **Fold Change**
  - PC
  - PCO
  - PE
  - PEO
  - PI
  - PS
  - Chol
  - TAG
  - PI
  - PS
  - Chol
  - TAG
  - PI
  - PS
  - Chol
  - GM2
  - GM3
  - SM

- **Legend**
  - WT: Wild Type
  - Cav1Δ: Cav1 knockout
  - control: Control condition
  - hypo: Hypocondition
Figure 6: Migration and invasion of Hs578t cells treated with EVs.

A

B

C

D

PBS control hypo WT Cav1−/−

% wound closure

0 3 6 9 12 15 18 21

0 3 6 9 12 15 18 21

0 0.5 1.0 1.5

0 0.5 1.0 1.5

0 0.5 1.0 1.5

**
Supplementary figure 1: Validation of mechanical stress models.

A

isotonic medium:
resting cell

hypotonic medium:
swollen cell

B

carlson

CD63 Cav1

hypo

recovery

C

cavin1

D

cells

agarose

96-well plate

24h

spheroid

cells
dextran

ECM

E

10

6 min

1 day

5 days

F

G

Cavaeles per um2

control 5 min 1 day 5 days

91
Supplementary figure 2: Effect of mechanical stress on EV secretion in additional cell lines.

A

Hs578t

WT  Cav1Δ  cavin1Δ

B

Hs578t

WT  Cav1Δ  cavin1Δ

C

MDA-MB-231

WT  Cav1Δ  Cav1Δ

D

HeLa

WT  cavin1Δ  cavin1Δ

Control  Hypo  Control  Hypo  Control  Hypo  Control  Hypo  Control  Hypo

particles/mL

2x10^9

4x10^9

6x10^9

8x10^9

0

2x10^9

4x10^9

6x10^9

8x10^9

0

2x10^9

4x10^9

6x10^9

8x10^9

0

2x10^9

4x10^9

6x10^9

8x10^9

0

2x10^9

4x10^9

6x10^9

8x10^9

0

2x10^9

4x10^9

6x10^9

8x10^9

0

2x10^9

4x10^9

6x10^9

8x10^9

0

2x10^9

4x10^9

6x10^9

8x10^9

0

2x10^9

4x10^9

6x10^9

8x10^9
Supplementary figure 3: Cav1 localization at MVBs after mechanical stress.
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4. Further investigating the involvement of Cav1 in the dynamics of EVs

4.1 Effect of cancer-derived EVs on CAF-mediated immunosuppression

Among the various components of the TEM, CAFs have emerged as key players in cancer progression. CAFs are a type of activated fibroblast that interact with cancer cells (Gascard & Tlsty, 2016). CAFs act as master regulators of the TME, through the release of soluble factors, regulation of extracellular matrix composition and involvement in cell-to-cell communication, all contributing to the progression of cancer (Kalluri, 2016). CAFs are involved in the secretion of a range of growth factors such as TGF-\(\beta\), fibroblast growth factor (FGF), Platelet-derived growth factor (PDGF), and hepatocyte growth factor (HGF), along with cytokines and chemokines that activate cancer cell growth (Mhaidly & Mechta-Grigoriou, 2020). They also promote angiogenesis, alter ECM properties, as well as enhance the invasiveness and migration of cancer cells by producing proteases such as MMPs and cathepsins which remodel the ECM (Xouri & Christian, 2010). CAFs also induce epithelial-mesenchymal transition (EMT), which is essential in promoting cancer stem cell-like features and often leads to increased metastasis (Jia et al., 2020).

CAF’s contribution to cancer cell invasion and metastasis is further facilitated by their ability to communicate with immune cells. They modulate the recruitment, differentiation, and function of immune cells present in the TME, promoting an immunosuppressive environment that favors cancer progression (Mhaidly & Mechta-Grigoriou, 2020).
Recent studies have shown that cancer cells can transfer EVs to CAFs, and vice versa, thereby facilitating communication (Naito et al., 2022). These EVs can carry bioactive molecules that modulate the behavior and function of recipient cells (Figure 17).

**Figure 17. EV-mediated crosstalk between cancer cells and cancer-associated fibroblasts.** Schematic representation of the intercellular crosstalk between cancer cells and CAFs via EVs. Cancer cells release bioactive molecules in EVs that are taken up by surrounding CAFs. Such molecules include TGF-β, ITGB4, p53, mRNAs and miRNAs. The uptake of cancer-derived EVs leads to specific changes in the recipient cells, including the induction of mitophagy and glycolysis, the development of myofibroblast features, alterations in the extracellular matrix, and the expression of inflammatory genes. Cancer-derived EVs also induce distinct CAF subtypes and contribute to the heterogeneity of CAFs. On the other hand, CAFs themselves utilize EVs to transfer various functional molecules to cancer cells. These functional molecules include CD81, CD9, FAP, miRNAs, IncRNAs, transposable RNAs, and metabolites. The transfer of these molecules from CAFs to cancer cells leads to the acquisition of more aggressive phenotypes by cancer cells (Naito et al., 2022).

EVs from CAFs have been shown to influence breast cancer cell behavior through the transfer of proteins and miRNA to promote EMT and facilitate
metastasis by promoting phenotypes such as increased migration, proliferation and invasion (Chen et al., 2017; Liu et al., 2020; Luga et al., 2012). Conversely, EVs derived from cancer cells can promote the activation and transformation of CAFs into a more supportive phenotype for tumor growth and metastasis.

Extensive research has led to the characterization and comparison of four subtypes of CAFs in breast cancer, with the identification of a specific subpopulation of CAFs, which express the fibroblast activation protein (FAP) marker, that are linked to immunosuppression and resistance to immunotherapy (Feig et al., 2013). This specific subset of CAFs, now called CAF-S1, were shown to increase attraction, survival, and population of CD4+ CD25+ FOXP3+ regulatory T cells (Tregs) in the tumor microenvironment through the release of CXCL12 and upregulation of different molecules including OF40L, JAM2, PD-L2, CD73, DPP4 and B7H3, leading to immunosuppression (Costa et al., 2018) (Figure 18). Furthermore, using single-cell RNA sequencing on CAF-S1, they found clusters of myofibroblasts that express high levels of extracellular matrix proteins and modulators of the immune system, including the immunosuppressive cytokine TGF-β. These particular CAFs create a localized immunosuppressive environment that enables tumor growth and metastasis (Kieffer et al., 2020).
Figure 18. Schematic representation of CAF subtypes and T cell recruitment.

A. CAF heterogeneity (CAF-S1-4) in breast cancer and their markers. B. CAF-S1-enriched breast cancer show high FOXP3+ and low CD8+ T cells compared with CAF-S4-enriched breast cancer. C. CAF-S1 promotes an immunosuppressive environment by attracting CD4+CD25+ T cells, retention of T cells, promoting their survival and differentiation into CD25+FOXP3+ T cells (Costa et al., 2018).

It is crucial to understand the mechanisms underlying this phenotypic transformation, including the impact of signaling molecules and cross-talk between CAFs and cancer cells. Clarifying the mechanisms by which CAFs acquire immunosuppressive traits can provide insights into the progression
and metastasis of cancer. Furthermore, understanding the interplay between CAFs and cancer cells can help identify key molecules or signaling pathways involved in CAF-mediated metastasis, paving the way for the development of targeted therapies.

Considering that breast cancer tumors are subjected to mechanical stress while growing, we hypothesized that the release of breast cancer cell-derived EVs could have an impact on the phenotypes of CAFs. Our objective was to test whether EVs from WT Cav1 depleted breast cancer cells, in resting conditions or under mechanical stress could promote the acquisition of these immunosuppressive traits in CAFs. The following experiments were performed in collaboration with Dr. Fatima Mechta-Grigoriou.

We first evaluated the effect of mechanical stress on the release of EVs from WT or Cav1−/− MDA-MB-231 triple negative breast cancer cells. As with the other cell lines evaluated, we observed a significant increase in the amount of EVs produced after mechanical stress, and this increase was completely inhibited in Cav1−/− cells (Figure 19A). Having confirmed this, we evaluated the effect of these EVs in a well-established assay to test CAF immunosuppressive ability. Briefly, CAFs were isolated from primary breast cancers and pre-incubated for 24 hours with EVs from WT, Cav1−/− or Cav1 rescued MDA-MB-231 cells, under resting or mechanically stressed conditions. CAFs were thoroughly washed to remove any excess EVs, and CD4+ CD25+ T cells were plated overnight on top of the CAFs. The next day,
T cells were analyzed by flow cytometry for the FOXP3 marker, indicating the degree of immunosuppression.

We found that incubation of T cells with CAFs without EV pre-treatment induced a ~2-fold increase in the number of FOXP3 positive T cells compared to T cells alone, corroborating the immunosuppressive effect of CAFs, in line with previous reports (Costa et al., 2018). Interestingly, pre-treatment of CAFs with EVs from WT MDA-MB-231 cells in resting conditions completely abolished this increase in the number of FOXP3 positive T cells, presenting a phenotype comparable to T cells without CAFs. However, CAFs pre-treated with EVs from mechanically stressed WT MDA-MB-231 cells were able to maintain the immunosuppressive phenotype of CAFs by increasing the number of FOXP3 positive T cells (Figure 1B). This increased immunosuppressive phenotype of CAFs observed after pre-treatment with mechanically stressed cells is in line with previous studies. In a liver cancer model, it was showed that EVs released from cancer cells subjected to shear stress, were able to activate CAFs by upregulating the expression of FAP, and alpha-smooth muscle actin (α-SMA) through the activation of insuling growth factor (IGF) pathway (Feng et al., 2022). These data demonstrate that mechanically stressed-induced EVs from cancer cells are more efficient to activate CAFs than EVs from cancer cells in resting conditions.
Figure 19. Effect of mechanical stress on breast cancer EV release and CAF immunosuppressive capabilities.

A. Quantification of EVs released by WT, Cav1−/− and Cav1 rescue MDA-MB-231 cells after osmotic shock, graph shows total particle number secreted measured by NTA. B. Detection by flow cytometry of CD4+ CD25+ FOXP3+ T cells after incubation with CAFs pretreated with EVs from MDA-MB-231 cells WT, Cav1−/− or Cav1 rescue, in resting conditions or after mechanical stress. *p < 0.05, **p <0.01.

Regarding the role of Cav1 in this immunosuppressive effect, CAFs pretreated with EVs from Cav1−/− MDA-MB-231 cells induced an increase in the number of FOXP3 positive T cells compared to the effect observed with EVs from WT cells regardless of the mechanical stress, but to a lesser extent than the effect observed with EVs from WT cells after mechanical stress (Figure 19B).

Currently, increasing evidence shows that EVs derived from tumor cells mediate and promote the conversion and activation of CAFs from heterogeneous origins. In a model of gastric cancer, it was shown that cancer-derived EVs containing miR-21-5p had the ability to trigger peritoneal mesothelial cells to undergo mesothelial-to-mesenchymal transition. Additionally, these EVs were also found to induce endothelial-to-
mesenchymal transition and convert endothelial cells to CAFs through the TGF-β/Smad pathway in gastric cancer *in vivo* (Li et al., 2018). The role of cancer derived EVs in CAF activation has also been reported in breast cancer models. EVs released by breast cancer cells and containing microRNA miR-105 had the ability to induce a specific metabolic program in CAFs, which included enhancing glucose and glutamine metabolism to provide nutrients to adjacent cancer cells (Yan et al., 2018). Furthermore, triple negative breast cancer cell-derived EVs can convert normal fibroblasts into CAFs, promoting tumor growth *in vivo* (Baroni et al., 2016).

Counterintuitively, our data shows that EVs from breast cancer cells do not enhance the immunosuppressive effect of CAFs over T cells. This may be explained by the fact that our model for the evaluation of immunosuppression involves the use of the already activated and immunosuppressive CAFs-S1 purified from breast cancer samples. Moreover, our co-culture model is devoid of cancer cells, therefore, any element of the secretome of cancer cells that may contribute to the CAF mediated immunosuppression is absent. We speculate that using a subpopulation of CAF that is not immunosuppressive *per se*, as is the case of CAF-S4 for example (Costa et al., 2018), we might find that breast cancer EVs act as expected and activate these CAFs towards a more immunosuppressive phenotype.

Overall, our results indicate that EVs from cancer cells, particularly those derived from mechanically stressed cells, can influence the pre-disposed immunosuppressive phenotype of CAFs. Moreover, Cav1 may contribute to the modulation of this effect, although other factors may also be involved.
Further research is needed to fully understand the mechanisms underlying these findings and explore their potential implications in cancer immunotherapy.

4.2 Role of Cav1 in EV uptake by receiving cells

EVs are now well known to be essential mediators of intercellular communication, delivering a plethora of bioactive molecules to recipient cells, including proteins and genetic material (Tkach & Théry, 2016). The biological outcomes of this cell-to-cell communication is determined by the efficient uptake of EVs by recipient cells.

This intricate process is thought to involve various pathways and sequential steps, including docking and signaling, EV-plasma membrane fusion or intact EV uptake, and EV degradation or fusion inside endosomes to finally release its contents in the cell (Figure 20). Understanding these mechanisms enables us to dissect the intricate interplay between EVs and recipient cells, providing critical insights into their physiological and pathological implications (Russell et al., 2019).
Figure 20. Potential outcomes of EV-plasma membrane docking at the recipient cell.
Schematic representation of EV-cell interaction. 1. After EV-PM docking, signaling pathways can be activated following interactions from the membrane contact. 2. EV-PM fusion can occur, leading to the release of the EV cargo directly into the recipient cell cytosol. 3. Endocytosis through different mechanisms can lead to the uptake of whole EVs. 4. Once inside endosomal structures, EVs can be degraded by fusion of endosomes with lysosomes. 5. Fusion of the EV membrane with the endosomal membrane can lead to the release of the EV cargo to the cytosol (Modified from Bonsergent et al., 2021).

The initial step of EV uptake involves the interaction between surface molecules on EVs and specific receptors or ligands on the membrane of the recipient cell. This docking process enables the attachment and binding of EVs to the recipient cells, followed by internalization. Experiments based on treatment of EVs with proteinase K showed a notable decrease in their uptake by cancer cells, indicating the crucial role of proteins in EV uptake.
Different types of proteins have been identified as key regulators of EV uptake. Tetraspanins, which are abundant in EVs, have been suggested to interact with various receptors on recipient cells. Tetraspanins facilitate EV binding and uptake and mediate the targeted delivery of EVs to specific cells or tissues. For instance, the tspan8-ITGα4 complex found on cancer EVs binds to CD54 in recipient cells specifically directing EVs to endothelial and pancreatic cells, while EVs carrying the tetraspanins CD9 and CD81, in conjunction with CD11a, CD54 and phosphatidylserine are able to specifically bind to dendritic cells expressing αvβ3 integrin (Morelli et al., 2004; Rana et al., 2012). Notably, tetraspanins interact with a variety of receptors on recipient cells, including integrins. Activated T cells and dendritic cells can capture EVs through expression of the integrin LFA-1 (Nolte-'t Hoen et al., 2009; Segura et al., 2007). Inhibition of αvβ3 integrin binding, using a specific inhibitor, disrupted tumor EV adhesion and uptake by epithelial cells (Altei et al., 2020). Intriguingly, this integrin was found to be present on the EVs rather than on the recipient cell surface, adding a layer of complexity to the process. Depletion of integrins ITGβ4 and ITGβ5 in EV-secreting cells resulted in a sharp decrease of the tropism of these EVs towards the lung and liver, respectively. EVs containing ITGβ5, when administered to cells, resulted in an upregulation of several pro-metastatic S100 genes which were not upregulated in ITGβ5-depleted EVs (Hoshino et al., 2015). These findings suggest that downstream signaling pathways may be triggered after EV binding to the plasma membrane.

EV-bound proteins activate signaling cascades within recipient cells, triggering intracellular events that modulate the cellular response to EV
uptake. These signaling events can involve the recruitment of adaptor proteins, activation of downstream kinases, and modulation of cytoskeletal dynamics. EVs have been shown to carry components of the Wnt signaling pathway, such as Wnt protein and Frizzled receptor, which can activate the Wnt signaling pathway in recipient cells upon EV uptake, leading to diverse cellular responses (Korkut et al., 2009). Studies have demonstrated that glioblastoma-derived EVs activate ERK 1/2 and HSP27 downstream signaling. This activated signaling pathway serves as a positive feedback loop that enhances the uptake of EVs (Svensson et al., 2013).

Following docking, EVs can directly fuse with the recipient cell plasma membrane, merging their lipid bilayers and delivering their cargo directly into the cytoplasm. This process relies on the interaction between specific fusogenic proteins on the EV and recipient cell membranes. Alternatively, EVs can be internalized via endocytic pathways, ultimately leading to the formation of endosomes. Once within the endosomes, EVs can fuse with the endosomal membrane, releasing their contents into the cytoplasm (Mulcahy et al., 2014).

When EVs are not able to fuse directly with the plasma membrane of recipient cells, several processes are responsible for EV uptake, including phagocytosis, macropinocytosis, clathrin-mediated endocytosis, and caveolin-mediated endocytosis (Delenclos et al., 2017). Studies have shown that pharmacological inhibition of the caveolar protein Cav1 leads to a reduction in EV uptake in lung cancer and pancreatic cells (Javeed et al., 2015; Wei et al., 2017). Interestingly, in a model of ischemia, hippocampal neurons upregulate the expression of Cav1 to capture EVs and generate a neuroprotective effect, mitigating ischemia-induced apoptosis (Yue et al., 2019). Often, Cav1-
dependent EV uptake has been called caveolae-dependent endocytosis by some researchers, without extensive exploration into the mechanism of this endocytosis pathway (Nanbo et al., 2013). However, contradictory results have been reported regarding the role of Cav1 in EV uptake. One study found that Cav1 negatively regulates EV uptake by suppressing ERK1/2 signaling, and downregulating Cav1 actually increased EV uptake (Svensson et al., 2013). These discrepancies suggest that specific proteins or molecules on the surface of EVs might be necessary for their uptake, indicating the possibility of targeted or specific uptake by recipient cells. It is important to note that the often use of non-Cav1 specific pharmacological inhibitors, such as filipin (a cholesterol sequestering probe), dynasore (a dynamin inhibitor), or nystatin (a sterol binding agent), may provide results that reflect the impairment of membrane integrity rather than the effect of Cav1 deficiency on EV uptake.

Therefore, our objective was to investigate the role of caveolae and/or Cav1 in EV uptake in cancer cells using genome edited cells. The following experiments were performed in collaboration with Dr. Gregory Lavieu.

To test the role of Cav1 in EV uptake we used two different engineered HeLa cells lines as EV donors, one stably expressing NanoLuc luciferase-tagged Hsp70. Hsp70 is a known EV cargo that in combination with the high sensitivity engineered NanoLuc reporter in EVs allows to monitor EV uptake in recipient cells with a high signal-to-noise ratio as already reported (Bonsergent et al., 2021), and one cell line stably expressing a GFP-tagged Hsp70 that allows us to follow the uptake of GFP positive-EVs by immunofluorescence (Bonsergent & Lavieu, 2019).
As receiving cells, we used the previously used WT HeLa cells. We measured the levels of Cav1 and cavin1 in the receiving WT HeLa cells, and western blot analysis showed that Cav1 was expressed at very low levels. We took advantage of this low expression to investigate the effect of different levels of caveolar components on EV uptake. For this, we overexpressed Cav1 and/or cavin1 in these cells (Figure 21A). Remarkably, we observed that
overexpression of Cav1 alone or with cavin1 increased EV uptake significantly. Overexpression of cavin1 in receiving cells had no effect on EV uptake (Figure 21B).

It is worth noting that the kinetics of EV uptake followed an inverse exponential-like function, with a constant EV uptake rate (~1% spontaneous rate per 1 h) that persists for 6 to 7 hours of incubation, until it reaches a plateau thereafter. This kinetics are in line with previous results based on the same construct to monitor EV uptake (Bonsergent et al., 2021).

The highest amount of internalized EVs was observed when Cav1 was overexpressed alone. Cavin1 being required for caveolae morphogenesis and stabilization at the plasma membrane, suggesting that the increase of EV uptake by receiving cells is likely due to an increase in the pool of non-caveolar Cav1, rather than an increase in the overall number of caveolae (Figure 21B).

To verify this hypothesis, we evaluated the uptake of EVs by cavin1−/− MEF cells expressing high or low levels of Cav1. In the absence of cavin1, these cells cannot assemble caveolae. We found that the uptake of HeLa derived EVs was significantly increased in receiving MEF cells with higher levels of Cav1 (Figure 21C). Thus, it confirms that the efficiency of uptake is correlated with the level of non-caveolar Cav1 present in receiving cells.

Finally, we measured the uptake of GFP-tagged EVs in Cav1−/− or cavin1−/− receiving HeLa cells. In line with our previous results, we observed that depletion of Cav1 significantly reduces the uptake of EVs compared to WT cells, further confirming the crucial role of Cav1 in this process. Cavin1 depletion reduced slightly the uptake of EVs, yet this reduction was not
significant. Considering that downregulation of cavin1, which is co-transcriptionally regulated with Cav1, leads to downregulation of Cav1 as well, we believe that it explains the reduced uptake of EVs in cavin1 depleted cells (Hill et al., 2008) (Figure 21D).

Caveolae have long been associated with endocytosis, not only of EVs but also of other molecules (Pelkmans, 2005). There has however been a recent change of paradigm based on several studies showing that caveolae are quite static at the plasma membrane and do not efficiently endocytose cargo. Also, it has become evident that there are no specific cargoes that depend strictly on caveolae for endocytosis. Caveolae endocytosis would mainly serve as a means to control their density at the plasma membrane. The consensus to date is that endocytosis is not their primary function (Parton et al., 2020). Our results therefore confirm this assumption as the uptake of EVs does not rely on caveolae, but on the level of non-caveolar Cav1.

Outside of caveolae, Cav1 has been shown to form oligomerized microdomains called Cav1 scaffolds. These stable structures can be present in different sizes (S1A, S1B, and S2) and are proposed to have signaling properties similar to the ones associated with Cav1, including signaling and endocytosis (Khater et al., 2019; Lajoie et al., 2009). It has been proposed that these Cav1 scaffolds could modulate clathrin-independent endocytosis through an indirect mechanism by modifying the lipid composition of raft nanodomains (Lajoie & Nabi, 2007). Furthermore, cells lacking Cav1 or cavin-1 show an upregulated clathrin-independent carriers/GPI-AP enriched early endosomal compartment (CLIC/GEEC) endocytic pathway, conversely,
upregulating Cav1 or Cav3 results in the inhibition of the CLIC/GEEC endocytic pathway (Chaudhary et al., 2014). We cannot rule out the possibility that these pathways might be involved in EV uptake.

Cav1 has been shown to recruit specific species of lipids in the plasma membrane, including phosphatidylserine, sphingomyelin and certain phospholipids, creating particular lipid nanodomains (Prakash et al., 2021; Sonnino & Prinetti, 2009). These nanodomains at the plasma membrane can differentially cluster transmembrane proteins, proteins with specific binding motifs for cholesterol, or proteins with preferential affinities towards certain features like acyl chains (Harayama & Antonny, 2023; Sezgin et al., 2017). We can hypothesize that the lipid-protein nanodomains formed by Cav1 scaffolds could cluster specific species of proteins in the recipient cell membrane that can interact with surface molecules on the EV favoring docking, fusion, or clathrin-independent endocytosis, resulting in enhanced uptake. Additional experiments are needed to test this hypothesis.

Altogether, our results reveal the critical role played by Cav1 in the enhancement of the uptake of EVs by receiving cells. The kinetics of EV uptake and the limited role of cavin1 suggest a more nuanced regulation of this process, which may involve other factors that require further investigation.
5. MATERIALS AND METHODS

Cell lines
HeLa, MEF and MDA-MB-231 cells and sublines were cultured in Dulbecco's modified Eagle's medium (DMEM Glutamax, Gibco), with 10% FBS (FBS, Gibco), and 100U/ml penicillin and 100 μg/ml streptomycin (Gibco). Cell lines were grown at 37°C, under 5% CO2.

Antibodies and plasmids
Primary antibodies used include rabbit anti-cav1 (BD Transduction Laboratories, cat. no. 610059; 1:2,000 for WB and 1:200 for IF), rabbit anti-cavin1 (Proteintech. cat. no. 18892-1-ap. 1:1,000 for WB), rabbit anti-GFP (Invitrogen, cat. no. A11122. 1:100 for IF). Secondary antibodies used include mouse-HRP (Jackson ImmunoResearch, cat. no. 715-035-151; 1:5,000 for WB) and rabbit-HRP (Jackson ImmunoResearch, cat. no. 711-035-152; 1:5,000 for WB), mouse-Alexa 488 (Invitrogen, cat. no. A21202; 1:200 for IF), mouse-Cy3 (Jackson ImmunoResearch, cat. no. 715-166-150; 1:200 for IF), rabbit-Alexa 488 (Invitrogen, cat. no. A21206; 1:200 for IF), rabbit-Cy3 (Jackson ImmunoResearch, cat. no. 111-166-045; 1:200 for IF).

Mechanical stress models
To induce swelling of cells in 2D cultures we used hypo-osmotic shock. Hypo-osmotic shock was performed on cells by using the corresponding growth medium diluted appropriately in deionized water (1:9 dilution for 30 mOsm hypo-osmotic shock).
**EV isolation**

For the hypo-osmotic shock experiments, $6 \times 10^6$ of cells were seeded per T75 culture flask. After 24 h, cells were washed 1 X with PBS (Gibco) and 12 ml of serum free iso or hypo-osmotic culture media. Media was changed after 5 min to iso-osmotic, serum free, culture media and incubated for the corresponding time periods. Culture supernatant was collected and was subjected to serial centrifugations (2,000 g for 10 min, 11,000 g for 30 min at 4°C), followed by ultracentrifugation at 100,000 g for 90 min at 4°C (45Ti or TLA110 rotors, Beckman Coulter). The pellet containing EVs was washed in cold 1x PBS (Gibco) and ultracentrifuged again at 100,000 g for 90 min at 4°C.

**EV characterization by NTA**

NTA was performed using ZetaView PMX-120 (Particle Metrix) equipped with a 488 nm laser, with software version 8.05.02 to measure the concentration and distribution size of particles by evaluating the Brownian motion in a light scattering system. The samples were diluted in 1X PBS (Gibco) to obtain a concentration of $10^6$ to $10^9$ particles/ml per field.

**CAF immunosuppressive phenotype characterization**

Fresh human samples (tumors and juxta-tumors) were obtained from the operating room, after the specimen's macroscopic examination and selection of areas of interest for diagnosis by a pathologist. BC samples were cut into small fragments (around 1 mm$^3$) and digested in CO2-independent medium (Gibco, #18045-054) supplemented with 5% fetal bovine serum (FBS, PAA,
A11-151, 2 mg/ml collagenase I (Sigma-Aldrich, #C0130), 2 mg/ml hyaluronidase (Sigma-Aldrich, #H3506) and 25 μg/ml DNase I (Roche, #11284932001) for 45 min at 37°C with shaking (160 rpm). After tissue digestion, cells were filtered using a cell strainer (40 μm, Fischer Scientific, #223635447) and resuspended in PBS (Gibco, #14190) supplemented with 2 mM EDTA (Gibco, #15575) and 1% Human serum (BioWest, #S4190-100) to a final concentration at approximately 5x10^5 cells in 50 μl. EVs were added on top of the CAFs in serum-free media for a final concentration of 1 μg protein/mL. After 24 hours, CAFs were washed with 1x PBS (Gibco) and X00,000 CD4+ CD25+ T cells were seeded on top. After 24 hours of coculture, cells were prepared for flow cytometry analyses.

**Western blotting**

Cell extracts were separated by SDS-PAGE, transferred to nitrocellulose, blocked in 1x PBS containing 3% BSA or 5% nonfat milk according to the antibody used. Membranes were probed overnight at 4°C. Bound antibodies were developed using SuperSignal™ West Femto Maximum Sensitivity Substrate (Thermo Scientific) and the ChemiDoc Touch Imaging System (BioRad). Protein bands were quantified by densitometric analysis using the ImageJ.

**Uptake assays**

For the NanoLuc-Hsp70 uptake assay, acceptor cells were seeded 24 h before the uptake experiment, at 20,000 cells per well in a 96-well plate. NLuc-Hsp70 EV input was added in serum-free DMEM for a final...
concentration of 1 μg protein/mL. Cells were incubated with EVs at 4 °C for 0–16 h. After incubation, cells were washed with PBS and then lysed in lysis buffer, prior to transferring in white 96-well plates. Finally, 50 μL of Nano-GloTM reagent (Promega, Wisconsin, USA) was added on each well and luminescence activity was read using iD3 SpectraMax microplate reader (Molecular Devices, California, USA) or Centro LB 960 microplate luminometer (Berthold, Germany).

For the GFP-Hsp70 uptake assay, acceptor cells were seeded 24 h before the uptake experiment, at 100,000 cells per well in a 24-well plate on the top of coverslips. Acceptor cells were incubated with GFP-Hsp70 EVs at a final concentration of 1 μg protein/mL for up to 16 h at 37 °C. Then cells were washed with PBS, fixed 15 min RT with PBS 4% Paraformaldehyde and permeabilized 30 min RT in PBS 1% bovine serum albumin (BSA) 0.1% Triton-X-100. Immunofluorescence was performed as already described. Image analysis and colocalization quantification were performed using ImageJ software (NIH, Maryland, USA).

**Immunofluorescence**

After the treatments, cells were fixed with 4% paraformaldehyde (EMS) for 15 min at room temperature. Cells were incubated for 1 h in a blocking solution: 1x PBS with 0.1% triton and 0.3% BSA. Then primary and secondary antibodies were successively incubated during 1 h each at RT in 1x PBS containing 0.1% triton and 0.1% BSA. Coverslips were then mounted on microscope slides with Fluoromount G (Invitrogen). Images were acquired on
a Zeiss LSM 780 confocal microscope with a 63x/1.46 Oil objective. At least 10 cells per replicate were imaged.

**Statistical analysis**
All analyses were performed using GraphPad Prism version 6.0 to 8.0, (GraphPad Software). Two-tailed (paired or unpaired) Student’s t-tests were used when comparing only two conditions. For more than two conditions, the Kruskal–Wallis test, ordinary one-way ANOVA or two-way ANOVA were used. Significantly different comparisons of means are marked on the graphs with asterisks. Error bars denote SEM or SD.
6. DISCUSSION

6.1 Mechanical strains and EV secretion

We were able to demonstrate in a robust manner the effect of mechanical stress on EV secretion. Both 2D osmotic shock and 3D compression models of mechanical strain, as demonstrated in our study, caused an increase in the secretion of EVs from HeLa, Hs578t and MDA-MB-231 human cancer cells, moreover, we show for the first time that this mechanically induced increase in EV secretion is Cav1 dependent.

Various systems have been identified to stimulate EV secretion, molecular disruption in the forms of small molecule modulators or inhibition of lysosomal pathways, as well as external factors and inducers such as starvation or hypoxia have been shown to increase EV secretion (Figure 22) (Wang et al., 2023). Physical perturbations have also been shown to stimulate EV secretion, with shear stress being a reliable and scalable method. For instance, cells grown on 3D scaffolds and subjected to fluid shear stress (0.5-6 mL/min direct flow) showed up to a 100-fold increase in the number of EVs detected by NTA after incubation in a bioreactor. However, analysis of EV cargo indicates that total protein content can decrease after mechanical stress (Guo et al., 2021; Patel et al., 2019). Another model of mechanical stress, cell stretching, has been shown to increase the secretion of a subpopulation of EVs with an altered miRNA cargo and differential activity on recipient cells. This subpopulation has been observed to promote the induction of proliferation and myogenic differentiation on myoblasts, or to inhibit bone marrow macrophage osteoclast
differentiation via inhibition of signaling pathways (Mullen et al., 2023; Xiao et al., 2021).

One reason for the recent increased interest in boosting EV secretion is their potential as therapeutic agents. In fact, in the last few years, multiple novel EV-based therapies have been developed as an alternate strategy to cell therapies for treating diverse medical conditions. These therapies offer unique advantages, including lower immunogenicity, improved safety and regulatory aspects, and the ability to serve as readily available products. However, to achieve industrial-scale production of EVs, poor yield and scalability must be resolved (Debbi et al., 2022). To this end, several reports have already characterized the therapeutic properties of large scale mechanical stress induced production of EVs. Stem cells grown on bioreactors have been reported to secrete EVs with altered miRNA and cytokine cargo that are able to confer specific therapeutic properties to receiving cells such as increased angiogenesis, neurogenesis and axonal sprouting (Cha et al., 2018; Guo et al., 2021). Furthermore, EVs secreted by natural killer cells grown in bioreactors were functionally active in killing melanoma and liver cancer cells in both 2D and 3D culture conditions in vitro, as well as suppressing melanoma growth in vivo (Wu et al., 2022).
Figure 22. Different types of approaches described to date for boosting EV secretion.
Schematic representation of the different methods for increasing EV secretion for clinical use including physical signals such as mechanical loading, 3D culture, ultrasound and electrical stimulation, molecular interference, environmental factors, external inducers like liposomes, and other environmental factors like hypoxia, starvation, and hyperglycemia (Wang et al., 2023).

Despite the advances in understanding the effects of mechanical stress on EV secretion, the underlying mechanism of this increase remains poorly understood. Although there are several documented effects of mechanical perturbations, alterations in the endosomal pathway, changes in membrane properties, and alterations in the cytoskeleton dynamics, the exact mechanism of EV secretion under mechanical stress remains elusive.

Of note, some reports have highlighted the potential relationship between increased EV secretion and the Yes-associated protein (YAP) pathway. The YAP pathway is a key signaling pathway involved in mechanotransduction, translating mechanical cues into cellular responses. Activation of the YAP pathway has been linked to various cellular processes, including proliferation,
differentiation and has been implicated in various diseases, including cancer (Cunningham & Hansen, 2022).

A recent study found that breast cancer cells grown in stiff substrates present a 2.5-fold increase in EV secretion compared to cells grown in soft substrates, showing that this effect is dependent on the transcriptional co-activator YAP, which is regulated by the Hippo signaling pathway and is known for its role in mechanotransduction and transcriptional activation of genes. By inhibiting YAP interaction with TEAD and its translocation to the nucleus using the pharmacologic inhibitor Verteporfin it was observed that the increased EV secretion was absent. Moreover, these EVs were found to confer pro-migratory and invasiveness traits to receiving cells (Patwardhan et al., 2021). Another study that used shear forces to induce increased EV secretion found that upon mechanical stress YAP is robustly translocated to the nucleus, and inhibition of this translocation with Verteporfin reduced the number of secreted EVs (Guo et al., 2021).

In all these studies, the increased EV secretion was evaluated after long times of exposure to mechanical strains (1-3 days), whereas in our models of mechanical strain, we observed that the increased EV secretion happens in as short time as 1 hour after as evaluated by NTA. Furthermore, the increased MVB-PM fusion activity observed in our experiments suggest an increase in the secretion of exosomes just minutes after the mechanical stress. Considering this, we don’t believe the translocation of YAP and subsequent gene transcription regulation is involved in this rapid reaction. Nevertheless, we could hypothesize that the YAP dependent regulation of increased EV secretion could
act as a secondary “line of defense” to sustained mechanical strains the cell could be subjected to.

Recently, the interaction between the YAP pathway and caveolae has been described to be important to respond to mechanical strains. YAP has been shown to be key mediators of the gene expression of both Cav1 and cavin1 proteins, without YAP, the cell is not able to form caveolae at the plasma membrane. Moreover, the activation of YAP is triggered by the caveolar-mediated response to shear stress (Figure 23) (Rausch et al., 2019). Analyzing the potential involvement of the hippo pathway in our models of mechanical stress could be of interest.

Figure 23. Interaction between the Hippo pathway and caveolae mechanics.
Schematic representation of regulation caveolae abundance by the Hippo pathway by the YAP/TAZ-TEAD mediated regulation of caveolar genes. At the same time, caveolae facilitate the mechanical stress mediated signaling via the Hippo pathway (Rausch et al., 2019).
6.2 Caveolae-free Cav1, at the plasma membrane and beyond

We show that upon disassembly of caveolae, triggered by mechanical stress, a population of Cav1 translocates from the plasma membrane towards endosomal compartments. At the same time, we demonstrated that both Cav1 and the ESCRT-0 complex are strictly required for the increased EV release after mechanical stress. Yet the precise mechanism by which Cav1 translocates to the MVB and acts together with ESCRT-0 is not yet fully understood.

Phosphorylation of Cav1 has been identified as a critical regulatory mechanism that affects various cellular processes. Among the different phosphorylation events, the specific phosphorylation of tyrosine 14 (Y14) on Cav1 has received significant attention due to its functional implications in multiple cellular functions. The phosphorylation of Y14 in Cav1 has been associated with cell migration, dynamics of focal adhesions, disassembly of caveolae, and subsequent endocytosis, among other processes (Joshi et al., 2008). Interestingly, Y14 phosphorylation of Cav1 has been observed in models of mechanical strain, as is the case of cyclic stretching, where this phosphorylation plays a crucial role in activating downstream signaling pathways and promoting the expression of caveolar genes, resulting in an increase in caveolae numbers at the plasma membrane (Joshi et al., 2012; Zhang et al., 2007). Furthermore, when exposed to shear flow, Cav1 is phosphorylated at Y14 and is involved in facilitating actin reorganization (Radel & Rizzo, 2005). In our models of mechanical strain, we did not observe an increase in Y14 phosphorylation. We hypothesize that this discrepancy is due to the type of mechanical strain utilized.
in our models of 2D osmotic shock and 3D compression, though achieving similar caveolae disassembly outcomes, may trigger distinct downstream events leading to altered Y14 phosphorylation dynamics. Additionally, the cell type studied may also play a role in contributing to the observed differences in Cav1 phosphorylation dynamics following mechanical strain.

This dynamic regulation of caveolae turnover through Y14 phosphorylation provides cells with the ability to modulate the abundance of caveolae on the plasma membrane, thereby influencing intracellular trafficking and downstream signaling. Importantly, Y14 phosphorylation of Cav1 has been implicated in key aspects of tumor dynamics, including enhanced growth, motility, adhesion, and increased metastatic potential in cancer cells (Goetz et al., 2008; Joshi et al., 2008; Ortiz et al., 2016).

In addition to phosphorylation, another important post-translational modification that plays a crucial role in regulating the function and localization of Cav1 is ubiquitination. Ubiquitination is a process that involves the covalent attachment of ubiquitin molecules to a substrate protein, typically marking it for degradation or sorting to specific subcellular structures. Multiple studies have highlighted the importance of ubiquitination in regulating the intracellular localization, stability, and function of Cav1.

Ubiquitination of Cav1 can occur at any of its 20 ubiquitination sites. One important site of Cav1 ubiquitination is the N-terminal region, which has been demonstrated to serve as a translocation signal for Cav1 to relocate towards ILVs in endolysosomal structures, therefore, serving as a signal for its degradation via the proteasome pathway. Mutation of these specific N-terminal ubiquitination sites resulted in enhanced stability of Cav1 (Hayer et al., 2010).
Another study shed light on the involvement of ubiquitination in the sorting of cargo into ILVs within MVBs. It was found that ubiquitination of Cav1 in the N-terminal region acts as a signal for its sorting into the ILVs. Again, mutations in the ubiquitination sites affected the incorporation of Cav1 into the MVBs and altered the sorting of cargo into the ILVs, highlighting the significance of ubiquitination in intracellular trafficking and signaling (Kirchner et al., 2013). Furthermore, at the ILVs, Cav1 can aid in the sorting of specific cargo, including adhesion proteins and ECM components, to be released in exosomes (Albacete-Albacete et al., 2020; Campos et al., 2018).

In our experimental investigation, we aimed to explore the translocation mechanism of Cav1 from the plasma membrane to endosomal compartments in response to mechanical stress. Given the importance of phosphorylation and ubiquitination modifications in regulating Cav1 dynamics, we hypothesized that these post-translational modifications could play a role in this translocation process. To investigate this, our initial approach involved assessing the levels of Cav1 Y14 phosphorylation and ubiquitination following osmotic shock. Western blot analysis of both Y14 phosphorylation or ubiquitination of Cav1 revealed no differences at 5 or 10 minutes after mechanical stress (Figure 24).

It is important to note that both phosphorylation and ubiquitination modifications are highly dynamic events. Therefore, capturing these modifications can be challenging, making it critical to use appropriate methods and controls for their accurate detection and interpretation.
Figure 24. Phosphorylation and ubiquitination of Cav1 after mechanical stress. 

A. (Left) Western Blot analysis of phospho-Cav1 (Y14) and total Cav1 in WT HeLa cells. (Right) Protein signal quantification graphs normalized to the stain free gel. 

B. (Left) Immunoprecipitation of Cav1 and western Blot analysis of Cav1 and ubiquitin in WT HeLa cells. (Right) Protein signal quantification.

To overcome these challenges associated with the dynamic nature of phosphorylation and ubiquitination modifications, we devised a strategy to address the functional consequences of these modifications on Cav1. Specifically, we decided to express a Y14 phosphorylation mutant (Cav1-Y14F) or an ubiquitin mutant (Cav1-K*R) form of Cav1 in Cav1 depleted cells (Hayer et al., 2010; Joshi et al., 2008). Following the reexpression, we subjected the cells to mechanical stress and quantitatively assessed the EV release using NTA. This experimental setup allowed us to investigate the impact of specific phosphorylation or ubiquitination modifications of Cav1 on EV release under mechanical stress conditions.
Interestingly, expression of the phosphorylation or ubiquitination mutant did not restore the cavin1 protein levels as compared to WT HeLa cells, suggesting reduced levels of caveolae at the plasma membrane (Figure 25A). We observed that when we expressed the Cav1 mutants in Cav1⁻/⁻ cells, the total number of secreted EVs detected by NTA were reduced drastically, showing a 3-fold reduction as compared to WT and Cav1⁻/⁻ HeLa cells in resting conditions and under mechanical stress (Figure 25B).

Figure 25. Phosphorylation and ubiquitination of Cav1 importance in EV release after mechanical stress.
A. Western Blot analysis of Cav1, cavin1 and tubulin in WT, Cav1⁻/⁻ HeLa cells an Cav1⁻/⁻ HeLa cells transfected with an ubiquitin mutant (K*R) or phosphorylation mutant (Y14F) Cav1. B. Quantification of EVs released by WT, Cav1⁻/⁻ HeLa cells and Cav1⁻/⁻ HeLa cells expressing the Cav1 mutants in response to hypo-osmotic shock. Graph shows the number of total secreted EVs.

The nature of this extraordinary reduction in EV release when cells were transfected with the mutant forms of Cav1 raises more questions than answers. First, could the transfection of cells itself be affecting the release of EVs? We used a lipid based transfection reagent, this type of transfection can affect the
lipid content of cells and their plasma membranes, therefore, affecting their topology and potential reaction to mechanical stress. Furthermore, transfecting cells can affect their viability, for example, long exposures to lipid based transfection reagents can induce reduced cell proliferation, toxicity due to the positively charged lipids used, and eventually, increased cell death (Li & Szoka, 2007).

As part of a post-doctoral project, we plan to further investigate the potential role of ubiquitination and phosphorylation of Cav1 in the enhanced EV secretion after mechanical stress. For example, another strategy to evaluate the involvement of ubiquitination on this process is to abolish the ubiquitination process from its start, by the use of ubiquitin-like modifier activating enzyme 1 (UBA1) inhibitors like TAK-234. This inhibitor stably binds to UBA1 and the ubiquitin substrate, preventing UBA1 from releasing ubiquitin, therefore, inhibiting all further downstream ubiquitin activation. If used in a timely manner, without provoking the over accumulation of ubiquitinated cargo that can become toxic to the cell, we will be able to evaluate if ubiquitination is a process needed for the increased EV release after mechanical stress (Hyer et al., 2018).

6.3 Exocytosis and endocytosis: A tense relationship

Regulating the physical properties of cells, including their shape and volume, is crucial for maintaining cellular homeostasis and enabling various physiological processes. Indeed, changes in membrane tension have been observed in various physiological contexts.
For example, during cell migration, the plasma membrane undergoes dynamic changes in tension to facilitate cell shape changes and movement (Sheetz & Dai, 1996). This tension regulation ensures that the cells can move efficiently through tissues or substrates and respond appropriately to external physical cues (Sens & Plastino, 2015). Additionally, in processes such as cell division, changes in membrane tension are essential for proper cell shape changes during cytokinesis (Reichl et al., 2005).

Endocytosis and exocytosis events have emerged as crucial regulators of plasma membrane tension homeostasis. These processes work together to dynamically adjust the plasma membrane area, ensuring the maintenance of tension equilibrium at the plasma membrane. During endocytosis, vesicles bud inwards from the plasma membrane, leading to a reduction in the overall plasma membrane area and an increase in plasma membrane tension. Conversely, during exocytosis, intracellular vesicles fuse with the plasma membrane, resulting in an increase in plasma membrane area and a decrease in plasma membrane tension. Consequently, changes in plasma membrane tension reciprocally influence exocytosis and endocytosis events. Generally, higher tension levels facilitate exocytosis, enabling the delivery of additional lipids and proteins to expand the surface area of the membrane (and conversely blocks endocytosis) (Dai et al., 1997). On the other hand, lower tension levels promote endocytosis, removing excess membrane from the surface (Apodaca, 2002; Gauthier et al., 2011; Mao et al., 2021).

Increases in membrane tension by hypo-osmotic shock has been characterized to induce a two-phase reaction. Initially, when there is an increase in plasma
membrane tension, cells respond by utilizing reservoirs in the form of invaginations, such as caveolae, and ruffles to expand the surface area of the membrane. Subsequently, there is a secondary reaction characterized by myosin contraction accompanied by a significant increase in vesicle exocytosis. Importantly, the initial phase has been described to last no more than 1 minute, while the second phase can last more than 15 minutes (Gauthier et al., 2009, Gauthier et al., 2011).

In our experiments using hypo-osmotic shock, we identified two major events. Firstly, we have observed that caveolae, which act as plasma membrane reservoirs, quickly sense the heightened plasma membrane tension and flatten out. This allows cells to utilize these reservoirs to increase the surface area and alleviate the increased tension (phase 1) (Figure 26A). Notably, this flattening of caveolae leads to an enrichment of non-caveolar Cav1 or Cav1 scaffolds at the plasma membrane, which have shown to be much more diffusive at the plasma membrane than caveolae, based on unpublished data from our lab. Consequently, these Cav1 scaffolds can be subjected to endocytosis, as supported by our findings of enriched Cav1 in endosomal compartments after hypo-osmotic shock (Figure 26B).

Secondly, we have demonstrated that the surge in plasma membrane tension following hypo-osmotic shock induces an increase in the fusion of MVBs with the plasma membrane. This, in turn, leads to a sustained increase in the secretion of EVs (phase 2). Altogether, the phenotype that we observe upon hypo-osmotic shock in our experiments fits perfectly with the two-phase model of response to hypo-osmotic shock (Figure 26C).
Figure 26. Schematic representation of the two-phase tension buffering model.

A. Increase in plasma membrane tension triggers phase 1 where the cell utilizes the reservoirs of plasma membrane to increase the surface area of the membrane, as reflected by the rapid flattening of caveolae. Caveolae flattening generates the production of Cav1 scaffolds. B. Cav1 scaffolds are endocytosed towards endosomal compartments (i.e. MVBs). C. There is an increase in exocytosis events as reflected in the increase in MVB-PM fusion and secretion of EVs.

It is important to note that the results obtained from our experiments using hypo-osmotic shock represent extreme conditions of membrane tension changes. Cells in physiological conditions are unlikely to experience such drastic changes in tension. Instead, it is likely that there is a delicate balance between high and low membrane tension promoting cycles of exocytosis and endocytosis. The interplay between these processes allows cells to finely regulate membrane tension according to their physiological needs and as a result the production of sEVS. Further investigation is needed to explore the intricate dynamics of membrane tension regulation in normal cellular processes.
and how imbalances in this regulation may contribute to various physiological and pathological conditions, such as cancer.
7. CONCLUSIONS AND PERSPECTIVES

To conclude, while caveolae have historically been associated with numerous cellular functions, increasing evidence since the seminal discovery in 2011 regarding their role in cell mechanics suggests that this may be their primary function. Recent evidence has shown that Cav1 can also exist as oligomers outside of caveolae, referred to as non-caveolar Cav1 or Cav1 scaffolds (Khater et al., 2019; Wong et al., 2021). This paradigm shift has highlighted the need to differentiate the functions of caveolae from those of non-caveolar Cav1. Our study offers new insights into the regulation of EV dynamics by Cav1. We demonstrate that upon being released from caveolae at the plasma membrane, non-caveolar Cav1 controls EV secretion through ESCRT-0 and increased MVB-PM fusion, leading to exosome secretion. Additionally, our findings underscore the physiological importance of this enhanced EV release mediated by Cav1, as evidenced by changes in the composition and cargo of secreted EVs and the acquisition of increased metastatic traits by recipient cells. Nevertheless, further research is required to fully elucidate the intracellular mechanisms underlying this Cav1-dependent effect. Moreover, our study also revealed that the uptake of EVs by recipient cells is dependent on Cav1 and independent of caveolae.

Overall, our comprehensive investigation has yielded compelling and robust evidence elucidating the intricate mechanisms underlying altered EV dynamics within the mechanical environments of the cell. By delving into this relatively unexplored domain of significant importance, we have greatly contributed to the existing knowledge base. This work not only expands our understanding of EV
biology but also lays the groundwork for further exploration into the potential therapeutic applications of modulating EV dynamics in various physiological and pathological contexts. In sum, our research fills a critical gap in the field and provides a stepping stone for future investigations in this captivating area of research.
8. ANNEX

Investigating the cell-to-cell communication through EVs in the nervous system

The following research article describing an intricate process of crosstalk through EVs between neurons and glial cells was in part developed before the start of my PhD project in the team of Felipe Court (Center for Integrative Biology, Universidad Mayor. Santiago, Chile). It was continued and finished for publication in collaboration after I had begun my thesis in my current lab.


Neuronal activity-dependent ATP enhances the pro-growth effect of repair Schwann cell extracellular vesicles by increasing their miRNA-21 loading

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Functional recovery after peripheral nerve injuries is critically dependent on axonal regeneration. Several autonomous and non-cell autonomous processes regulate axonal regeneration, including the activation of a growth-associated transcriptional program in neurons and the reprogramming of differentiated Schwann cells (dSCs) into repair SCs (rSCs), triggering the secretion of neurotrophic factors and the activation of an inflammatory response. Repair Schwann cells also release pro-regenerative extracellular vesicles (EVs), but is still unknown whether EV secretion is regulated non-cell autonomously by the regenerating neuron. Interestingly, it has been described that nerve activity enhances axonal regeneration by increasing the secretion of neurotrophic factors by rSC, but whether this activity modulates pro-regenerative EV secretion by rSC has not yet been explored. Here, we demonstrate that neuronal activity enhances axonal regeneration by increasing the secretion of neurotrophic factors by rSC, and that nerve activity enhances axonal regeneration by increasing the release of rSC-derived EVs and their transfer to neurons. This effect is mediated by activation of P2Y receptors in SCs after activity-dependent ATP release from sensory neurons. Importantly, activation of P2Y in rSCs also increases the amount of miRNA-21 present in rSC-EVs. Taken together, our results demonstrate that neuron to glia communication by ATP-P2Y signaling regulates the content of SC-derived EVs and their transfer to axons, modulating axonal elongation in a non-cell autonomous manner.

KEYWORDS
Schwann cell, extracellular vesicles, ATP, purinergic receptors, axonal growth, axonal regeneration, miRNA-21
Introduction

The nervous system function is critically dependent on glial cells, which regulate several neuronal functions. In both the central and peripheral nervous systems (CNS and PNS, respectively), glial cells modulate nerve conduction velocity, provide metabolic support to axons and synapses, and respond to tissue injury by activating mechanisms aimed to restore homeostasis (Jäkel and Dimou, 2017; Barros et al., 2018). In the PNS, regeneration of injured axons depends on the reprogramming of Schwann cells from a differentiated phenotype (dSC) to a repair Schwann cell phenotype (rSC), specialized for clearing myelin debris, recruiting macrophages, and guiding regenerating axons to their targets by secretion of neurotrophic factors and contact-mediated signaling events (Chen et al., 2015; Gomez-Sanchez et al., 2015; Jessen and Mirsky, 2016; Szepesi et al., 2018; Jessen and Arthur-Farraj, 2019). In addition, neuronal responses can be modulated by the horizontal transfer of RNA and proteins by means of extracellular vesicles (Rajendran et al., 2014). Astrocyte-derived extracellular vesicles regulate synaptic activity by miRNA transfer to neurons (Chaudhuri et al., 2018), and microglia-derived extracellular vesicles stimulate neurotransmission (Antonacci et al., 2012). We have demonstrated that rSC-derived exosomes enhance axonal regeneration in vitro and in vivo (Lopez-Verrilli et al., 2013), an effect dependent on the reprogramming of dSC into a repair phenotype (Lopez-Leal et al., 2020). Exosomes correspond to small extracellular vesicles (EVs) or nano-vesicles (50–120 nm) released after the fusion of multivesicular bodies (MVBs) with the plasma membrane (Théry et al., 2002). During their formation, exosomes are loaded with proteins, lipids, mRNA, and non-coding RNAs (Jeppesen et al., 2019). Exosomes are enriched in specific repertoires of miRNAs, showing an asymmetric distribution when compared to their cells of origin (Valadi et al., 2007; Villarroya-Beltri et al., 2013). Secretion of exosomes is regulated by several mechanisms, including the phenotypic state of the cell, as well as activation by non-cell autonomous mechanisms (Mathieu et al., 2019). In the nervous system, exosome secretion by oligodendrocytes is enhanced by the neurotransmitter glutamate released by neurons (Frühbeis et al., 2013), suggesting that nerve activity might modulate exosome secretion by glial cells. Interestingly, nerve activity enhances axonal regeneration and functional recovery in the PNS (Haastert-Talini and Grothe, 2013). The electrical stimulation of the sciatic nerves of the transected mice increases axonal regeneration across the injured site by 30–50% (Singh et al., 2012), and in a rat model of femoral nerve regeneration, brief periods of electrical stimulation enhance axonal regeneration and muscle reinnervation in a TTX-dependent manner (Al-Majed et al., 2000). Nevertheless, whether neuronal activity modulates the release of pro-regenerative exosomes by rSC after nerve injury has not been investigated.

Using in vitro models of neuronal and glial cultures, we demonstrate that sensory neurons activate small EV secretion by rSC through an activity-dependent release of ATP, acting over P2Y receptors in rSCs. Strikingly, activation of this signaling pathway not only enhances the release of rSC EVs but also modifies their miRNA content, increasing the EV expression of the pro-regenerative miRNA-21. Taken together, our results identify a signaling mechanism by which neurons modulate the quantity and quality of glial-derived EVs, enhancing their regenerative capacity.

Methods

Schwann cell primary culture

SC primary cultures were obtained from newborn Sprague Dawley (SD) rat sciatic nerves as previously described (Wilby et al., 1999; De Gregorio et al., 2018). Briefly, the perineurium was removed, and the nerve was dissociated in 0.05% trypsin/1% collagenase type 1 solution. Cells were plated on laminin (40 ng/mL; Millipore)-treated flasks in Dulbecco’s Modified Eagle’s Medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (FBS, Invitrogen) and 10% penicillin-streptomycin (Invitrogen). The following day, cells were treated with 10 mM cytosine arabinoside (Sigma). After 1 week in culture, contaminant fibroblasts were eliminated by complement-mediated cell lysis using an anti–CD90 antibody (Invitrogen) and rabbit complement (Sigma). SCs were maintained in DMEM-10%, FBS-1%, penicillin-streptomycin supplemented with 2 μM forskolin (Millipore), and 20 mg/ml bovine pituitary extract (Invitrogen). SCs were then frozen in 10% DMSO and 90% DMEM and passed up to five times. For each independent biological replicate, two newborn SD rat sciatic nerves from the same rat were used for the preparation of the cell culture.

Schwann cell stimuli, EV purification, and analysis

SC primary cultures supplemented with DMEM containing 2 mM forskolin, 20 mg/ml bovine pituitary extract, and 10% EV-free FBS (obtained by serum ultracentrifugation at 100,000 g for 2 h) (SC media) were stimulated with different reagents, such as 50 μM ATP (Sigma), 2 μM ionomycin (Sigma), 300 μM suramin, 10 μM BBG, and 100 mM EDTA (Sigma), for 5 min. Then the media was replaced with fresh SC media to eliminate all traces of reagents. The cells were incubated for different time points (1, 3, 6, 8, 16, and 24 h), and the supernatant was subjected to serial centrifugations (2,000 g for 10 min and 11,000 g for 30 min at 4°C), followed by ultracentrifugation at 100,000 g for 60 min at 4°C (T865 rotor, OTD Combi
Sorvall ultracentrifuge, Dupont). The pellet containing EVs was washed in cold 0.1 M phosphate buffer saline, pH 7.4 (PBS), and ultracentrifuged again at 100,000 g for 60 min at 4°C. In each EV preparation, the concentration of total proteins was quantified by NanoOrange protein quantitation kit (Invitrogen) and stored at −20°C for later use. For the analysis of EVs, the pellets were resuspended in 1 ml of PBS, and the concentration and the particle size distribution were analyzed under a Nanosight NS3000 (Malvern).

**DRG explants culture and axonal elongation assay**

Dorsal root ganglia (DRG) were obtained from day 16 SD rat embryos as previously described (López-Leal et al., 2018). Briefly, E16 rat embryos were decapitated, and the limbs and organs were removed. The spinal cord with DRG was dissected and placed on coverslips or glass plates coated with poly-L-lysine (100 ng/mL; Sigma) and collagen (50 µg/mL; Invitrogen). DRG were maintained in Neurobasal medium (Invitrogen) supplemented with 2% B27 (Invitrogen), 2 mM L-glutamine, 50 ng/mL human nerve growth factor (NGF; Sigma) and placed on coverslips or glass plates coated with poly-L-lysine and PBS (control) or EVs from SC in different conditions were added daily for 4 days. Then, the pool of DRG were homogenized in TRIzol reagent (Life technologies) and placed on coverslips or glass plates coated with poly-L-lysine or a negative control A inhibitor (YI00199006) (Exiqon) in Optimem media (Gibco). The media was replaced after treatment. DRG explants were treated daily with 0.5 µM ATP after 8 h, and DRG explants were treated daily with 0.5 µg of these EVs. Axonal elongation was measured on day 4 after treatment.

**mRNA and miRNA qPCR**

The expression levels of mRNA were evaluated by plating DRG explants, PBS (control), or EVs from SC in different conditions, and were added daily for 4 days. Then, the pool of DRG was homogenized in TRIzol reagent (Life technologies) for the extraction of total RNA by the Trizol method. The aqueous phase was precipitated with 1 volume of isopropanol, 0.1 volumes of 3 M sodium acetate, and 10 µg of glycogen overnight at −20°C. cDNA synthesis was performed with 5x iScript Reverse Transcription Supermix (BioRad). RNA levels of each sample were evaluated by real-time PCR using 5x HOT FIREpol Evagreen qPCR Mix Plus (Medibena). The mixture was run in a real-time PCR thermal cycler (CFX96 Touch Real-Time PCR Detection System, BioRad). Thermal cycling parameters were 15 min at 95°C, followed by 40 cycles of 15 s at 95°C, 20 s at 60°C, and 20 s at 72°C. At the end of the program, melting curve analysis was performed from 60 to 95°C. The following primers were used: PTEN: Forward AAGGACGGACTGGTGTAA and Reverse CCTGAGTTGAGAGTAGAT, SPRY2: Forward GCAGATAGCGGGTGGG and Reverse CCTCACAGGGGTCAACTC, TIMP3: Forward CTGGAGCCCTGGGCACTG and Reverse CCGATCAGGTGTCGGAGT, and GAPDH: Forward TCCCTCAAGATTTCAGCAG and Reverse AGATCCACACGGATACTT. For relative comparison, the Ct value was analyzed with the ΔΔCt method of normalizing.

The miRNA levels of miRNA-21 were analyzed in SCs, and their EVs were treated with or without 50 µM ATP. SC and SC-derived EVs were homogenized in Qiazol reagent (Qiagen) for the extraction of total RNA based on the miRNAsey Mini kit (Qiagen) protocol. MicroRNA qPCR for miRNA-21 was performed using the MystiCq MicroRNA Quantitation System (Sigma) according to the manufacturer’s protocol; briefly, miRNA cDNA of 20 ng of RNA was synthesized using the MystiCq microRNA cDNA synthesis mix (Qiagen). The miRNA levels of each sample were determined by real-time PCR using MystiCq microRNA SYBR Green qPCR ReadyMix (Qiagen). The mixture was run in a real-time PCR thermal cycler (Lightcycler System; Roche Diagnosis Corp).

**Functional assays**

For the gain and loss of function assays, we used miRNA mimics and inhibitors (Exiqon) for miRNA-21 from *Rattus norvegicus*. First, DRG explants were transfected with lipofectamine 2000 (Thermo Fisher) with the miRNA-21 mimic 1 day after plating the DRG, and axonal elongation was quantified 4 days post-transfection. For the loss-of-function assay, SCs were transfected with lipofectamine 2000 with 50 nM LNA-enhanced antisense miRNA inhibitor of miRNA-21 or a negative control A inhibitor (YI00199006) (Exiqon) in Optimem media (Gibco). The media was replaced after 4 h with SC media. Then, EVs were purified by ultracentrifugation from SCs incubated with or without 50 µM ATP after 8 h, and DRG explants were treated daily with 0.5 µg of these EVs. Axonal elongation was measured on day 4 after treatment.
Extracellular vesicle internalization assay

SCs were transduced with a lentivirus coding for CMV-Palm-eGFP (Lai et al., 2015). EVs were purified from SC incubated with or without 50 μM ATP for 8 h by differential ultracentrifugation and quantified by NanoOrange protein assay. DRG were treated with 5 μg of EVs and were fixed 4 h later.

Co-culture of schwann cells and sensory neurons

SCs were transduced with a lentivirus coding for CMV-Palm-eGFP (Lai et al., 2015). Twenty-four hours after the transduction process, SCs were trypsinized and seeded over DRG plated 7 days before and left for 3 days before the experiments were performed. Co-cultured cells were treated with apyrase 1.5 U/mL (Sigma) or 0.5 μM TTX, and the EV release from the SC and internalization by the axon were measured in a Leica TCS SP8 spectral confocal microscope, measuring the colocalization of the GFP-tagged EVs with the axon NFM signal.

Immunofluorescence

The culture medium was removed from SC or DRG cultures, washed three times with PBS, and fixed with 4% PFA for 15 min at RT. Blocking and permeabilization were done with 0.1% Triton X-100 and 2% fish skin gelatin in PBS for 2 h at RT. Then, the cells were incubated with the primary antibody in 0.1% Triton X-100 and 1% fish skin gelatin in PBS and incubated overnight at 4°C. Then, the primary antibody was removed, the cells were washed three times with PBS, and the secondary antibody diluted in 0.1% Triton X-100 and 1% fish skin gelatin in PBS was incubated for 2 h at RT. Afterward, the secondary antibody was removed, and the cells were washed two times with PBS and one time with DAPI stain in PBS (100 ng/ml). Then, the cells were washed once more in PBS, and coverslips were mounted with fluoromount-G (EMS).

Statistical analysis

All data are presented as mean ± SEM from the indicated number of experiments. Statistical analysis was performed using Student’s t-test, linear regression, or ANOVA with Tukey’s multiple comparison tests.

Results

Neuronal activity increases extracellular vesicle release by rSC and their transfer to axons in an ATP-dependent manner

As activity-dependent ATP secretion by peripheral neurons has been associated with the activation of signaling pathways in SCs (Negro et al., 2016; Rodella et al., 2017), we investigated the effect of nerve activity on small EV release by SCs and their transfer to axons. To this end, we used sensory neurons from dorsal root ganglia (DRG), which have been previously demonstrated to have spontaneous activity in vitro (Matsuka et al., 2008; Mutafova-Yambolieva and Durnin, 2014). Indeed, purified DRG neurons in vitro release ATP to the culture medium, which is decreased more than 2-fold after blocking action potentials using the voltage-dependent sodium channel blocker tetrodotoxin (TTX, Figure 1A). We then evaluated whether this TTX-dependent ATP secretion induces a change in the release and uptake of SC-derived EVs. rSCs expressing GFP-labeled EVs were seeded over DRG neurons, and the GFP signal associated with axons was analyzed using a custom-developed particle detection method from confocal-derived images (Supplementary Figure 1 and Methods section). In neurons co-cultured with rSC for 72 h, extensive GFP-positive puncta colocalized with axons immunolabeled against neurofilament protein (Figures 1B,C). After blocking neuronal activity using TTX, rSC-derived GFP signal associated with axons decreases by several folds (Figures 1B,C), and hydrolyzing extracellular ATP using apyrase diminishes the amount of rSC-derived EVs in axons by a magnitude similar to that of TTX (Figures 1B,C). Importantly, TTX or apyrase does not inhibit EV release by rSC (Supplementary Figure 2), suggesting that spontaneous neuronal activity enhances EV release by rSC through a mechanism associated with activity-dependent ATP release.

ATP enhances the pro-growth capacities of rSC-derived extracellular vesicles

As rSC-derived EV secretion can be modulated by activity-dependent ATP release from sensory neurons, we next used purified rSC in order to study the dynamics of this phenomenon and their effect on axonal growth. To this end, rSCs were stimulated with ATP, and the number of released EVs was quantified using nanoparticle tracking analysis (NTA) after differential ultracentrifugation of the conditioned media (see Methods section). In control rSC cultures, EV content in the culture medium reaches a plateau after 3 h. In contrast, EVs from ATP-stimulated rSCs continue to accumulate after 3 h,
FIGURE 1
sEV transfer from rSC to axons in co-culture is inhibited upon ATP depletion and action potential inhibition. The involvement of neuronal ATP and spontaneous activity in the transfer of EVs from rSC to axons was analyzed in a co-culture system. (A) DRG neurons from primary culture were seeded in white bottom culture plates and left to grow for 7 days; ATP release from neurons was analyzed by luminescence with the ATPlite detection kit in a luminescence spectrometer. (B) DRG neurons co-cultured with rSC expressing CD63-GFP to visualize EVs were analyzed for EV transfer by immunofluorescence and detection of GFP signal in axons. Scale bar, 20 µm. (C) Colocalized particles were quantified as described in Supplementary Figure 1 and plotted as particle density per volume of axons. In all quantifications, the average and SEM values of at least three independent experiments are shown (*p < 0.05).

reaching a 3-fold increase compared to control at 16 h post-stimulation (Figure 2A). Ionomycin, which transiently increases intracellular Ca^{2+} in rSC (not shown), has no noticeable effect on EV secretion (Figure 2A). We then study if this ATP-dependent increase in EV accumulation is associated with changes in the rate of EV release by rSC. For this, rSCs were treated with vehicle, ATP, or ionomycin, and 1-h conditioned media was collected at different time points post-stimulation. As expected, in control conditions, the rate of EV release was constant for up to 24 h, and stimulation with ionomycin led to an increase in EV release only in the first hour (Figure 2B), which probably corresponds to the ready-releasable pool of MVBs present in rSCs. Remarkably, an acute 5-min ATP treatment leads to a long-lasting 3-fold increase in the rate of EVs released by rSC until 8 h post-treatment (Figure 2B). Associated with this increase in EV release, rSC at 8 h post-ATP treatment showed an increase in the presence of enlarged clusters of the MVB marker CD63 compared to vehicle-treated cells (Figures 2C, D). Importantly, changes in the rSC EV secretion rate triggered by ATP were not accompanied by major alterations in the EV size distribution as revealed by NTA (Figure 2E).

As we have previously demonstrated that rSC-derived exosomes increase axonal growth and regeneration of sensory neurons (Lopez-Verrilli et al., 2013), we tested the pro-growth effect of EVs obtained from control and ATP-stimulated rSC. We first tested the effect using EVs secreted from equal volumes of conditioned media of control and ATP-treated rSC. DRG devoid of SC were daily treated with vehicle, EVs from control, or ATP-treated rSC, and the axonal extension was evaluated (Figure 2F). EVs derived from HEK cells were used as an additional control. As expected, control rSC-derived EVs enhanced axonal regeneration when compared to vehicle or HEK-derived EVs (Figure 2F). Interestingly, EVs derived from ATP-rSC showed an even higher pro-regenerative effect (Figures 2F,G). Since ATP-treated rSCs released a higher number of EVs (Figures 2A, B), we performed the same assays using comparable amounts of EVs for the different conditions (assessed by protein content or EV number analyzed by NTA, see
Supplementary Table 1). Remarkably, when normalized, ATP-treated rSC-derived EVs still possess a higher pro-growth capacity than control rSC or HEK-derived EVs (Figures 2H, I). Importantly, EVs from control or ATP-treated rSC were similarly internalized by DRG axons (Supplementary Figure 3). Taken together, these results demonstrate that ATP enhances the rate of EV release by rSCs and modifies the pro-growth capacity of these EVs.
ATP-dependent enhancement of the pro-growth capacity of rSC extracellular vesicles is associated with the activation of P2Y receptors in SCs

Extracellular ATP can activate metabotropic or ionotropic purinergic receptors, both expressed by SCs (del Puerto et al., 2013). Therefore, we used a pharmacological strategy to evaluate the pathway by which ATP enhances rSC-EV secretion rates and their pro-growth properties. First, we analyzed the effect of the P2X7 and P2Y receptor antagonists BBG and suramin, respectively, on EV release rate. Although both BBG and suramin do not change the basal release rate of rSC-EVs, the ATP-induced increase of EV release was completely abrogated by suramin (Figure 3A). Next, we analyzed the effect of purinergic receptor inhibition on the pro-growth effect of EVs secreted by ATP-treated rSC. Indeed, EVs from suramin-treated ATP-rSC lose their pro-growth activity over sensory neurons (Figures 3B,C). Together, these data show that ATP activates P2Y metabotropic receptors in Schwann cells, leading to an increase in the rate of EV release, as well as an enhancement in their pro-growth effect over peripheral neurons. Considering the inhibitory effect of suramin over the rate of EV secretion and their pro-growth capacity in ATP-treated rSC, we next evaluated the effect of purinergic inhibition in a co-culture system as described above. In agreement with the previous results, suramin strongly inhibited the transfer of GFP-labeled EVs from SCs (Figures 3D,E). Taken together, these results support our hypothesis that neuron-derived ATP released under spontaneous activity acts through P2Y receptors in rSC, and not P2X, to induce the release and transfer of EVs from rSC to axons.

The enhanced growth effect of extracellular vesicles from ATP-activated rSC depends on the increased expression of miRNA-21

We have already demonstrated that miRNA contained in rSC exosomes enhances axonal growth (López-Leal et al., 2020). Therefore, we hypothesized that miRNAs could be implicated in the pro-growth effect of ATP-treated SCs. We first used ultraviolet (UV) treatment to photochemically damage RNA molecules contained in rSC EVs (Eldh et al., 2010; Zhang et al., 2016). We observed that UV exposure completely abolished the increase in axonal growth of ATP-rSC-derived EVs (Figures 4A,B). We also previously demonstrated that miRNA-21 contained in rSC exosomes mediates their pro-growth effect over sensory neurons (López-Leal et al., 2020). Importantly, miRNA-21 contained in rSC-EVs showed a 1.7-fold increase in ATP-treated rSC compared to non-treated rSC (Figure 4C). miRNA-21 targets various genes involved in the regulation of neurite elongation and axonal growth, such as PTEN, SPRY2, and TIMP3 (Duraikannu et al., 2019; Jamsuwan et al., 2020; Borger et al., 2022); therefore, we evaluated whether ATP-rSC-derived EVs regulate the expression of these genes in DRG neurons. Indeed, we observed a 70% decrease in the expression of PTEN, a 15% decrease in the expression of SPRY2, and a 30% decrease in the expression of TIMP3 in neurons treated with ATP-rSC-derived EVs compared to neurons treated with control rSC-derived EVs (Figure 4D). To evaluate the participation of miRNA-21 in axonal growth, we first transfected DRG neurons with a miRNA-21 oligonucleotide mimic. Indeed, miRNA-21 overexpression in DRG increases axonal growth when compared to the non-targeting miRNA-39-3p (Figures 4E,F). We next assessed the participation of rSC miRNA-21-containing EVs in the elongation of axons. For this, a miRNA-21 inhibitor was transfected into rSCs, and secreted EVs were used for growth assays. Depleting miRNA-21 from ATP-stimulated rSCs causes a decrease in growth enhancement provided by the secreted EVs (Figures 4G,H). In summary, these results demonstrate that ATP increases the presence of miRNA-21 in rSC-derived EVs, which is responsible for their pro-growth effect on DRG neurons.

Discussion

The role of EVs in intercellular communication and delivery of active molecules has been widely described in the nervous system (Lopez-Verrilli et al., 2013; Prada et al., 2018). Furthermore, exosome release from glial cells has been linked to synaptic activity and neurotransmitter release from neurons in the central nervous system (Frühbeis et al., 2013). In this work, we have been able to thoroughly characterize the mechanism by which neuron-evoked ATP is released into the extracellular space, and how it modulates the secretory activity of glial cells. We have also shown that ATP-rSCs enhance neurite elongation and axonal growth, both in co-culture with sensory neurons and in vivo, and our results provide new insights into the mechanisms underlying these effects.

Our previous studies demonstrated that SC-derived exosomes induce an increase in the axonal elongation of sensory neurons (Lopez-Verrilli et al., 2013), but the mechanism that causes this increased neurite growth remained a mystery. Recent studies have shown that ATP is released by degenerating cerebellar granular neurons and spinal cord motor neurons. Moreover, ATP is capable of triggering Ca\(^{2+}\) spikes, cyclic AMP production, and activating the ERK/CREB pathway in SCs co-cultured with these neurons (Negro et al., 2016; Rodella et al., 2017). It is important to note that SCs express ionotropic and metabotropic purinergic ATP receptors, including P2X7, P2Y1, and P2Y2 (Fields and Stevens, 2000). The involvement of these purinoreceptors in EV dynamics has been described in various models. It has been shown that the activation of the P2X7 receptor is able to induce the release of large and small EVs in microglia (Bianco et al., 2009; Asai et al., 2015). Furthermore,
P2Y family of receptors is involved in the increased sEV release and axonal elongation effect of ATP on rSC. To analyze to which receptor ATP binds to exert its effect on rSC we pharmacologically inhibited P2X7 or P2Y receptors and analyzed the sEV released by NTA and their effect on axonal growth in DRG after ATP treatment. (A) Conditioned media from 8 h after ATP and drug treatment were measured by NTA to analyze the rate of release of sEV from rSC. (B,C) The effect of sEV from rSC treated with or without ATP in the presence of BBG or suramin on neurons was analyzed by daily treatment of DRG with 5 µg of sEV. The elongation effect on DRG was visualized on day 4 by immunofluorescence using neurofilament medium (NFM) immunostaining in axons. Scale bar, 500 µm. (D,E) Co-cultured DRG neurons with rSC expressing CD63-GFP to visualize EVs were analyzed for EV transfer by immunofluorescence after incubation with BBG or suramin and detection of GFP signal in axons. Scale bar, 20 µm. In all quantifications, average and SEM of at least three independent experiments are shown (***p < 0.0001; **p < 0.001; *p < 0.05).

the microglial-EVs released upon ATP stimulation have been associated with various phenotypes in neurological diseases, such as traumatic brain injury or multiple sclerosis (Matute et al., 2007; Verderio et al., 2012). On the other hand, the P2Y family of receptors has been described to have a positive role in platelet-EV formation and release, in a multi-step process involving an increase in intracellular calcium concentration, decrease in intracellular cAMP concentration, and activation of
miRNA-21 is a key participant in the axonal elongation effect of rSC-derived sEV on DRG neurons. (A,B) RNA participation in the axonal elongation effect of sEV over DRG neurons was first evaluated by UV irradiation. Axonal growth was analyzed on day 4 after adding rSC-derived sEV daily from SC treated with or without ATP to the DRG neurons. For RNA inactivation, EVs were previously UV irradiated. The axonal elongation of DRG induced by sEV was visualized by immunofluorescence using neurofilament medium (NFM) immunostaining in axons. Scale bar, 1,500 \( \mu \text{m} \).

(C) miRNA-21 content in rSC-derived sEVs was assessed by qPCR in sEV samples from control or ATP-stimulated rSCs. (D) Expression of PTEN, SPRY2, and TIMP3 was measured by qPCR from DRG neurons after treatment with sEV derived from control or ATP-treated rSC. (E,F) The involvement of miRNA-21 was assessed by transfection of miRNA-21 inhibitors or mimics in rSC. miRNA-21 was directly transfected in DRG neurons, and the axonal growth area was analyzed 4 days after transfection, and the miRNA mimic mir-39-3p with no known rat mRNA target was used as a control. The increased axonal growth induced by transfection of miRNA-21 was visualized by immunofluorescence using neurofilament medium (NFM) immunostaining in axons. Scale bar: 1,500 \( \mu \text{m} \). (G,H) SCs were transfected with miRNA-21 inhibitors, sEVs were purified, DRG were treated daily with these sEVs, and the axonal growth area was analyzed 4 days after transfection. The effect of the inhibitors on the sEVs and their axonal elongation effect were visualized by immunofluorescence using neurofilament medium (NFM) immunostaining in axons. Scale bar: 1,500 \( \mu \text{m} \). In all quantifications, average and SEM of at least three independent experiments are shown (\# \( p < 0.0001 \); ** \( p < 0.001 \); * \( p < 0.05 \)).
a signaling cascade including PI3K, which leads to the activation and phosphorylation of various target proteins that eventually lead to the increased platelet-EV formation (Gasecka et al., 2020). Interestingly, we show that the increased release of SC-derived EVs induced by ATP is not dependent on P2X receptors but rather dependent on the P2Y family of receptors and lasts for several hours, which suggest that there is a long-lasting effect, consistent with the activation of intracellular pathways.

Changes in the RNA cargo of EVs have been reported in several cell types after induction by certain stimuli. Cardiomyocytes subjected to ischemia change the miRNA cargo of their EVs promoting cardiac angiogenesis (Ribeiro-rodrigues et al., 2017). TNFα and IL-1β have been shown to modify a specific set of miRNAs in EVs derived from astrocytes to diminish the activity of target neurons (Chaudhuri et al., 2018). To date, only a handful of studies have demonstrated that ATP is able to impact EV composition. It has been shown that microglia-derived EVs present a distinct protein content after a prolonged ATP exposure compared to EVs from non-stimulated cells (Drago et al., 2017). Furthermore, EVs from ATP-stimulated microglia have a stronger impact on the activation state of recipient cells (Drago et al., 2017).

Even though the mechanism by which ATP, through P2Y receptors, leads to a change in the composition of EV miRNAs remains to be explored, different sorting mechanisms for the asymmetric miRNA expression in EVs have been described, including those controlled by the recognition of specific miRNA motifs by loading proteins, as well as regulation by the cellular levels of the target transcripts (Villarroya-Beltri et al., 2013; Santangelo et al., 2016).

Among the miRNAs that are more abundant in ATP-stimulated SC-derived EVs, we found miRNA-21. miRNAs have already been shown to be involved in the regulation of neuron growth. During the early stages of brain development, miRNA-29c is expressed and promotes neurite outgrowth by decreasing PTEN expression (Zou et al., 2015). We have previously described that SC reprogramming into repair cells modifies the exosomal cargo, promoting neurite growth in receiving neurons. Furthermore, this effect is dependent on the selective loading of miRNA-21 into the secreted exosomes (López-Leal et al., 2020). Interestingly, PTEN and SPRY2 are miRNA targets of miRNA-21, and DRG treated with EVs derived from ATP-stimulated SC show a decrease in the levels of PTEN and SPRY2. We hypothesize that this decrease in PTEN and SPRY2 expression may be partially responsible for the axonal elongation effect seen on EV-treated DRG. Interestingly, this effect has already been reported in the growth of sensory neurons in vitro and in vivo regeneration after spinal cord injury, where miRNA-21 promotes neurite outgrowth by regulating PTEN and SPRY2 (Jiang et al., 2017; Jansuwan et al., 2020).

It has been demonstrated that nerve stimulation activates pro-regenerating programs in both neurons and glial cells (Willand et al., 2016), including an increase in the secondary messenger cAMP in peripheral neurons (Aglah et al., 2008; Udina et al., 2008) and the upregulation of neurotrophic factors by neurons and SCs (Al-Majed et al., 2000; Huang et al., 2010; Sharma et al., 2010; Wan et al., 2010). In the PNS, neurons release ATP during the firing of action potentials, and after nerve injury, this ATP then activates purinergic receptors in SCs, which lead to Ca2+ spikes in these cells and cause the activation of ERK/CREB, a signaling pathway with a key role in peripheral nerve regeneration (Negro et al., 2016). Our work establishes an unexpected mechanism by which neurons can modulate axonal elongation in a non-cell autonomous fashion, which involves the modulation of the miRNA cargo of SC-derived EVs. Overall, our data provide evidence about new checkpoints that could serve to develop strategies to enhance axonal elongation in conditions where regeneration is greatly impaired.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material; further inquiries can be directed to the corresponding author.

Ethics statement

The animal study was reviewed and approved by CBB, Universidad Mayor, Santiago, Chile.

Author contributions

CS: planning and executing experiments and writing the manuscript. RC, RL-L, RR, and DN: planning and executing experiments. UW, CL, and FC: planning experiments. All authors contributed to the article, reviewed the final manuscript and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.
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Supplementary material
The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fncel.2022.943506/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Particle transfer detection protocol. (A) Images were taken using a size of 1,024 × 512 pixels, and each stack was taken every 0.2 µm with a speed of 200 Hz. Background levels were normalized using the software LAS X based on the control image corresponding to not infected (GFP-palp SC and DRG co-cultures incubated with the primary anti-GFP and secondary antibody Alexa 488). The value of filter settings was 7, which allowed the total subtraction of the non-specific signal. Each image was deconvolved in 3D in the software LASA using a total of 10 iterations, under the blind method, and then the images were analyzed using IMARIS 9.2.1 software. Scale bar, 25 µm. (B) For each channel, a surface was created using the voxel number algorithm. The SC surface reconstruction (green channel) was performed with a detail of 0.4 µm and a threshold setting of 0.5, and subsequently, a mask that contains the voxels outside of this surface was used for analysis. The axon surface analysis (red channel) was performed with a detail of 0.2 µm and a threshold setting of 5, and a mask of this was created for analysis. The voxels outside the surface mask of the green channel were colocalized with the surface of the red channel mask, adjusting the threshold to a value of 2 for all images. The colocalization mask was analyzed with the spot detection tool, detecting particles between 80 and 400 nm. The particle density was determined by taking the number of spots and dividing for the axonal volume. Scale bar, 20 µm.

SUPPLEMENTARY FIGURE 2
TTX and apyrase treatments do not affect exosome release from rSC. rSCs were treated with ATP, TTX, and/or apyrase, and the conditioned media from each condition was centrifuged to purify EVs. Samples were measured by NTA to analyze the release of particles in 1 h. In all quantifications, average and SEM values of at least three independent experiments are shown (*p < 0.05).

SUPPLEMENTARY FIGURE 3
Internalization of rSC exosomes by DRG neurons. Exosomes were isolated from control or ATP-treated rSCs transfected with CMV-palp-eGFP to visualize EVs. (A) DRG explants were treated with 5 µg of EVs for 3 h, washed, and immunostained against neurofilament heavy chain (NF-H, red) and GFP (green). Scale bar, 2 µm. (B) The axon-exosome colocalization percentage was obtained from deconvolved z-stack confocal images by measuring the eGFP mean staining area colocalized with the NF-H staining. Scale bar, 15 µm. In all quantifications, average and SEM values of at least three independent experiments are shown.

SUPPLEMENTARY TABLE 1
Protein quantification by NanoOrange and particle count by Nanosight.

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ABSTRACT

Extracellular vesicles (EVs) are released by most cell types and present in all human bodily fluids. EVs contain genetic material and proteins that can be transferred to and generate an effect in other cells. Caveolin-1 (Cav1) is a key component of the small invagination of the plasma membrane called caveolae, where it functions as mechano-sensors and membrane tension buffering device. Cav1 expression is often increased in the late stages of cancer. Interestingly, high levels of Cav1 have been found in EVs of patients with advanced cancer. Given the importance of mechanical forces in the microenvironment of cancer cells, we hypothesized that caveolae and/or Cav1 may represent key players in the regulation of EV biology and cancer progression under mechanical strain. To test this hypothesis, we subjected different cancer cell lines, having (WT) or deleted for Cav1 expression (KO) to 2D or 3D systems of mechanical stress. We found a striking increase in the release of EVs after mechanical stress both in 2D and 3D models. This increase was strictly dependent on the presence of Cav1 and correlated with enhanced fusion of multivesicular bodies to the plasma membrane. Lipidomic analysis revealed differences in the lipid composition of EVs in Cav1KO cells and after mechanical stress, suggesting a difference in the properties of these EVs. Finally, EVs from mechanically stressed cells were shown to promote enhanced migration and invasion phenotype in breast cancer cells, whereas EVs from Cav1 depleted cells did not. These data allow us to conclude that mechanical stress is associated with increased release of EVs and the acquisition of metastatic traits in receiving cells in vitro, with Cav1 being a key player of this process.

KEYWORDS

Caveolae, extracellular vesicles, mechanical stress, cancer