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DECODING ADAPTIVE CELLULAR MECHANISMS OF CISPLATIN CHEMORESISTANCE IN OVARIAN CANCER CELLS

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EVALUACIÓN

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Become the laboratory by standing still, or sitting on the cushion provided. Proceed to do nothing. Relax your posture and attitude, and observe, with a light touch, whatever comes into experience. That's the experiment. Note the specific manifestation of mind as if they were data. Repeat as many times as you can this gesture of full presence, of mindfulness. The laboratory is now portable and you may carry it with you wherever you go. Keep track of your findings!

The Portable Laboratory. Francisco J. Varela.

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RESUMEN

El cáncer de ovario (OvCa) es una enfermedad agresiva cuyo tratamiento habitual es la terapia basada en cisplatino (CDDP). Sin embargo, entre los distintos tipos de cánceres tratados con CDDP, el OvCa es el que más desarrolla quimioresistencia a esta droga, planteándose que los exosomas podrían jugar un papel central en la adquisición de esta propiedad. Los exosomas se forman como vesículas intraluminales (ILVs) dentro de cuerpos multivesiculares (MVBs) organelos que participan al menos en tres vías; 1) la fusión con la membrana plasmática (PM) para la secreción de exosomas, proceso regulado por proteínas de la familia RAB GTPasas, siendo la RAB27A la más estudiada; 2) la vía degradativa donde los MVBs fusionan con los lisosomas, un evento controlado por la RAB7; y 3) la vía de anfisomas donde los MVBs se fusionan con los autofagosomas, organelos híbridos que participan en secreción de exosomas (fusión con la PM) y en degradación (fusión con lisosomas). Distintos estudios indican que una pérdida de la función lisosomal pudiera potenciar la secreción de exosomas, sin embargo se desconoce si la adquisición de quimioresistencia del OvCa al CDDP es el resultado de una fina regulación de estos eventos celulares se desconoce. En este trabajo de tesis se caracterizaron las diferentes estructuras asociadas a los eventos mencionados anteriormente, los MVBs, lisosomas, y anfisomas, mediante diferentes estrategias tanto de biología celular, molecular y bioquímicas, y se compararon entre las células de OvCa sensibles al CDDP (A2780) y resistentes al CDDP (A2780cis). Nuestros resultados muestran que las células A2780cis tienen un mayor número de MVBs/ILVs y estructuras tipo anfisomas. Además, las A2780cis poseen niveles elevados de RAB27A y proteínas asociadas a la maquinaria ESCRTs, en comparación con las células A2780. Contrariamente, pudimos definir que las células A2780cis tienen una actividad lisosomal disminuida, explicada en parte por un número reducido de lisosomas, y de niveles de RAB7. En conjunto, nuestros resultados sugieren que la resistencia al CDDP pudiera ser el resultado de una mayor secreción de exosomas vía MVBs/ILVs y/o anfisomas como consecuencia de una disfunción lisosomal. Interesantemente, el silenciamiento de RAB27A en las células A2780cis causó un incremento robusto en la función lisosomal, sugiriendo la activación de un mecanismo compensatorio en respuesta a una reducida secreción vía MVBs y/o anfisomas. Sorprendentemente, esta reversión fenotípica del estado funcional de los lisosomas promueve quimiosensibilidad al CDDP en las células A2780cis. Además, ya que las células A2780cis presentan un incrementado número de estructuras tipo anfisomas, evaluamos el efecto de la reducción de estos organelos mediante el silenciamiento de FIP200, un gen clave en la biogénesis de los autofagosomas. Inesperadamente, el silenciamiento de FIP200 causó una reducción de los niveles de RAB27A y de estructuras CD63, junto a un aumento en los niveles de RAB7, similar a lo observado al bloquear la secreción de exosomas. Este resultado nos permite proponer una estrategia contra la quimioresistencia al CDDP en el OvCa por afectar la secreción de exosomas a través de la inhibición en la biogénesis de autofagosomas. Con todos

estos hallazgos este trabajo de tesis doctoral ha permitido definir que la quimioresistencia de células OvCa a CDDP es controlada por la actividad lisosomal, organelos que definirían el potencial secretor de exosomas vía MVBs y/o anfisomas. Molecularmente, se pudo determinar que estos procesos están regulados por un fino equilibrio entre los niveles de RAB27A/RAB7. La identificación de blancos moleculares que cambien este equilibrio, tal como ocurre con el silenciamiento de FIP200, abre una posibilidad de considerar la disrupción de estos procesos como un potencial blanco terapéutico que revierta la adquisición de la quimioresistencia al CDDP en el OvCa.

Palabras claves: Quimioresistencia, Exosomas, Lisosomas

ABSTRACT

Ovarian cancer (OvCa) is an aggressive disease usually treated with cisplatin-based therapy (CDDP). However, among the different types of cancers treated with CDDP, OvCa is the most common type of cancer that develops chemoresistance to this drug, where exosomes are proposed to play a central role in the acquisition of this property. Exosomes are formed as intraluminal vesicles (ILVs) within multivesicular bodies (MVBs) organelles that participate in at least three pathways; 1) the fusion with the plasma membrane (PM) for exosomal secretion, a process regulated by proteins of the RAB GTPase family, where RAB27A is the most studied; 2) the degradative pathway where MVBs fuse with lysosomes, an event controlled by RAB7; and 3) the amphisome pathway where MVBs fuse with autophagosomes, hybrid organelles involved in exosome secretion (fusion with the PM) and degradation (fusion with lysosomes). Different studies indicate that a loss of lysosomal function could enhance exosome secretion; however, whether the acquisition of OvCa chemoresistance to CDDP is the result of a fine regulation of these cellular events is unknown. In this thesis work, the different structures associated with the aforementioned events, MVBs, lysosomes, and amphisomes were characterized by different cell biological, molecular, and biochemical strategies and compared between CDDP-sensitive (A2780) and CDDP-resistant (A2780cis) OvCa cells. Our results show that A2780cis cells have a higher number of MVBs/ILVs and amphisome-like structures. In addition, A2780cis possess elevated RAB27A levels and ESCRTs machinery-associated proteins compared to A2780 cells. Conversely, we were able to define that A2780cis cells have diminished lysosomal activity, explained in part by a reduced number of lysosomes and levels of RAB7. Taken together, our results suggest that CDDP resistance could be the result of increased exosome secretion via MVBs/ILVs and/or amphisomes as a consequence of lysosomal dysfunction. Interestingly, RAB27A silencing in A2780cis cells caused a robust increase in lysosomal function, suggesting the activation of a compensatory mechanism in response to a reduced secretion via MVBs and/or amphisomes. Surprisingly, the reversal phenotype of the lysosomal status promoted chemosensitivity to CDDP in A2780cis cells. Furthermore, since A2780cis cells exhibit an increased number of amphisome-like structures, we evaluated the effect of a reduction in these organelles by FIP200 silencing, a key gene in autophagosome biogenesis. Unexpectedly, FIP200 silencing caused a downregulation in the levels of RAB27A and CD63 structures, together with an increase in the RAB7 levels that are similar to our findings on the blockage of exosomes secretion. This result allows us to propose a strategy against CDDP chemoresistance in OvCa by affecting exosome secretion through the inhibition of autophagosome biogenesis. With all these findings, this doctoral thesis work enables us to define that the chemoresistance of OvCa cells to CDDP is controlled by lysosomal activity, organelles that would define the exosome secretory potential via MVBs and/or amphisomes. Molecularly, we determined these processes are regulated by a fine balance between RAB27A/RAB7 levels. The

identification of molecular targets that caused a change in this balance, as occurs with FIP200 silencing, opens a possibility to consider the disruption of these processes as a potential therapeutic target to reverse the acquisition of CDDP chemoresistance in OvCa.

Keywords: Chemoresistance, Exosomes, Lysosomes

1 INTRODUCTION

1.1 OVARIAN CANCER AND TREATMENT

The World Health Organization (WHO) defines cancer as a large group of diseases that can start in almost any organ or tissue when abnormal cells grow uncontrollably, with the ability to invade and spread to other organs. In 2018, WHO reported that around 18.1 million women worldwide had cancer, and 9.6 million died due to this disease (Bray et al., 2018).

1.1.1 Cancer development

The origin of cancer is diverse and, in many cases, unknown. However, several aspects are involved, including genetic mutation to epimutation, from a viral infection to exposure to chemical or physical agents. Also, health risk factors and lifestyle could facilitate the emergence and development of cancer (Diori Karidio & Sanlier, 2021). The development of this disease, termed carcinogenesis, is a multistep and multifactorial molecular process that involves interactions between cells and the environment of the organism (Cerda-Troncoso et al., 2021; Diori Karidio & Sanlier, 2021). Carcinogenesis consists of three different stages: initiation, promotion, and progression (Marongiu et al., 2018; Pitot, 1993). The first stage implicates irreversible genetic variations in normal cells, promoted by a carcinogen, followed by a reversible process regulated by epigenetic modifications, stimulating the clonal expansion of the altered cells, initiates to express a malignant phenotype and acquisition of aggressive characteristics (Conti, 2010; Oliveira et al., 2007; Pitot, 1993). In the progression stage, altered cells increase the incidence of genetic abnormalities that contribute to developing several cellular capacities, including resistance to cell death, metabolic reprogramming, high proliferative capacity, induction of angiogenesis, evasion of the immune system, and activation of the metastatic flux (Hanahan & Weinberg, 2011). Additionally, new emergent biological processes such as cellular plasticity, the microbiota composition, and senescent program activation contribute to the cancer malignancy and its progression (Hanahan, 2022). The process of

metastases is a major cause of death from cancer that involves the capacity of cancer cells to migrate and invade the surrounding tissues, colonizing and growing in the new site, through intravasation in the circulatory or lymphatic system, survival to the circulation, and new site extravasation (Fares et al., 2020; He et al., 2018; Nguyen et al., 2009).

1.1.2 Ovarian Cancer

Ovarian cancer (OvCa) is one of the most common gynecologic cancers (Reid et al., 2017) and represents 47% of deaths associated with genital cancer in women (Berek et al., 2021). In Chile, it has been reported that OvCa is the ninth leading cause of death among the different types of cancer in women, with a linear increase in deaths associated with this cancer in the last 15 years (Cuello F, 2013). This cancer has a lower prevalence but is three times more lethal. The high mortality rate of ovarian cancer is caused by asymptomatic and secret growth of the tumor (Momenimovahed et al., 2019).

Several risk factors are associated with ovarian cancer where more than one-fifth are hereditary. Between 65–85 percent of cases are associated with a germline mutation in *BRCA* genes. In parallel, around 36 percent of ovarian cancer cases are related to genetic syndromes, such as Lynch syndrome and Li-Fraumeni syndrome (Berek et al., 2021; Roett & Evans, 2009; Toss et al., 2015). Also, other risk factors linked to physiological, health, and lifestyle conditions have been summarized (Momenimovahed et al., 2019; Reid et al., 2017; Roett & Evans, 2009). According to its cellular origin, this cancer is classified into three types: epithelial (85 to 95 percent of incidence), stromal (5 to 8 percent of incidence), and germinal (3 to 5 percent of incidence) (Roett & Evans, 2009).

The Federation of Gynecology and Obstetrics (FIGO) has classified epithelial ovarian cancer into four stages, from I to IV, according to the localization of the tumor and its spread from where it originated (Berek et al., 2021). Around two-thirds of all epithelial ovarian cancers are in Stage III (the tumor involves one or both ovaries or fallopian tubes, with confirmed spread to the peritoneum outside the pelvis and metastasis to the retroperitoneal lymph nodes) or Stage IV (distant

metastasis in the extra-abdominal and parenchymal liver or spleen involvement, excluding peritoneal metastases) at diagnosis (Berek et al., 2021; Katopodis et al., 2019).

1.1.3 Cisplatin Treatment for Ovarian Cancer

The current treatment for patients with ovarian cancer involves primary debulking surgery followed by platinum-based and/or taxane-based combination chemotherapy (Kim et al., 2018; Roett & Evans, 2009). For platinum-based, cisplatin (CDDP) is the most used chemotherapy. It was the first FDA-approved platinum compound for cancer treatment in 1978 (Dasari & Bernard Tchounwou, 2014; Helm & States, 2009). Cisplatin structurally is a coordinated compound with square planar geometry. This entry to the cells is by passive diffusion through the plasmatic membrane (PM), or by facilitated diffusion through copper transporters Ctr1 and Ctr2, and organic cation transporters OCT1 and OCT2 principally (Makovec, 2019). Once internalized into cells, CDDP is spontaneously activated through aquation reactions involving substituting cis-chloro groups with water molecules. As a product of these reactions, the CDDP is transformed into an electrophilic compound, acquiring the affinity towards sulfhydryl groups on proteins and nitrogen donor atoms in nucleic acids (Galluzzi et al., 2012; Tchounwou et al., 2021).

The mechanisms of action of CDDP could be divided into nuclear and cytoplasmic sections. In terms of nuclear effects, aquated CDDP binds to DNA on purine bases, forming DNA–DNA inter-and intra-strand adducts (Galluzzi et al., 2012), causing significant alteration of the DNA, blockage cell division, and promoting the induction of apoptosis. It has been reported that apoptosis-induction by CDDP is principally through p53, p38 mitogen-activated protein kinase (p38 MAPKs), c-jun N-terminal kinases (JNK), or c-Abl activation (Alderden et al., 2006; Tchounwou et al., 2021).

Concerning cytoplasmic effects, CDDP can generate ROS directly through mitochondrial damage (through binding to mitochondrial DNA, by the alteration of the function of voltage-dependent anion channel) or by induction of lipid

peroxidation that increases carbonylation of proteins and induces oxidative damage of cell membranes, promoting apoptosis (Galluzzi et al., 2012; Tchounwou et al., 2021). Induction of Ca^{+2} release from the endoplasmic reticulum by CDDP treatment activates the calcium-dependent protease Calpain and consequently the Caspase-3 (Al-Bahlani et al., 2011). Similarly, the depolarization of the mitochondrial inner membrane induced by CDDP promotes Cytochrome-C release and subsequently Caspase-3 activation. Finally, the activation of this Caspase promotes apoptosis (Tchounwou et al., 2021).

Initially, OvCa patients have an excellent response to CDDP, however, in ovarian cancer patients, it has been observed that a significant percentage of initially sensitive tumors develop chemoresistance (Galluzzi et al., 2012; D. W. Shen et al., 2012). This has led to a decrease in the clinical effectiveness of CDDP, and the ovarian cancer survival rate has not improved significantly (Kim et al., 2018; D. W. Shen et al., 2012). Therefore, efforts are addressed to investigate and understand the mechanisms of CDDP chemoresistance in ovarian cancer related to cellular adaptive processes, knowledge that could help to the development of new therapies to be used as co-treatment.

1.2 CHEMORESISTANCE TO CDDP IN OVARIAN CANCER

The use of chemotherapeutics focuses on the ability of these drugs to decrease tumor size or induce short-term remission by induction of apoptosis or controlling cell division (Mollaei et al., 2021). Cancer cells may acquire resistance to chemotherapy as an adaptive mechanism to survive (Hasan et al., 2018; Qin et al., 2020), but chemoresistance is a significant contributor to the drastic number of deaths in all cancers (Hayatudin et al., 2021). In terms of CDDP chemoresistance, epithelial ovarian cancer can be classified into two groups: CDDP-resistant, which corresponds to patients relapsing before six months following the treatment completion; and CDDP-sensitive, which corresponds to patients relapsing at least six months after the completion of treatment (Binju et al., 2019; Davis et al., 2014). The CDDP-sensitive patients with relapse can be re-treated with CDDP-based therapy with good clinical effectiveness. However, it

has been identified that half of these newly treated patients develop CDDP resistance (Binju et al., 2019; Davis et al., 2014; Kim et al., 2018).

The acquisition of chemoresistance in the tumor through different cellular and molecular mechanisms produces changes in cell identity. These changes are given by adaptive processes that evade the effects of chemotherapy, known as cancer cell plasticity (Qin et al., 2020; Tchounwou et al., 2021; Yuan et al., 2019). Consistent with this, cellular and molecular mechanisms of chemoresistance to cisplatin in ovarian cancer can include CDDP reduction entry inside cells; DNA repair systems, metabolic reprogramming, cell death inhibition; epigenetic response; and adaptive cellular processes (Ai et al., 2016; Chan et al., 2021; Chapman-Rothe et al., 2013; Du et al., 2016; Fraser et al., 2008; Green & Ferguson, 2001; Hudson et al., 2016; Norouzi-Barough et al., 2018; Patch et al., 2015; Schneiderman et al., 1999; Stordal et al., 2012; Strathdee et al., 1999; Tchounwou et al., 2021; Tong et al., 2019; Xie et al., 2021; Zeller et al., 2012).

1.2.1 Adaptive cellular processes

In recent years, the role of various adaptive cellular processes has been explored in chemoresistance to cisplatin in ovarian cancer. One mechanism is related to Gap junctions (GJs), PM channels that participate in cell-cell communication (Hervé & Derangeon, 2013). GJs consist of two hemichannels known as connexons, composed of six transmembrane proteins called connexins (Cxs). Interestingly, A2780cis CDDP-resistant ovarian cancer cells show a higher expression of Cx32 at the PM than CDDP-sensitive cells (W. Wu et al., 2017), facilitating the efflux of CDDP to the extracellular milieu (Y. Zhang et al., 2019).

Another adaptive response is related to the mitochondrial function. Cisplatin promotes mitochondrial damage in ovarian cancer (Galluzzi et al., 2012; Tchounwou et al., 2021). Intriguingly, CDDP-resistant ovarian cancer cells have an increase in mitochondrial function compared to sensitive cells, thus reducing the effect of CDDP on these organelles, which in part can be explained by an increased mitochondrial turnover rate through mitophagy (Zampieri et al., 2020). Together with the role of mitochondria, CDDP also impacts the function of the

endoplasmic reticulum (ER). CDDP induces dysregulation in ER calcium homeostasis promoting stress-triggered apoptosis (Mandic et al., 2003), due to the close contact between the ER and mitochondria, ER-stress promotes a flow of calcium from ER to mitochondria, causing a mitochondrial calcium overload and amplification of pro-apoptotic signaling (Sano & Reed, 2013). In this way, CDDP-resistant ovarian cancer cells showed tolerance to calcium outflow from the ER by the induction of ER-stress by cisplatin compared with the respective sensitive cells, probably due to a decrease in ER-mitochondrial contacts or by an increased mitochondrial turnover (XU et al., 2015; Zampieri et al., 2020).

Among all the adaptive cellular mechanisms described another pathway that has become relevant in CDDP resistance in ovarian cancer is macroautophagy (usually called autophagy). This catabolic pathway engulfs cytoplasmic materials into double-membrane structures named autophagosomes, which subsequently are fused with lysosomes to form autolysosomes, where the materials are degraded by acidic hydrolytic enzymes (Cerda-Troncoso et al., 2021). Autophagy can regulate cancer therapy efficacy and resistance (Poillet-Perez et al., 2021). For example, mitophagy (selective autophagy of mitochondria) improves mitochondrial turnover to evade the toxic effect of CDDP on this organelle in cisplatin-resistant ovarian cancer cells (Zampieri et al., 2020). ***However, the role of autophagy in chemoresistance to cisplatin in ovarian cancer is still poorly understood.***

1.3 EXTRACELLULAR VESICLES IN CHEMORESISTANCE

In addition, cells can secrete cellular material in response to cytotoxic compounds such as CDDP offering another mechanism for chemoresistance. The secretion of cellular material is mediated by a heterogeneous group of extracellular vesicles (EVs) that eliminate unneeded compounds from the cell, or transfer nucleic acids, lipids, or proteins as a mechanism for cell-cell communication through nucleic acids, lipids, or proteins (Van Niel et al., 2018). Based on their biogenesis, EVs can be classified into apoptotic bodies, 50 nm to 5 μm vesicles released

undergoing apoptosis; ectosomes, with a range of 100 nm to 1 μm in diameter, formed by direct outward budding from the PM; and exosomes, 30–100 nm vesicles, originated from the endocytic compartment and secreted upon fusion of these compartments with the PM (Doyle & Wang, 2019; Mathieu et al., 2021; Van Niel et al., 2018). Exosomes are nanosized extracellular vesicles of an endosomal origin, constitutively released from cells (Steinbichler et al., 2019; Van Niel et al., 2018). In several cancer cell models an increase in exosome secretion in response to the chemotherapy has been observed, probably by promoting their biogenesis and activating its secretion (Bandari et al., 2018; Lv et al., 2012; Y & J, 2021). In chemoresistance, EVs including exosomes could play a role in homeostasis maintenance through the expulsion of cytotoxic components produced by chemotherapy, or even releasing the chemotherapeutic drug into EVs, but also transferring chemoresistance through microRNAs, long non-coding RNAs (LncRNAs), drug efflux receptors, and proteins related to tumor progression into neighboring sensitive cells (Mosquera-Heredia et al., 2021; Steinbichler et al., 2019; S. Yu et al., 2015; He-da Zhang et al., 2018). Recently, the term chemo-EVs, that corresponds to EVs (including exosomes, referred as Chemo-Exosomes), secreted in response to chemotherapy has been introduced (Bandari et al., 2020). In ovarian cancer, CDDP promotes the secretion of chemo-exosomes from CDDP-resistant cells as a mechanism of drug expulsion (Safaei et al., 2005). However, the cellular and molecular mechanisms that mediate the enhancement of secretion of exosomes or chemo-exosomes from chemo resistant ovarian cancer cells are poorly understood (Cossart & Helenius, 2014; Elkin et al., 2016; Kiss & Botos, 2009; Mayor et al., 2014). Thus, the study of these emerging mechanisms opens a door to the development of new therapies to curb CDDP chemoresistance in ovarian cancer.

1.3.1 Biogenesis of exosome in endosomes

The exosomes are formed as intraluminal vesicles (ILVs) by the process of inward budding in the endosomal membrane during the maturation of multivesicular bodies (MVBs) and are secreted due to its fusion with the PM (Klumperman &

Raposo, 2014; Van Niel et al., 2018). Because MVBs are part of the endolysosomal network (Klumperman & Raposo, 2014), it is necessary to comprehend this cellular network to understand the regulation of biogenesis and secretion of exosomes.

The endolysosomal network is required for multiple functions and control of homeostasis maintenance (Klumperman & Raposo, 2014). This network started with early endosomes (EEs), which receive almost all components internalized from the cell surface in the endocytosis (Cossart & Helenius, 2014; Elkin et al., 2016; Kiss & Botos, 2009; Mayor et al., 2014). EEs have a structure with tubular and vacuolar domains, with a mosaic of sub-domains in their limiting membrane (Huotari & Helenius, 2011). EEs could be fused homeotypically or maturate to form late endosomes (LEs), also referred to as MVBs (Klumperman & Raposo, 2014; Rink et al., 2005), through a mechanism dependent on RAB5 GTPase (Langemeyer et al., 2018; Zerial & McBride, 2001).

As mentioned, the formation of ILVs (future exosomes) begins in EEs and continues in the endosome maturation process to MVBs/LEs with the result that these structures contain more ILVs (Huotari & Helenius, 2011; Klumperman & Raposo, 2014; Van Niel et al., 2018; Vietri et al., 2020). The biogenesis of ILVs/exosomes is mediated by a mechanism dependent on the endosomal sorting complex required for transport (ESCRT) and ESCRT-independent (Van Niel et al., 2018).

In ESCRT-dependent, this machinery plays a key role in ILVs/exosome biogenesis and protein sorting to these vesicles (Vietri et al., 2020). In the cytosolic surface of the EEs membrane enriched in phosphatidylinositol 3-phosphate [PtdIns(3)P] (Kutateladze, 2006; Raiborg et al., 2013), clathrin forms a coat to deform the membrane to facilitate clustering of cargos by ESCRT-0, composed of HRS and STAM1/2 (Huotari & Helenius, 2011; Hurley & Hanson, 2010; Raiborg et al., 2002; Vietri et al., 2020; Wollert & Hurley, 2010). The protein sorting for the inclusion into ILVs/exosomes is marked by lysine-63-linked polyubiquitin chains recognized by HRS (Raiborg et al., 2002; Vietri et al., 2020). After, ESCRT-I (TSG101, VPS28, VPS37, MVB12A) is recruited, enhancing

cargo clustering, principally through ubiquitin-binding domains (UBA) of TSG101 (Hurley & Hanson, 2010; Vietri et al., 2020; Wollert & Hurley, 2010); and promoting ESCRT-II (VPS22, VPS25, VPS36) recruitment, which together initiates inclusion of cargos through membrane buds (Boura et al., 2012; Hurley & Hanson, 2010; Vietri et al., 2020; Wollert & Hurley, 2010). Finally, ESCRT-I through ESCRT-II or the adaptor protein Bro1/ALG-2-interacting protein X (ALIX) promotes nucleation and stabilization that drives the polymerization of ESCRT-III (CHMP2A/B, CHMP3, CHMP4A/B/C CHMP6) filaments (Christ et al., 2017; Friand et al., 2015; Hurley & Hanson, 2010; Vietri et al., 2020). These filaments interact with the endosomal membrane, facilitating neck constriction of buds and vesicle scission, ending the ILVs/exosome biogenesis (Christ et al., 2017; Vietri et al., 2020; Wollert & Hurley, 2010). After, ESCRT-III polymers are disassembled to be recycled by AAA+ ATPase Vps4 to disassemble ESCRT-III polymers (Hurley & Hanson, 2010).

In addition, three ESCRT-independent mechanisms have been described: Ceramide-Sphingomyelin, Tetraspanines, and Syndecan–Syntenin–ALIX dependent pathways (Andreu & Yáñez-Mó, 2014; Baietti et al., 2012; Friand et al., 2015; Verderio et al., 2018). The type-II sphingomyelinase in the membrane of endosomes hydrolyzes the sphingomyelin into ceramide (Verderio et al., 2018). The generation of ceramide enriched subdomain in endosomes promotes the curvature of the membrane generating ILVs (Goñi & Alonso, 2009). In the Ceramide-Sphingomyelin pathway, the activation of Sphingosine 1-Phosphate Receptor mediates the incorporation of cargos into ILVs (Kajimoto et al., 2013). The Tetraspanines (proteins with four transmembrane domains) in MVBs can form microdomains that promote inward budding and ILVs, incorporating proteins and RNA (Andreu & Yáñez-Mó, 2014; Van Niel et al., 2018). In the Syndecan–Syntenin–ALIX pathway, Syndecans (abundant ubiquitous transmembrane proteins) are recognized by Syntenin in EEs and during their maturation promote a cluster of Syndecan-Syntenin. This cluster mediates the inward budding of membrane and ILVs biogenesis through the interaction of Syntenin with ALIX and

the participation of some ESCRT-I, -II and -III proteins (Baietti et al., 2012; Friand et al., 2015).

1.3.2 RAB-GTPases and exosome secretion

The membrane trafficking steps, such as biogenesis, transport, tethering, and membrane fusion events are regulated by the RAB family of small GTPases. These molecules function as molecular switches, cycling between GTP-bound and GDP-bound states (Cavieres et al., 2020; Homma et al., 2021). These processes are controlled by guanine nucleotide exchange factors (GEFs), which catalyze the exchange of GDP for GTP; and GTPase-activating proteins (GAPs), which accelerate the intrinsic GTPase activity of a RAB, promoting the hydrolysis of GTP to GDP (Hutagalung & Novick, 2011; Stenmark, 2009). In the active state, each RAB associates with its specific subcellular compartment for the recruitment of specific effectors, orchestrating different membrane trafficking steps (Grosshans et al., 2006; Zhen & Stenmark, 2015).

The RAB11A, RAB22A RAB27A/B, RAB31, and RAB35 have been reported to mediate trafficking and fusion of endosomes structures with the PM for release of ILVs in the form of exosomes, under basal condition or specific stimulus (Jin et al., 2021; Messenger et al., 2018; Ostrowski et al., 2010; T. Wang et al., 2014; Wei et al., 2021). Among these, RAB27 is the most studied. This consists of two isoforms, RAB27A and RAB27B, encoded by different genes (Mitsunori Fukuda, 2013). RAB27 mainly regulates the secretion of exosomes derived from MVBs/LEs (M. Fukuda, 2008; Van Niel et al., 2018). It has been characterized that basal and induced exosome secretion is regulated by RAB27 (Dorayappan et al., 2018; Ostrowski et al., 2010). Several reports indicate that particularly the RAB27A is associated with poor cancer prognosis (Koh & Song, 2019; X. Li et al., 2017; Q. Wang et al., 2015; F. Yu et al., 2020), mediating tumor progression (Bobrie et al., 2012; Dorayappan et al., 2018; Feng et al., 2016; Hendrix & De Wever, 2013; Kren et al., 2020; J. Li et al., 2017; W. Li et al., 2013; Liu et al., 2017; J.-S. Wang et al., 2008; X. Wu et al., 2013; F. Yu et al., 2020), and participates in chemoresistance through exosome secretion in numerous cancers

(Hertzman Johansson et al., 2013; J. Li et al., 2017; X. Li et al., 2017; Liu et al., 2017). ***However, the role of RAB27A and the other RABs associated with exosome secretion has not been studied in CDDP-resistance in ovarian cancer.***

1.4 LYSOSOME STATUS AND AUTOPHAGOSOMES: NEW KEYS IN REGULATION OF EXOSOME SECRETION

The pathway of exosome secretion is interconnected with a degradative pathway of endosomes. The MVBs/LEs fused with the lysosomes, degrading the contents incorporated into ILVs (Eitan et al., 2016; Huotari & Helenius, 2011; Klumperman & Raposo, 2014). This trafficking is governed by RAB7A (Bucci et al., 2000; Hyttinen et al., 2013; Vanlandingham & Ceresa, 2009a) and contributes to the maintenance and biogenesis of lysosomes (Bae et al., 2019; Huotari & Helenius, 2011). The sorting mechanisms governing the differential delivery of MVBs/LEs to the PM or on route to lysosomes are unknown (Eitan et al., 2016). However, cumulative evidence in some pathologies suggests that inhibition of the delivery of MVBs/LEs to one direction may stimulate the other as a compensatory response (Adams et al., 2021; Eitan et al., 2016; Guix et al., 2021; D. Huang et al., 2022; Miranda et al., 2018; Ortega et al., 2019; Strauss et al., 2010; van de Vlekkert et al., 2019; Villarroya-Beltri et al., 2016; J. Zhang et al., 2021). Interestingly, CDDP-resistant cells have a decrease in lysosome function compared to sensitive cells (Guerra et al., 2019; Kalayda et al., 2008; Safaei et al., 2005). Instead, CDDP-resistant cells release a higher number of exosome secretion suggesting these two pathways are highly regulated (Safaei et al., 2005).

As mentioned in section 1.2.3, the autophagosome engulfed cytoplasmic materials send them to the lysosomes for their degradation by acidic hydrolytic enzymes (Cerda-Troncoso et al., 2021). For cargo recognition and incorporation into autophagosomes, cytoplasmic material is recognized by autophagy cargo receptors, such as p62/SQSTM1 and NBR1 (Mancias & Kimmelman, 2016). These receptors can bind to autophagosomes by interacting with the ATG8s

family proteins (LC3A, LC3B, LC3C, GABARAP, GABARPL1, and GABARAPL2), which are associated with the membrane of autophagosomes (Johansen & Lamark, 2020; Kabeya et al., 2004). The trafficking and fusion of autophagosomes with lysosomes are mediated by RAB7A (Hyttinen et al., 2013) and are highly dependent on their degradative status of lysosomes (X. Chen et al., 2021; Kawai et al., 2007; J. Wang et al., 2017; A. Yamamoto et al., 1998). However, autophagosomes can also be fused with MVBs/LEs, forming a hybrid organelle known as amphisomes (Berg et al., 1998; Ganesan & Cai, 2021), that finally fused with lysosomes for degradation. Alternatively, amphisomes can be fused with the PM mediating the secretion of the cytoplasmic content within autophagosomes together with exosomes. Interestingly, recent reports indicate that amphisomes could promote the exosome secretion (Hu et al., 2020; Peng et al., 2021) in a lysosomal dysfunction context (Peng et al., 2021). Moreover, in CDDP-resistant A2780cis ovarian cancer cells autophagosomes play a positive role in chemoresistance (Bao et al., 2015) by a mechanism poorly understood.

One relevant problem of cell biology related with deaths associated with ovarian cancer is the development of CDDP resistance, the main chemotherapeutic agent used for this cancer. With the presented evidence suggesting that MVBs/ILVs biogenesis and exosomal secretion could promote CDDP chemoresistance of OvCa cells, a process that could be controlled by the status of lysosomal, we propose the following hypothesis:

2 HYPOTHESIS

“The chemoresistance of ovarian cancer cells to CDDP is dependent on an increase in the secretory MVBs pathway as a consequence of reduced lysosomal function”

3 OBJECTIVE

Characterize the biogenesis of MVBs, their secretion, and the lysosomal compartments between CDDP-sensitive (A2780) and CDDP-resistant (A2780cis) OvCa cells.

3.1 SPECIFIC OBJECTIVES

- Analyze components of biogenesis and MVBs secretion between CDDP-sensitive (A2780) and CDDP-resistant (A2780cis) cells.

- Analyze components of lysosomes and their function between CDDP-sensitive (A2780) and CDDP-resistant (A2780cis) cells

- Evaluate the effect of disruption of MVBs secretion on CDDP resistance in CDDP-resistant (A2780cis) cells.

4 MATERIALS AND METHODS

4.1 MATERIALS

4.1.1 Biological Material

The ovarian cancer cell lines A2780 (CDDP-sensitive, cat#93112519) and A2780cis (CDDP-resistant, cat#93112517) were obtained from Sigma-Aldrich (St. Louis, MO, USA). The cell line Lenti-X™ 293T Cell Line (HEK293T, cat#632180) was obtained from Takara Bio USA, Inc (San Jose, CA, USA).

4.1.2 Chemical Reagents

Amersham™ Protran® Nitrocellulose (cat#GE1060002), Cisplatin (CDDP, cat#479306), Gelatin Type A from porcine skin (cat#G-2500), L-Poly-Lys (cat#P4707), 2-Mercaptoethanol (cat#M3148), Saponin (cat#S4521), Sulforhodamine B (SRB, cat#230162), puromycin dihydrochloride (cat#P8833), and protease inhibitors cocktail (cat#P8340) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Methanol (cat#1060092500) Acetic Acid (cat#100064.1000), Trichloroacetic acid (TCA, cat#100807.0250), and HEPES Free Acid (cat#391340) were purchased from Merck Millipore (Burlington, MA, United States). BCA Kit (cat#23225), DMEM without red phenol (cat#21063-029), Fetal Bovine Serum (FBS) free exosome (cat#A27-208-01), glycerol (cat#15514-011), LysoTracker™ Red DND-99 (cat#L7528), OptiMEM (cat#31985-070), One Shot™ Stbl3™ *E.coli* (cat#C737303), Pen/Strep (cat#15140-122), RNAi Max (cat#13778-075), TRIzol™ (cat#15596018), Trypsin-EDTA 0,05% (cat#25300-54), West Pico (cat#34577), West Dura (cat#34076) and 4′,6-diamidino-2-phenylindole (DAPI) (cat#D1306) were purchased from ThermoFisher Scientific (Waltham,126 MA, USA). Acrylamide: Bisacrilamide 29:1 30% (cat#BM0100), bromophenol blue (cat#A2-0395), $CaCl_2$ (cat#E506), $MgCl_2$ (cat#E255), Ponceau Red (cat#BM-1492), Winkler) were purchased from Winkler (Santiago, Chile). Ampicillin Sodium Salt (cat#A2260), Sodium dodecyl sulfate (SDS, cat#C15081006) and Triton X-100 (cat#T8655) were purchased from USBiological life science (Salem, Massachusetts, USA). Fluoromount-G

(cat#17984-25) and Paraformaldehyde 16% Solution EM grade (PFA cat#15710) were purchased from Electron Microscopy Science (Hatfield, PA 19440, USA). Glycin (cat#FER00G500G) and Tris Base (cat#FER00B500) were purchased from Fermelo Biotec (Santiago, Chile). RPMI (cat#SH30255.02) and Dulbecco's Modified Eagle Medium High Glucose (DMEM-HG cat# SH30243.02) were purchased from Cytiva (Marlborough, MA, USA). AccuRuler RGB Plus Ladder Protein (cat#02102-250) was purchased from MaestroGen (Hsinchu City, Taiwan, China). Ammonium peroxodisulfate (APS, cat#0486-256) was purchased from Avantor-VWR (Radnor, PA, USA). Bovine Serum Albumin (BSA, cat#BSA-05), was purchased from Rockland Immunochemicals (Pottstown, PA, USA). FBS (cat#04-127-1A) was purchased BI Biological Industries/Sartorius (Aubagne, France). dNTPs (dATP cat#U1205, dCTP cat#U1225, dGTP cat#U1215, and dTTP cat#U1235), Go taq G2 Flexi (cat#M780B), 5x Green Go taq Flexi Buffer (cat#M891A), and 25 mM MgCl2 (cat#A3803) was obtained from was obtained from Promega (Madison, WI, USA). Magic Red® (cat#6133) and 2'-[4'-etoxifenil]-5-[4-metilpiperazin-1-il]-2,5'-bis-1h- benzimidazole trihidrocloruro trihydrate (Hoechst, cat# 639) were purchased from Immunochemistry Technologies, LLC (Bloomington, IN, USA). LB Broth Powder Growth (Miller) (cat#12106-1) was obtained from MO BIO Laboratories, Inc (Carlsbad, CA, USA). LB-agar medium (cat#113002232) was purchased from MP Biomedical (Irvine, CA, USA). M-MuLV Reverse Transcriptase (cat#M0253S) was purchased from New England Bio-Labs® (Ipswich, MA, USA). Phosphate Buffered Saline (PBS, cat#46-013 CM) was purchased from Corning Inc. (Glendale, Arizona, USA). Tween-20 (cat#SC29113B) was purchased from ChemCruz™ Biochemicals-Santa Cruz Biotechnology (Dallas, TX, USA). SsoAdvanced Universal SYBR® Green Supermix kit (cat#172571) was obtained from Bio-Rad (Hercules, CA, USA).

4.1.3 Antibodies

The following monoclonal antibodies were used: mouse anti-ALIX (cat# sc-53540), mouse anti-RAB7 (cat# sc-376362), mouse anti-RAB11A (cat#SC166912), and β-actin (cat#SC47778) from Santa Cruz Biotechnology,

Dallas, TX, USA; mouse anti-CD63 (cat# ab8219) and rabbit anti-RAB22A (cat# ab137093) from Abcam, Cambridge, UK; rabbit anti-FIP200 (cat# 172501-AP) from ProteinTech, Rosemont, IL, USA; mouse anti-LAMP1 clone H4A3 (cat# DSHBH4A3) and mouse anti-LAMP2 clone H4B4-c (cat#DSHBH4B4) from Developmental Studies Hybridoma Bank, Iowa City, IA, USA; mouse anti-TSG101 (cat# 612696) and mouse anti-p62/SQSTM1 (cat# 810832) from BD Bioscience, Becton, NJ, USA. The following polyclonal antibodies: goat anti-CATHEPSIN-D (cat#AF1014) from R&D Systems, Minneapolis, MN, USA; rabbit anti-HGS (HRS) (cat#ab15539) from Abcam, Cambridge, UK; rabbit anti-LC3 (cat#2775S) and rabbit anti-RAB35 (cat# 9690) from Cell Signaling Technology, Danvers, MA, USA; rabbit anti-RAB27A (cat#168013) from Synaptic Systems, Göttingen, Germany. Horseradish peroxidase-conjugated secondary antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA, USA), Alexa fluorophore-conjugated secondary antibodies were purchased from Thermo Fisher Scientific.

4.1.4 Plasmids and oligos

The shRNA was used as a control for Luciferase in pLKO.1 (containing a puromycin resistance gene) obtained from MISSION® (Sigma-Aldrich, cat#SHC007). The shRNA for RAB27A was cloned in pLKO.1 and donated by Matias Ostrowski Ph.D., Buenos Aires, Argentina (Gerber et al., 2015). The following sequences were used for shRNA-Luciferase: 3’ CCGG**CGCTGAGTACTTCGAAATGTC**CTCGAG**GACATTTCGAAGTACTCAG CG**TTTTT-5’, and shRNA-RAB27A: 3’-CCGG**CGGATCAGTTAAGTGAAGAAA**CTCGAG**TTTCTTCACTTAACTGATC CG**TTTTT-5’. The plasmids pCMV-*VSV-g* (viral envelope vector, cat# 12260) and pS-*PAX2* (packaging vector, cat#8454) were obtained from Addgene (Watertown, MA, USA). The following siRNAs were ordered from Dharmacon/Horizon Discovery (Boyertown, PA, USA): siRNA non-target 5’-UUCUCCGAACGUGUCACGU-3’ and siRNA FIP200 5’-GGAGAUUUGGUACUCAUCAUCA-3’. The following primers for qPCR were

ordered from Integrated DNA Technologies IDT (Coralville, IA, USA). Mycoplasm detection: 5'-GGGAGCAAACAGGATTAG-3', 3'-TGCACCATCTGTCACTCTGTTAACCTC-5'; *GAPDH*: 5'-GGAAGATGGTGATGGGATTTC-3', 3'-GAAGGTGAAGGTCGGAGTCAA-5'; and *RAB27A*: 5'-ATGGAACGGTGTGTGGACAA-3', 3'-CCACATGCCCCTTTCTCCTT-5'.

4.2 METHODS

4.2.1 Cell culture

HEK 293T cell line obtained from Takara Bio were cultured in Dulbecco's modified Eagle's medium High Glucose (DMEM-HG; HyClone), supplemented with 10% heat-inactivated fetal bovine serum (FBS; BI Biological Industries), penicillin 100 U/mL, and streptomycin 100 mg/mL (Gibco). A2780 (CDDP-sensitive) and A2780cis (CDDP-resistant) cell lines were cultured in Roswell Park Memorial Institute 1640 (RPMI; HyClone), supplemented with 10% heat-inactivated fetal bovine serum (FBS; Life Technologies), L-glutamine 300 mg/mL, HEPES 25 mM, penicillin 100 U/mL and streptomycin 100 mg/mL (Gibco). Every two weeks in culture or every three passages, A2780cis cells were treated with 1 μM CDDP for 72 h, then CDDP was removed to seed the cells for experiments. The three cell lines were cultured at 37°C, in a humid environment and 5% CO_2 until the required density was obtained. Each 2-3 days, cells were sub-cultured using Trypsin (0.05% Trypsin, 1 mM EDTA) at 37°C. Alternatively, they were frozen in 1.5 mL of freezing medium (10 % v/v DMSO, 90 % v/v FBS) and stored in cryotubes at -80°C.

Periodically, the presence of mycoplasmas was evaluated by PCR. Briefly, 1 mL of culture medium was obtained from cells at 60-80% confluence. Then was centrifuged at 15,000 RPM for 5 min. After, 900 μL was discarded and then vortexed the remaining 100 μL. For PCR, 2 μL of sample were taken for the mix (1X green Go Taq Flexi buffer, 100 μM dNTPs, 200 μM of each primer, 3 mM $MgCl_2$, 0,1 μL Go taq G2 Flexi Enzyme, ultra-pure H_2O up to 20 μL). The thermal

profile of the reaction was: 95°C for 20 sec; 30 cycles of 95°C for 30 sec followed by 60°C for 30 sec and 68ºC for 1 min; 68ºC for 5 min; and hold time at 4ºC to its analysis by 2% agarose electrophoresis.

4.2.2 Bacterial transformation and plasmid DNA isolation

To prevent homologous recombination of Long Terminal Repeats (LTRs) found in pLKO.1 vector, we transformed the bacteria One Shot™ Stbl3™ *E.coli* (Invitrogen™, ThermoFisher), designed for this propose, according to the manufacturer's protocol. The transformed bacteria with pLKO.1 vector was grown in LB-Agar with 100 μg/mL Ampicillin 16 h at 37ºC. After an isolated colony of transformed bacteria was selected and inoculated in 2 mL of LB with 100 μg/mL Ampicillin. The inoculum was incubated at 37ºC in a shaker at 200 rpm for 8 h. Then, to plasmid DNA isolation, a second inoculum was made in 100 mL of LB with 100 μg/mL Ampicillin in a 1:1000 dilution from the 2 mL and was incubated at 37ºC in a shaker at 200 rpm for 16 h. The plasmid DNA isolation was performed with NucleoBond Xtra Midi kit (Machery-Nagel) according to the manufacturer's protocol. The isolated plasmid DNA was quantified using the spectrophotometer EPOCH2 microplate reader (BioTek).

4.2.3 Generation of stable Knockdown cell lines through shRNA Lentiviral Particles

We generated stable A2780cis ovarian cancer cell lines with reduced levels of RAB27A by introducing shRNA-containing lentiviral particles. shRNA against the luciferase gene was used as a control. Lentiviral particles were generated by co-transfection of HEK293T cells with pLKO.1-*shRNA* constructs (8 μg), pCMV-*VSV-g* (viral envelope vector, 4 μg), and pS-*PAX2* (packaging vector, 8 μg). For co-transfection, HEK293T cells were grown on a cell culture plate in DMEM supplemented with 10% FBS, 0.1 mM non-essential amino acids, 6 mM L-Glutamine, penicillin 100 U/mL, streptomycin 100 mg/mL, and 500 μg/mL Geneticin.

Transfection was performed with the calcium phosphate technique. Briefly, 4 h before transfection, the medium of the cells was changed. Plasmid DNAs were diluted in 500 µL of 0.25 M $CaCl_2$, making up the volume to 520 µL with nuclease-free water. Then, 520 µL of 2X HBS (50 mM HEPES Free Acid, 280 mM NaCl, 10 mM KCl, 1.5 mM Na_2HPO_4, 12 mM Dextrose, pH 7.1) was maintained under vortex while the dilution of plasmid DNAs in $CaCl_2$ was added dropwise. After the mix, HBS|$CaCl_2$|DNA was left incubating for 20 min at RT and added dropwise to the cells. Sixteen hours post-transfection, the medium of the cells was changed to OptiMEM (Gibco), without serum, penicillin 100 U/mL, and streptomycin 100 mg/mL. After 60 h post-transfection, the medium containing the virus produced by the cells was collected and centrifuged at 1000 rpm for 5 min to eliminate cellular debris. The supernatant was subsequently filtered on a 0.45 µm filter. Before virus concentration, 2 mL of 25% TNE-Sucrose was added to the ultracentrifuge tubes, and then the filtered medium containing the viruses was added. Ultracentrifugation was then performed at 28,000 rpm for 1:30 h at 4°C in a Hitachi P28S orbital rotor. The viral particles were then resuspended in PBS 1X and left in an orbital rotor at 4°C for 2 h, and storage to -80°C.

For transduction with lentivirus, A2780 and A2780cis cells were grown on a 24 well tissue culture plate overnight, then added 15 µL of respective concentrated lentivirus and cells cultivated at a temperature of 37°C, in a humid environment and 5% CO_2 until 80% density was obtained. Before, cells were sub-cultured using Trypsin (0.05% Trypsin, 1 mM EDTA) at 37 °C and seeded on a 6 well tissue culture plate. Once a density of 80% density was obtained, the cells were washed with PBS 1X, and fresh medium supplemented with 6 µg/mL puromycin was added for selection. The selected stable cells were expanded, and for maintenance, they were grown in a medium containing 3 µg/mL puromycin.

4.2.4 Transient Knockdown in cell lines through siRNA transfection

We generated transient A2780cis ovarian cancer cell lines with reduced levels of FIP200 by transfection with target siRNA target FIP200 mRNA (Gan et al., 2005). Non-targeting siRNA was used as a control. Transfections were carried out in 35

mm tissue culture plates using the Lipofectamine RNAiMax transfection reagent (Thermo Fisher Scientific) according to the manufacturer's protocol for two transfections with siRNA. After 72 h, cells were collected or treated for further analysis.

4.2.5 Preparation of protein extracts and western blotting

Cells were washed in cold phosphate buffer saline (PBS, corning) and subjected to lysis at 4°C in RIPA lysis buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 5 mM EDTA, 0.1% SDS, 1% Deoxycholate, 1% NP-40, pH 7.4) supplemented with a cocktail of protease inhibitors (Sigma-Aldrich). Lysates were sonicated at 4°C, at minimum amplitude for three seconds. After, all lysates were cleared by centrifugation at 16,000 g for 20 min, and protein concentration was determined with BCA Protein Assay Kit (Thermo Fisher Scientific, USA). Samples with equivalent amounts of protein were boiled for 5 min with Laemmli SDS-PAGE sample buffer and analyzed by SDS-PAGE. Proteins were electroblotted onto nitrocellulose membranes of 0.22 μm and incubated sequentially with primary and secondary antibodies for 1 h at room temperature or overnight at 4ºC. Chemiluminescence protein detection was performed using Pierce Western Blotting Substrate (Thermo Scientific). As an internal loading control, β-actin levels were examined on the same blots. Quantification of blots was carried out using FIJI software.

4.2.6 RNA isolation and relative quantitative real-time polymerase chain reaction

RNA was isolated from cells using TriZol (Invitrogen), following the manufacturer´s recommendations. RNA yield was quantified using the spectrophotometer EPOCH2 microplate reader (BioTek). Reverse transcription of RNA (2 μg) was undergone in a 25 μl reaction, using oligo(dT) and Moloney Murine Leukemia Virus Reverse Transcriptase Kit (M-MLV RT, NEB). For Relative Quantitative Real-Time PCR was performed using 5 μl of ssAdvanced Universal SYBR® Green Supermix (Bio-Rad), 1 μl cDNA (1:25), and 0.6 μl of 10

μM gene-specific primer mix for a total reaction volume of 12 μl. Quantification of gene expression was performed using the Rotor-Gene (QIAGEN). The thermal profile of the reaction was: 95°C for 1 min and 40 cycles of 95°C for 10 sec followed by 60°C for 15 sec and 72ºC for 20 sec. All samples were run, at least in triplicate. Amplification of the sequence of interest was normalized with an endogenous reference gene glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*). Values were expressed as *RAB27A* levels relative to *GAPDH* levels.

4.2.7 Transmission electron microscopy

Cells grown on 60 mm cell culture plates were processed in the advanced microscopy unit of the Pontificia Universidad Católica de Chile. Briefly, cells were fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.0) at room temperature overnight. Then, washed with a cacodylate buffer for 2 h with three changes. After, cells were postfixed with 1% aqueous osmium tetroxide for 2 h and then rinsed with double distilled water and stained in a block with 1% uranyl acetate for 90 min. They were dehydrated with a battery of acetone at increasing concentrations (50%, 70%, 95%, and 100% twice) for 20 min each. Cells were pre-included with epon:acetone 1:1 overnight and then embedded in pure epon. Polymerization was carried out in an oven at 60 °C for 48 hr. Finally, thin slices were obtained (80 nm) on a Leica Ultracut R ultramicrotome and stained with 4% uranyl acetate in methanol for 2 min and with lead citrate for 5 min. The sections were observed under a Philips Tecnai 12 microscope (Eindhoven, The Netherlands) at 80 kV.

4.2.8 Indirect immunofluorescence

Cells grown on glass coverslips were washed with PBS 1X and fixed in 4% (v/v) paraformaldehyde for 30 minutes or in methanol for 5 minutes at room temperature according to the antibody used. After fixation, cells were washed in PBS and permeabilized with 0.2% (v/v) Triton X-100 in PBS for 10 min at room temperature. Cells were incubated with the indicated primary antibodies diluted in immunofluorescence buffer (PBS containing 10% (v/v) FBS and 0.1% (w/v)

saponin for 30 min at 37 ºC. Coverslips were washed in PBS and incubated with the corresponding Alexa-conjugated secondary antibody diluted in immunofluorescence buffer for 30 min at 37 ºC. Cells were washed with PBS for nuclei staining and incubated for 10 min at room temperature with 0.1 μg/mL 4′6-diamidino-2-phenylindole (DAPI). After the final wash, coverslips were mounted onto glass slides with Fluoromount-G (Electron Microscopy Science, USA).

4.2.9 Fluorometric lysosomal assays with fluorescent probes

We evaluated the alteration of the pH of lysosomes using the LysoTracker Red DND-99 probe (Invitrogen). Cells grown on live-cell imaging plates before the assay was performed. After, cells were incubated with 100 nM of this probe in RPMI medium for the last 20 min of treatments at 37°C, in a humid environment and 5% CO_2. Cells were washed twice with PBS 1X, and the nucleus was stained with 2'-[4'-etoxifenil]-5-[4-metilpiperazin-1-il]-2,5'-bis-1h- benzimidazole trihidrocloruro trihydrate (Hoechst, Immunochemistry Technologies, LLC) for 10 min at 37°C, in a humid environment and 5% CO_2 and finally washed 3 additional times with PBS 1X. Cells were maintained in DMEM medium with 25 mM HEPES for image capture in fluorescent confocal microscopy under 577 nm excitation conditions.

In parallel, we measured the activity of Cathepsin B, a lysosomal member of the papain-like family of cysteine proteases, with MagicRed kit (Immunochemistry Technologies, LLC). Cells grown on live-cell imaging plates and previously to assay were treated with 5 μM CDDP for 24 h. Then, cells were loaded with MagicRed RPMI medium probe for the last 20 min of treatments at 37°C, in a humid environment, and with 5% CO_2. Cells were washed twice with PBS 1X, and the nucleus was stained with Hoechst (Immunochemistry Technologies, LLC) for 10 min at 37°C, in a humid environment and 5% CO_2, and finally washed 3 additional times with PBS. Cells were maintained in DMEM medium with 25 mM HEPES for image capture in confocal microscopy for image capture in fluorescent confocal microscopy under 577 nm excitation conditions.

4.2.10 Fluorescent confocal microscopy

Fluorescence microscopy images were acquired using a TCS SP8 laser-scanning confocal microscope (Leica Microsystems, Wetzlar, Germany) equipped with a 63x oil immersion objective (1.4 NA) running the Leica Application Suite LAS X software. We quantified the number of positive structures per cell, the average fluorescence intensity, and the area of positive structures of the signal of MagicRed, LysoTracker-Red, LAMP1, LAMP2, CD63 and LC3B as required. Briefly, 16-bit images were identical settings avoiding signal saturation, and measurements were executed using ICY software (Quantitative Image Analysis Unit, Institut Pasteur, http://icy.bioimageanalysis.org/). A pipeline was created to completely automate image analysis by using the following sequential plugins: active contours (cell segmentation), hk-means (threshold detection), and wavelet spot detector (spot detection). For quantification of Manders coefficient colocalization, we used FIJI software version 2.1.0 (http://imagej.net/ software/fiji/) (Schindelin et al., 2012) plus the Just Another Colocalization Plugin (JACoP, 2.1.1 version) (BOLTE & CORDELIÈRES, 2006), adjusting the threshold of the images respect to control.

4.2.11 Isolation of extracellular vesicles

Extracellular vesicles (EVs) derived from A2780, and A2780cis cells without treatment or with 5 μM cisplatin treatment were isolated from conditioned medium (CM) by differential centrifugation. Approximately $13x10^6$ cells were seeded on 150 cm^2 plates in RPMI-1640 medium supplemented with 5% exosome-free FBS (A2780, A2780cis) or RPMI-1640 supplemented with 5% exosome-free FBS and 5 μM CDDP (A2780cis+CDDP). 24 h post-seeding the CM was collected and centrifuged at: (i) 300 × g for 5 min at 4°C, (ii) 2,000 × g for 20 min at 4°C, (iii) 10,000 × g for 35 min at 4°C with Himac P70AT rotor, (iv) 100,000 × g for 75 min at 4°C with Himac P70AT rotor. After each centrifugation step, the pellet was discarded, and the supernatant was used for the next step. The resulting pellet from step four was washed in PBS (filtered on 0.2 μm filter) and ultracentrifuged at 100,000 × g for 75 min at 4°C with Himac P70AT rotor to remove contaminating

proteins. The resulting pellet was resuspended in PBS 1X and stored at -80 °C until further use.

4.2.12 Nanoparticle tracking analysis of isolated extracellular vesicles enriched in exosomes

The size distribution and concentration of isolated EVs derived from A2780, A2780cis, A2780cis treated with cisplatin (A2780cis+CDDP) were evaluated by nanoparticle tracking analysis (NTA). The EVs were diluted in PBS and analyzed on the Nanosight NS300 (Malvern, Universidad de los Andes) using a 532 nm laser and a 565 nm filter, and camera level parameters of 9 and detection threshold of 3.

4.2.13 Sulforhodamine B assay for the determination of the lethal dose 50 (LC_{50})

Cells were grown on 96 well tissue culture plates. 6 h after seeding, the different doses of CDDP were added and cells cultivated at 37 °C, in a humid environment and 5% CO_2 for 24 h. Then, cells were fixed in a final concentration of 20% trichloroacetic acid at 4ºC for 1 h. After fixation, cells were washed in distilled H_2O four times, discarding the complete volume. Subsequently, cells were stained with 100 μL of 0.4% SRB (in 0.1% acetic acid) at room temperature for 1 hour. Cells were washed with 1% acetic acid four times. Stained cells were dried overnight, and then 100 μL of 10 mM Tris-base was added and left in orbital shaking for 30 min. After, the absorbance was then measured at 492 nm in a spectrophotometer EPOCH2 microplate reader (BioTek). The LC_{50} values associated with the cytotoxic effects of CDDP were calculated using Prism 9.0 (GraphPad Software, San Diego, CA, USA) using a non-linear regression model and variable slope model (log(agonist) vs. response). Two-way ANOVA analysis was performed to evaluate the differences between cell viability curves.

4.2.14 Quantifications and Statistical Analysis

Densitometric quantification of the immunoblot signal was estimated using FIJI software (Schindelin et al., 2012), version 2.1.0 (http://imagej.net/ software/fiji/). For each condition, protein bands were quantified from at least three independent experiments.

Data analysis from densitometric quantifications, real-time qPCR, electron microscopy transmission, fluorescence confocal microscopy, and SRB-LC_{50} were performed using Microsoft Excel 2021 (Microsoft Corporation) and Prism 9.0 (GraphPad Software, San Diego, CA, USA) for macOS Big Sur to generate corresponding charts and perform statistical analyses. Results are represented in graphs depicting the mean ± standard deviation (SD) or standard error of the mean (SEM) as indicated. Statistical significance was determined by non-parametric paired or parametric unpaired t-test, as indicated in each figure. P-values of *$p<0.05$, **$p<0.01$, and ***$p<0.001$ were regarded as statistically significant and are indicated in the figures.

5 RESULTS

5.1 A2780CIS CDDP-RESISTANT OVCA CELLS HAVE AN INCREASED MACHINERY FOR ILVS/EXOSOME BIOGENESIS.

Chemoresistant cells develop adaptative mechanisms to evade the cytotoxic effects produced by chemotherapy (S. H. Chen & Chang, 2019). In the CDDP-resistant cellular model of OvCa, it has been suggested that the secretion of exosomes (Endosomal EVs < 200 nm) is implicated in the chemoresistance process, an event that could be exacerbated with the impairment of the lysosome function (Guerra et al., 2019; Safaei et al., 2005). To understand the role of exosomes in chemoresistance in OvCa, we used A2780 CDDP-sensitive OvCa cells and A2780cis CDDP-resistant OvCa cells. We initially characterized the number of CD63 structures, one of the most accepted markers for MVBs/ILVs organelles that originated exosomes (Mathieu et al., 2021). By immunofluorescence we observed a higher number of CD63 structures in A2780cis cells compared to A2780 cells (Fig 1A, B). Consistently, we found an increase in the average intensity of CD63 in A2780cis compared to A2780 cells (Fig. 1B). Interestingly, the CD63 positive structures showed a higher area in A2780cis compared to A2780 cells (Fig. 1A, B), a phenotype that suggests an enhancement in ILVs biogenesis (Peng et al., 2021). Next, by transmission electron microscopy, we investigated the number of MVBs and ILVs per cell comparing these two different cell types. Again, we observed A2780cis cells have an increase in the number and area of MVBs (Fig. 1C, D), confirming our findings with CD63 in Fig. 1A, B. Additionally, A2780cis cells have an increase in the number of ILVs per MVBs compared to A2780 cells (Fig 1C, E). Due to these findings, we anticipated that A2780cis cells would have increased levels of the machinery involved in MVBs/ILVs biogenesis. To test this, we measured the levels of proteins involved in ILVs/exosome including ALIX, HRS, and TSG101 protein markers. Western blot analysis showed that a 1.62 ± 0.37-fold increase of ALIX, 1.40 ± 0.11-fold increase of TSG101, and 2.61 ± 0.18-fold increase of HRS proteins in A2780cis cells have compared to A2780 cells (Fig. 1F, G). These data

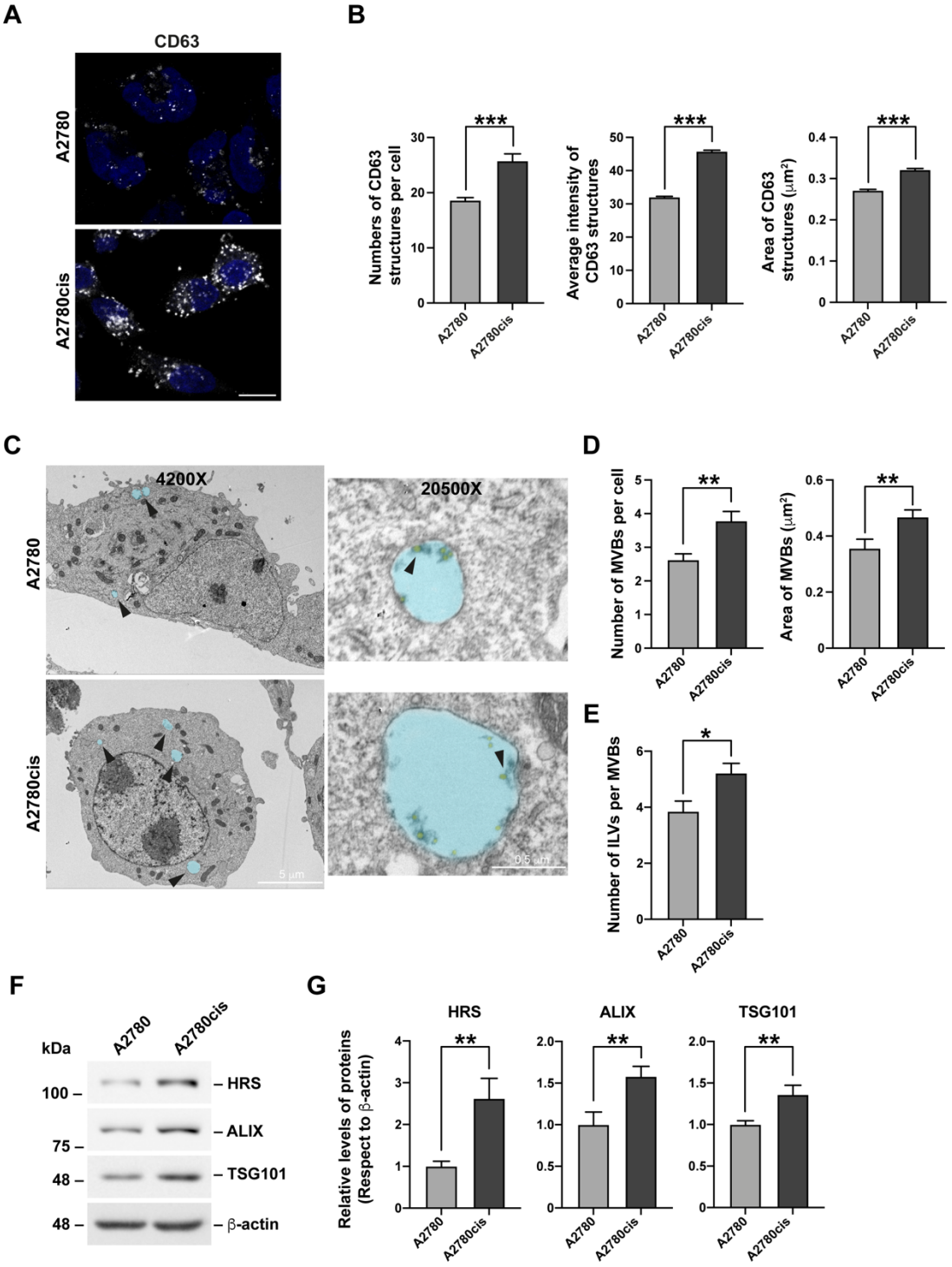

A
CD63
A2780
A2780cis
B
Numbers of CD63 structures per cell
Average intensity of CD63 structures
Area of CD63 structures (μm²)
***
A2780
A2780cis
C
4200X
20500X
A2780
A2780cis
5 μm
0,5 μm
D
Number of MVBs per cell
Area of MVBs (μm²)
**
E
Number of ILVs per MVBs
*
F
kDa
100 –
75 –
48 –
48 –
– HRS
– ALIX
– TSG101
– β-actin
G
HRS
ALIX
TSG101
Relative levels of proteins (Respect to β-actin)
**

Figure 1. Increased number of MVBs in CDDP-resistant OvCa cells. **(A)** Immunofluorescence detection of CD63 structures in A2780 and A2780cis, bar 10 μm. **(B)** Number, average intensity, and area of structure analysis of CD63 from (A) with SEM error bar; ***P<0.001; parametric unpaired t-Test. **(C)** Ultrastructural analysis of MVBs and ILVs from Transmission Electron Microscopy of A2780 and A2780cis. **(D)** Semiquantitative analysis of the number of MVBs per cell and their area of A2780 and A2780cis from (C), with SEM error bar; **P<0.01; Non-parametric t-Test. **(E)** Semiquantitative analysis of ILVs per MVBs in A2780 and A2780cis from (C), with SEM error bar; *P<0.05; parametric unpaired t-Test. **(F)** Western blot of endogenous protein levels of HRS, ALIX, TSG101, and β-actin from A2780 and A2780cis cells. **(G)** Densitometric quantification of Western blot indicated in (B) with SD error bar; **P<0.01; non-parametric paired t-Test.

suggest that A2780cis CDDP-Resistant OvCa cells have an increased capacity for MVBs/ILVs biogenesis and exosomal secretion.

5.2 THE RAB27A GTPASE IMPLICATED IN EXOSOMAL SECRETION IS OVEREXPRESSED IN A2780CIS CDDP-RESISTANT OVCA CELLS

A2780cis cells have high levels of the cellular machinery needed for MVBs/ILVs biogenesis. Increased ILVs biogenesis does not implicate an increase in the exosome secretion because several other proteins are necessary to regulate exosome secretion by MVBs-PM fusion. To investigate whether the increased number of MVBs could indicate a higher capacity of these cells to secrete exosomes, we evaluated the protein levels of several RAB GTPases described as regulators of exosomal secretion (Jin et al., 2021; Messenger et al., 2018; Ostrowski et al., 2010; T. Wang et al., 2014; Wei et al., 2021). Among all GTPases tested including RAB11A, RAB22A, RAB27A, and RAB35, only RAB27A was found differentially high expressed in A2780cis cells compared to A2780 cells. Indeed, we observed a 6.00 ± 1.48-fold increase in the levels of RAB27A in A2780cis cells compared to A2780 cells (Fig. 2A, B). To explain this increase, we measured the mRNA levels of RAB27A by RT-qPCR comparing A2780 and A2780cis cells. Our results indicated a significant 3.48 ± 0.18-fold increase in the RAB27A mRNA in A2780cis cells compared to A2780 cells indicating the increase observed in RAB27A GTPase is due to a differential gene expression regulation (Fig. 2C). These findings strongly suggest expression of RAB27A is likely to be highly implicated in the acquisition of chemoresistance in A2780cis cells.

Next, due to the well-documented role of RAB27A in exosomal secretion, we investigated the quantity of exosomes secreted in A2780 and A2780cis cells. For this, we measured exosomal fractions secreted from these cell lines for 24 h under basal conditions. In addition to this, we analyzed the secretion of exosomes in A2780cis upon CDDP-treatment during 24 h. Treatment of CDDP in A2780 cells was not tested as a previous study demonstrated that CDDP does not induce secretion of exosomes in A2780 cells (Samuel et al., 2018). We referred to exosomes as those fractions enriched in small vesicles <200 nm (Fig. 2D), as

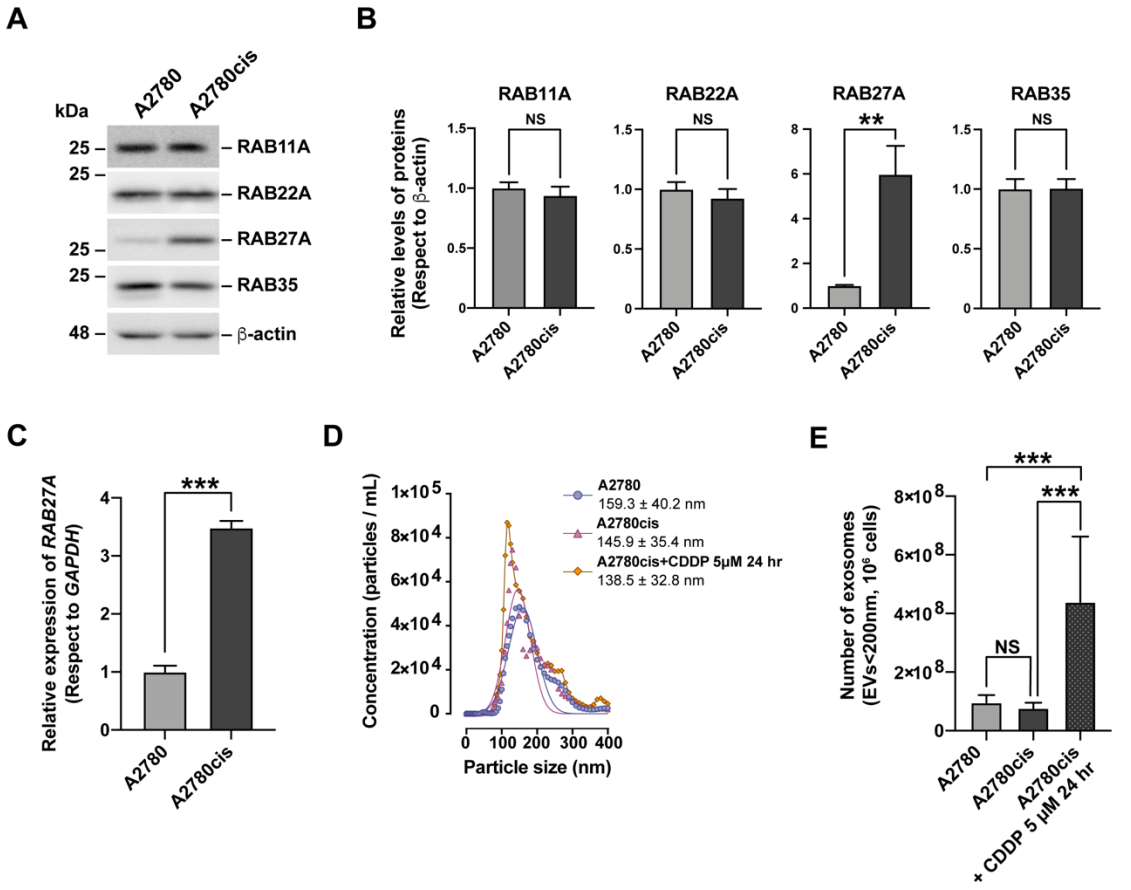


Figure 2. Increased levels of RAB27A in CDDP-resistant OvCa cells. **(A)** Western blot of endogenous protein levels of RAB11A, RAB22A, RAB27A, RAB35, and β-actin from A2780 and A2780cis cells. **(B)** Densitometric quantification of Western blot indicated in (A) with SD error bar; **P<0.01, NS: Not significant; non-parametric paired t-Test. **(C)** Analysis of RAB27A expression concerning GAPDH by qPCR from A2780 and A2780cis cells, with SD error bar; ***P<0.001; non-parametric paired t-Test. **(D)** Nano-tracking analysis from EVs isolated from A2780, A2780cis, and A2780cis cells treated with 5 μM of CDDP for 24 hrs., indicating the average particle size of the analyzed particles. **(E)** The number of exosomes (EVs<200 nm) per 10^6 cells from A2780, A2780cis, and A2780cis cells treated with 5 μM of CDDP for 24 hrs., with SD error bar; ***P<0.001, NS: Not significant; non-parametric paired t-Test.

previously described (Van Niel et al., 2018). Unexpectedly, we found A2780cis CDDP-resistant cells secreted exosomes at similar levels to A2780 CDDP-sensitive cells (Fig. 2E). However, a higher secretion of exosomes in response to CDDP was observed (Fig. 2E), indicating the pre-synthesized intracellular MVBs/ILVs organelles in A2780cis cells respond to specific stimulus, such as CDDP.

5.3 CDDP-RESISTANT A2780 OVCA CELLS SHOW AN IMPAIRED LYSOSOMAL FUNCTION IN COMPARISON TO QUIMIOSENSITIVE A2780 OVCA CELLS

Based on recent findings (Peng et al., 2021), the increased levels of molecular components for MVBs/ILVs biogenesis as well as the presence of larger MVB-like structures in A2780cis cells could be the result of disturbances in MVBs trafficking and function due to lysosomal dysfunction. In this context, it has been reported that C13 cells, a cellular model of OvCa CDDP-resistant cells, similar to A2780cis, have fewer and poorly functional lysosomes with respect to equivalent CDDP-sensitive cells (Guerra et al., 2019; Safaei et al., 2005). As a result of these antecedents, we investigated the levels and function of lysosomal organelles between A2780 and A2780cis cells. First, we evaluated the levels of the structural lysosomal membrane proteins including LAMP1 and LAMP2 by Western blot. We observed a significant 5.65 ± 1.47-fold decrease in LAMP1 in A2780cis cells compared to A2780 cells. Intriguingly, and contrary to the effect on LAMP1, we observed a significant 2.14 ± 0.06-fold increase in LAMP2 in A2780cis cells compared to A2780 cells (Fig. 3A, B). Three different isoforms due to an alternative splicing of LAMP2 have been described (Cuervo & Dice, 2000). Although LAMP2B has been implicated in the delivery of cargoes into MVBs/ILVs (Hung & Leonard, 2015; Z. Li et al., 2020; Liang et al., 2020) and in the biogenesis of exosomes (T. Yamamoto et al., 2022), with the tools available in our laboratory we decided to focus on the characterization of LAMP1 and Cathepsin D positive structures, two abundant proteins of lysosomal organelles (Lübke et al., 2009;

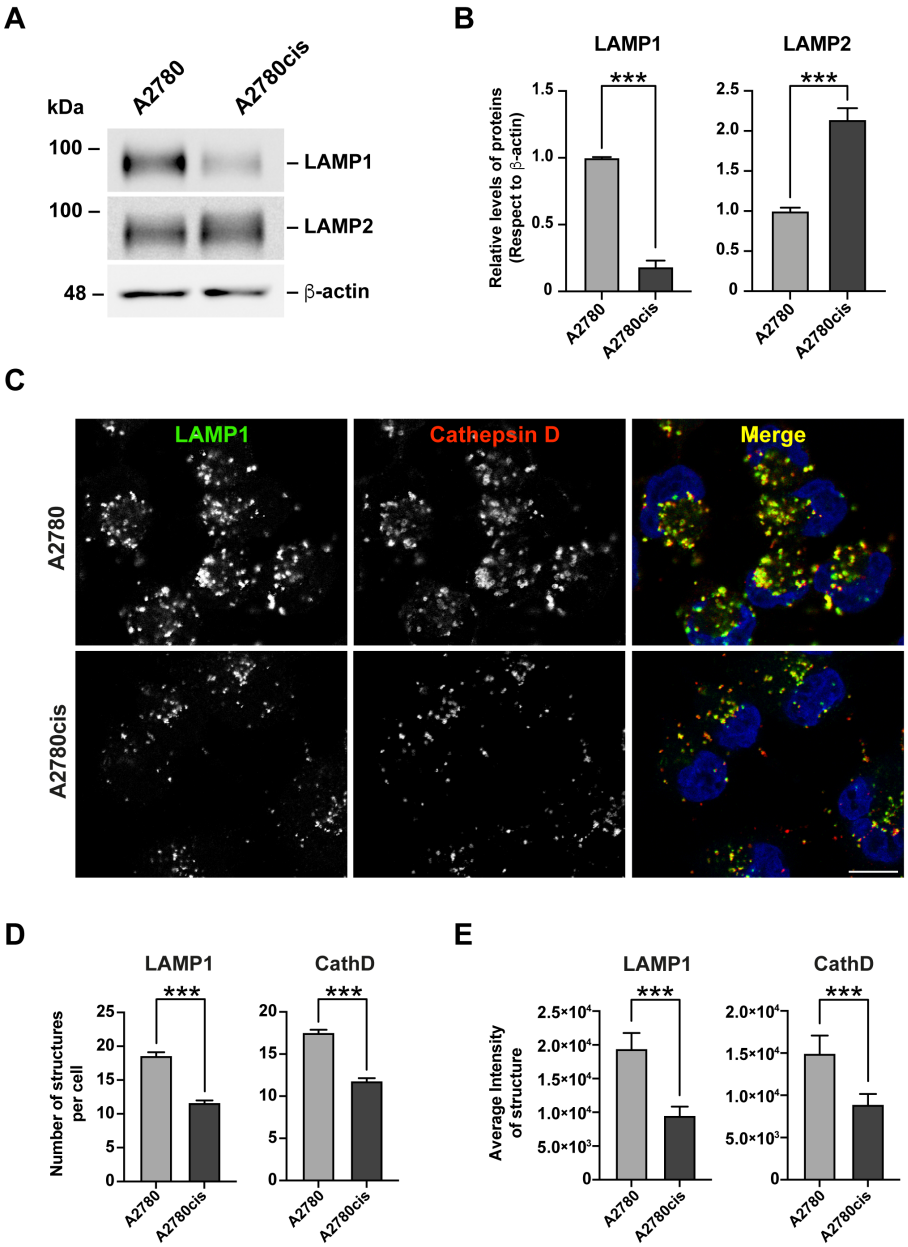

A
kDa
A2780
A2780cis
100 –
– LAMP1
100 –
– LAMP2
48 –
– β-actin
B
LAMP1
LAMP2
***
***
Relative levels of proteins (Respect to β-actin)
1.5
1.0
0.5
0
2.5
2.0
1.5
1.0
0.5
0
A2780
A2780cis
A2780
A2780cis
C
LAMP1
Cathepsin D
Merge
A2780
A2780cis
D
LAMP1
CathD
***
***
Number of structures per cell
25
20
15
10
5
0
20
15
10
5
0
A2780
A2780cis
A2780
A2780cis
E
LAMP1
CathD
***
***
Average Intensity of structure
2.5×10⁴
2.0×10⁴
1.5×10⁴
1.0×10⁴
5.0×10³
0
2.0×10⁴
1.5×10⁴
1.0×10⁴
5.0×10³
0
A2780
A2780cis
A2780
A2780cis

Figure 3. LAMP1 and Cathepsin D lysosomal-like structures are reduced in CDDP-resistant OvCa cells. **(A)** Western blot of LAMP1, LAMP2 (A, B, C isoform), and β-actin from A2780 and A2780cis cells. **(B)** Densitometric quantification of Western blot indicated in (A), with SD error bar, ***P<0.001; non-parametric paired t-Test. **(C)** Immunofluorescence detection of LAMP1 and Cathepsin D structures of A2780 and A2780cis cells; bar 10 μm. **(D)** The number of structures analysis of LAMP1 and Cathepsin D (CathD) from (C), with SEM error bar; ***P<0.001; parametric unpaired t-Test. **(E)** The average intensity of structure analysis of LAMP1 and CathD from (C), with SEM error bar; ***P<0.001; parametric unpaired t-Test.

Oberle et al., 2010; Pi et al., 2017). By immunofluorescence, we observed a strong decrease in LAMP1 and Cathepsin D structures in A2780cis compared to A2780 cells (Fig. 3C). Quantification analysis confirmed A2780cis shows a significant reduction in the number of LAMP1 and Cathepsin D positive structures, compared to A2780 cells (Fig. 3D). Similarly, the average intensity of LAMP1 and Cathepsin D positive structures was significantly reduced in A2780cis compared to A2780 cells (Fig. 3E). Next, we investigated whether these differences in LAMP1 and Cathepsin D lysosomal proteins were indicative of lysosomal function impairment. For this, we performed labeling of acidic compartments with the probe Lysotracker in living cells. Images obtained with confocal microscopy showed a significant reduction in the labeling of acidic structures in A2780cis compared to A2780 (Fig. 4A). Quantification analysis confirmed our findings, observing that A2780cis have a lower number of Lysotracker punctate structures compared to A2870 cells (Fig. 4B). Moreover, average intensity quantification also showed a significant decrease in this parameter in A2780cis compared to A2870 cells, an indicative of deacidification of acidic compartments (Fig. 4B).

Due to the fact that the acidic environment is critical for lysosomal hydrolase activities, we tested whether the reduction in pH in A2780cis cells caused a reduction in the activity of a lysosomal hydrolase. For this, live cells were labeled with the Magic Red probe, a tool used to quantify the activity of the lysosomal acidic hydrolase Cathepsin B (Kundu et al., 2018). Images obtained with confocal microscopy were analyzed to quantify average intensity of Magic Red positive punctate structures. This analysis showed a significant reduction in this parameter in A2780cis cells, with respect to A2780 cells (Fig. 4D). Finally, we tested the levels of RAB7, a GTPase that promotes cargo traffic to lysosomes (Bucci et al., 2000; Stroupe, 2018; Vanlandingham & Ceresa, 2009b). Surprisingly, we found that levels of RAB7 were significantly 1.77 ± 0.44-fold decreased in A2780cis compared to A2780 cells by Western blot (Fig. 4E, F). Together, all these findings demonstrate A2780cis cells have a reduced lysosomal function compared to

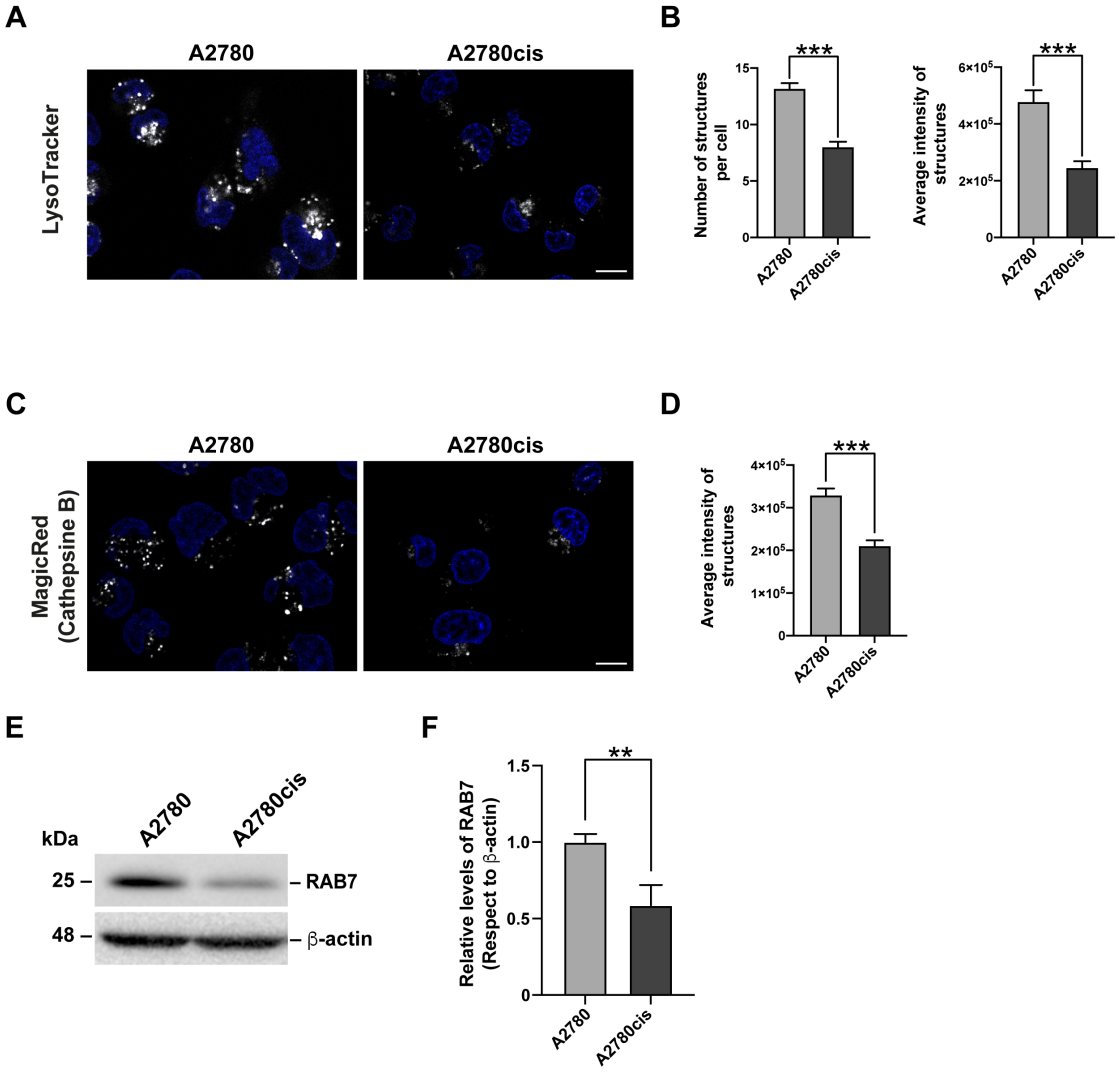

A
A2780
A2780cis
LysoTracker
B
Number of structures per cell
***
A2780
A2780cis
Average intensity of structures
***
A2780
A2780cis
C
A2780
A2780cis
MagicRed (Cathepsine B)
D
Average intensity of structures
***
A2780
A2780cis
E
kDa
A2780
A2780cis
25
RAB7
48
β-actin
F
Relative levels of RAB7 (Respect to β-actin)
**
A2780
A2780cis

Figure 4. Lysosomal acidity and function are reduced in CDDP-resistant OvCa cells. **(A)** Analysis of LysoTracker from A2780 and A2780cis cells; bar 10 μm. **(B)** The number and average intensity analysis of LysoTracker from (A), with SEM error bar; ***P<0.001; parametric unpaired t-Test. **(C)** Analysis of MagicRed probe from A2780 and A2780cis cells; bar 10 μm. **(D)** The average intensity analysis of MagicRed from (C); with SEM error bar; ***P<0.001; parametric unpaired t-Test. **(D)** Western blot of endogenous levels of RAB7A and β-actin from A2780 and A2780cis cells. **(E)** Densitometric quantification of Western blot indicated in (D), with SD error bar; **P<0.01; non-parametric paired t-Test. **(F)** Analysis of RAB27A expression concerning GAPDH by qPCR from A2780 and A2780cis cells, with SD error bar; ***P<0.001; non-parametric paired t-Test.

A2780 cells, a phenotype that could be related with a poor delivery of critical components by the reduction in key molecules such as RAB7.

5.4 RAB27A KD REESTABLISHES LYSOSOMAL FUNCTION IN CDDP-RESISTANT A2780CIS OVCA CELLS

Impairment of lysosomal activity correlated with an increased capacity of exosomal secretion has been described in some neurodegenerative diseases, suggesting the existence of a balance between lysosomal function and exosome secretion (Adams et al., 2021; Eitan et al., 2016; Guix et al., 2021; D. Huang et al., 2022; Miranda et al., 2018; Ortega et al., 2019; Strauss et al., 2010; van de Vlekkert et al., 2019; Villarroya-Beltri et al., 2016; J. Zhang et al., 2021). Our results indicate that A2780cis CDDP-resistant OvCa cells acquired the capacity to secrete more exosomes in response to CDDP probably as a compensatory response to lysosomal impairment. In this context, we investigated the potential effect of a blockage on exosomal secretion in A2780cis cells. Moreover, A2780cis cells have an increase in RAB27A expression, we performed stable silencing of RAB27A in A2780cis CDDP-resistant OvCa cells with the expression of a specific shRNA using lentiviral particle infection, as previously described (Blanc & Vidal, 2018; Bobrie et al., 2012; H. Huang et al., 2021; Ostrowski et al., 2010; Salimu et al., 2017). As control, A2780cis CDDP-resistant OvCa cells stably expressing a shRNA against luciferase named as shLuc cells were included. A2780/shLuc, A2780cis/shLuc, and A2780cis/shRAB27A puromycin resistant cells were tested for RAB27A expression by western blot. As expected, we observed a significant decrease of RAB27A protein levels in A2780cis/shRAB27A cells compared to A2780cis/shLuc cells, observing similar levels than in A2780/shLuc cells (Fig. 5A, B). Next, with the rational that RAB27A silencing could switch the trafficking of MVBs on route for secretion (positive to CD63), now to MVBs on route to lysosomes (positive to CD63 and Cathepsin D), we studied the colocalization of CD63 positive structures with Cathepsin D, a hydrolase enriched in lysosomal organelles. In fact, it is known CD63 has a lysosome targeting motif that regulates its sorting into MVBs/LEs (Mathieu et al., 2021; Takino et al., 2003). Interestingly,

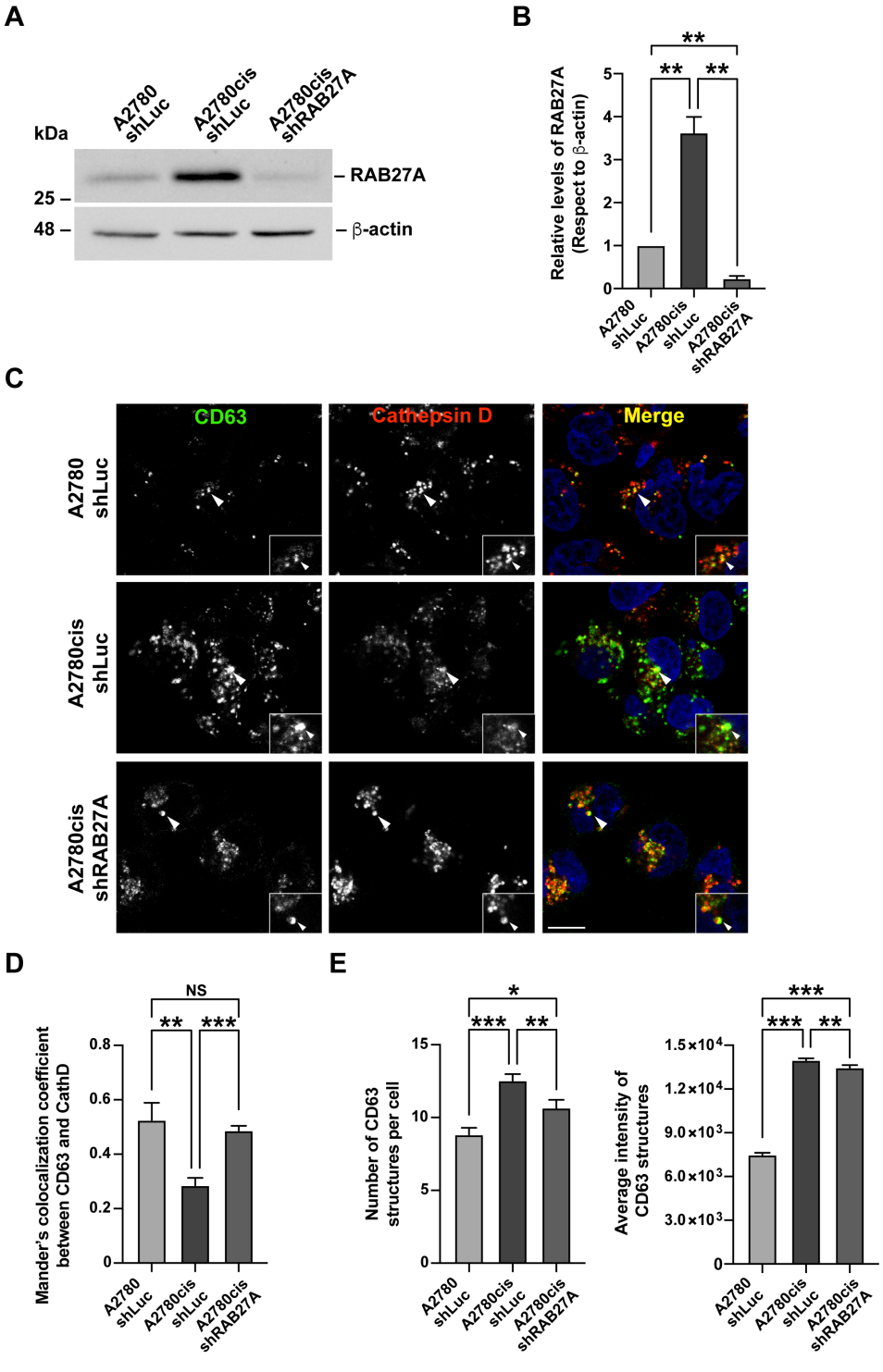

A
kDa
A2780 shLuc
A2780cis shLuc
A2780cis shRAB27A
25 –
48 –
– RAB27A
– β-actin
B
Relative levels of RAB27A (Respect to β-actin)
**
**
**
A2780 shLuc
A2780cis shLuc
A2780cis shRAB27A
C
CD63
Cathepsin D
Merge
A2780 shLuc
A2780cis shLuc
A2780cis shRAB27A
D
Mander's colocalization coefficient between CD63 and CathD
NS
**
***
E
Number of CD63 structures per cell
*
***
**
Average intensity of CD63 structures
***
***
**

Figure 5. RAB27A silencing expression promotes the endo-lysosomal pathway. **(A)** Western blot of endogenous protein levels of RAB27A and β-actin from A2780 shLuc, A2780cis shLuc, and A2780cis shRAB27A cells. **(B)** Densitometric quantification of Western blot indicated in (A), with SD error bar; **P<0.01, ***P<0.001; non-parametric paired t-Test. **(C)** Immunofluorescence detection of CD63 and Cathepsin D structures in A2780 shLuc, A2780cis shLuc, and A2780cis shRAB27A cells, bar 10 μm; arrowhead indicates colocalization spots. **(D)** Mander´s colocalization coefficient analysis between CD63 and CathD from (C), with SEM error bar; **P<0.01, ***P<0.001, NS: Not Significant; parametric unpaired t-Test. **(E)** The number and average intensity analysis of CD63 from (C), with SEM error bar; *P<0.05, **P<0.01, ***P<0.001; parametric unpaired t-Test.

this analysis showed that RAB27A silencing causes an increase in the colocalization of CD63 with Cathepsin D in A2780cis/shRAB27A cells, compared to A2780cis/shLuc (Fig. 5D), observing a similar pattern of colocalization to A2780/shLuc cells (Fig. 5D). Quantification analysis confirmed this conclusion, observing a significant increase in the colocalization between these two markers in A2780cis/shRAB27A compared to A2780/shLuc cells (Fig. 5D), reaching similar levels to those found in A2780/shLuc cells. In addition, we found that the increased number and average intensity of CD63 structures observed in A2780cis/shLuc cells, in comparison to A2780/shLuc cells, was significantly reduced upon RAB27A silencing (Fig. 5E). Together, our findings suggest RAB27A silencing in CDDP-resistant OvCa A2780cis cells revert the type of CD63-MVBs to a phenotype like the one observed in CDDP-sensitive OvCa A2780 cells.

Further, we investigated whether reduction in the levels of RAB27A could cause a recovery in lysosomal function. To this, we studied the levels of the membrane lysosomal protein LAMP1 in A2780/shLuc, A2780cis/shLuc, and A2780cis/shRAB27A puromycin resistant cells by western blot. We found RAB27A silencing promotes a significant increase of LAMP1 levels in A2780cis cells, like the levels found in A2780 CDDP sensitive cells (Fig. 6A, B), results that strongly indicate an enhancement in lysosomal function. To investigate this hypothesis, we first evaluated levels of RAB7, which has been shown to have a positive impact in lysosomal biogenesis (Bucci et al., 2000; Stroupe, 2018; Vanlandingham & Ceresa, 2009b). In agreement with LAMP1, we found silencing of RAB27A causes an increase in the levels of RAB7 protein in A2780cis/shRAB27A cells, compared to A2780cis/shLuc cells, to comparable levels found in A2780/shLuc cells (Fig. 6A, B). Next, we investigated whether a reduction in RAB27A protein levels could enhance lysosomal function measuring the number of LAMP1 and Cathepsin D positive structures. In agreement to our previous findings, we found a strong recovery in LAMP1 and Cathepsin D positive punctate structures in A2780cis/shRAB27A, like the levels found in A2780/shLuc cells (Fig. 6C). Quantification analysis confirmed this conclusion, observing

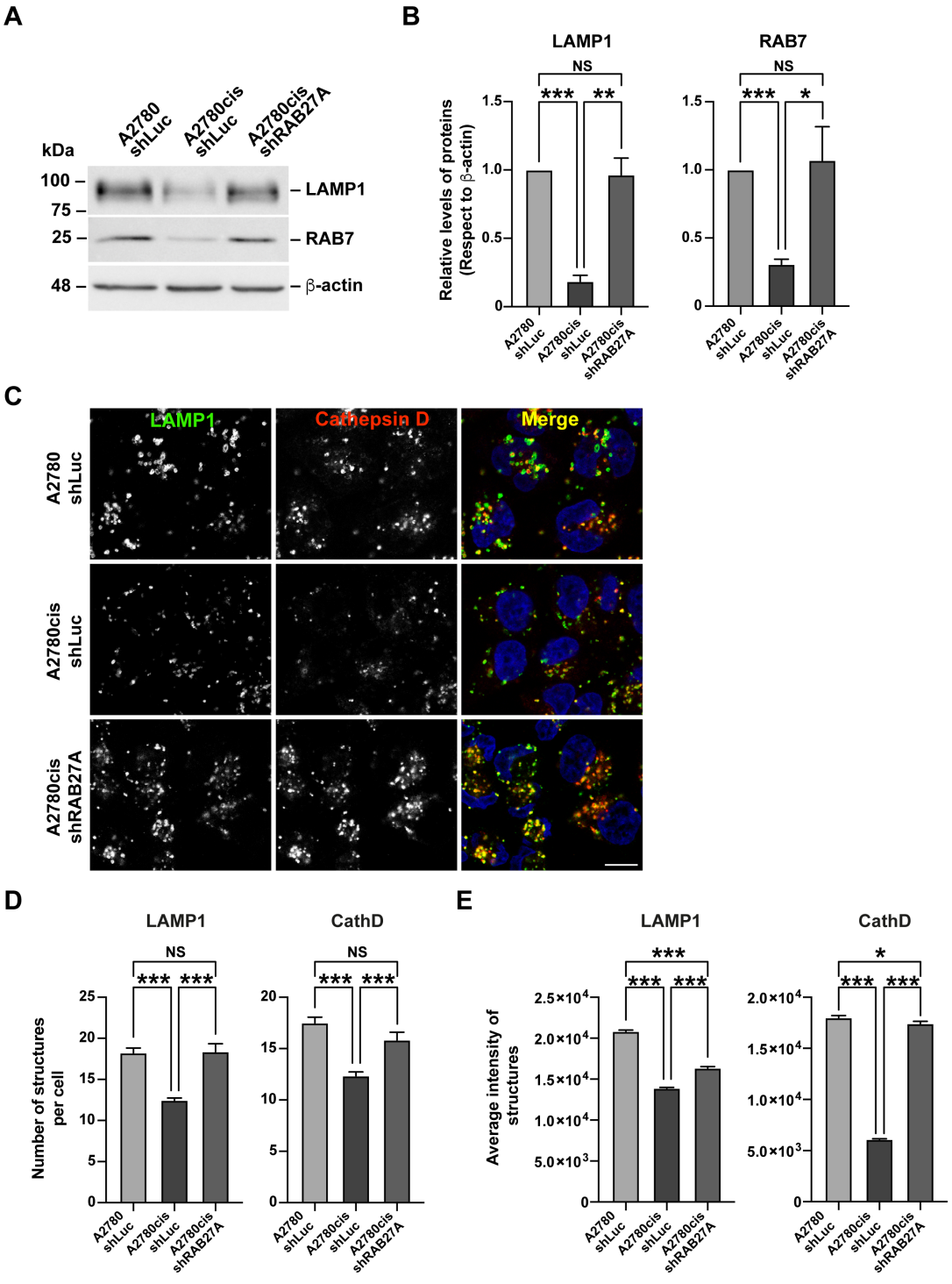

A
A2780 shLuc
A2780cis shLuc
A2780cis shRAB27A
kDa
100
75
25
48
LAMP1
RAB7
β-actin
B
LAMP1
RAB7
NS
***
**
*
Relative levels of proteins (Respect to β-actin)
1.5
1.0
0.5
0
A2780 shLuc
A2780cis shLuc
A2780cis shRAB27A
C
LAMP1
Cathepsin D
Merge
A2780 shLuc
A2780cis shLuc
A2780cis shRAB27A
D
LAMP1
CathD
NS
***
Number of structures per cell
25
20
15
10
5
0
E
LAMP1
CathD
***
*
Average intensity of structures
2.5×10^4
2.0×10^4
1.5×10^4
1.0×10^4
5.0×10^3
0

Figure 6. RAB27A KD causes a recovery of lysosome-like structures in CDDP-resistant OvCa cells. **(A)** Western blot of endogenous protein levels of LAMP1 and RAB7, and β-actin from A2780 shLuc, A2780cis shLuc, and A2780cis shRAB27A cells. **(B)** Densitometric quantification of Western blot indicated in (A) with SD error bar; *P<0.05, **P<0.01,***P<0.001, NS: Not Significant; nonparametric paired t-Test. **(C)** Immunofluorescence detection of LAMP1 and Cathepsin D structures in A2780 shLuc, A2780cis shLuc, and A2780cis shRAB27A cells; bar 10 μm. **(D)** The number of structure analysis of LAMP1 and CathD from (C), with SEM error bar; ***P<0.001, NS: Not Significant; parametric unpaired t-Test. **(E)** The average intensity of LAMP1 and CathD positive structures from (C), with SEM error bar; *P<0.05, ***P<0.001; parametric unpaired t-Test.

RAB27A silencing in A2780cis causes a significant increase in the number and average intensity of LAMP1 and cathepsin D positive structures, to comparable levels found in A2780/shLuc cells (Fig. 6D, E). Our findings demonstrate RAB27A silencing reverts the phenotype of CDDP-resistant OvCa A2780cis cells to a phenotype similar to the CDDP-sensitive OvCa A2780 cells.

Moreover, because the reduced number in acidic lysosomes and in Cathepsin B activity found in A2780cis, respect A2780 cells, we evaluated whether RAB27A silencing in A2780cis cells could rescue the number of acidic and active lysosomal organelles. As before, we used the LysoTracker probe to measure the number of acidic compartments. Surprisingly, we found RAB27A silencing causes a significant enhancement in the number of LysoTracker positive structures, reaching levels even higher than the number found in A2780/shLuc cells. Similarly, we observed that RAB27A silencing causes an increase in the average intensity of LysoTracker confirming the recovery in lysosomal acidity in A2780cis cells (Fig. 7A, B). In agreement with this conclusion, A2780cis/shRAB27A cells showed a significant increase in MagicRed average intensity compared to A2780cis/shLuc (Fig. 7C, D), confirming the rescue in lysosomal activity. Together, our findings uncovered that silencing of RAB27A expression is a powerful strategy to revert the RAB7 dependent endo-lysosomal pathway in A2780cis CDDP-resistant OvCa cells, opening an alternative to revert CDDP chemoresistance.

5.5 SILENCING OF RAB27A ACTS AS A CHEMOSENSITIZER STRATEGY IN CDDP-RESISTANT A2780CIS OVCA CELLS

The recovery in the RAB7 dependent endo-lysosomal pathway in A2780cis cells with RAB27A silencing suggests that this phenotype could have an impact on the sensibility of these to CDDP. To investigate this, we measured the Lethal $Dose_{50}$ (LD_{50}) to CDDP using the Sulforhodamine B (SRB) assay in A2780cis/shLuc and A2780cis/shRAB27 cells after 24 h of treatment. Our analysis indicates RAB27A silencing causes a significant negative impact on cell viability upon treatment with

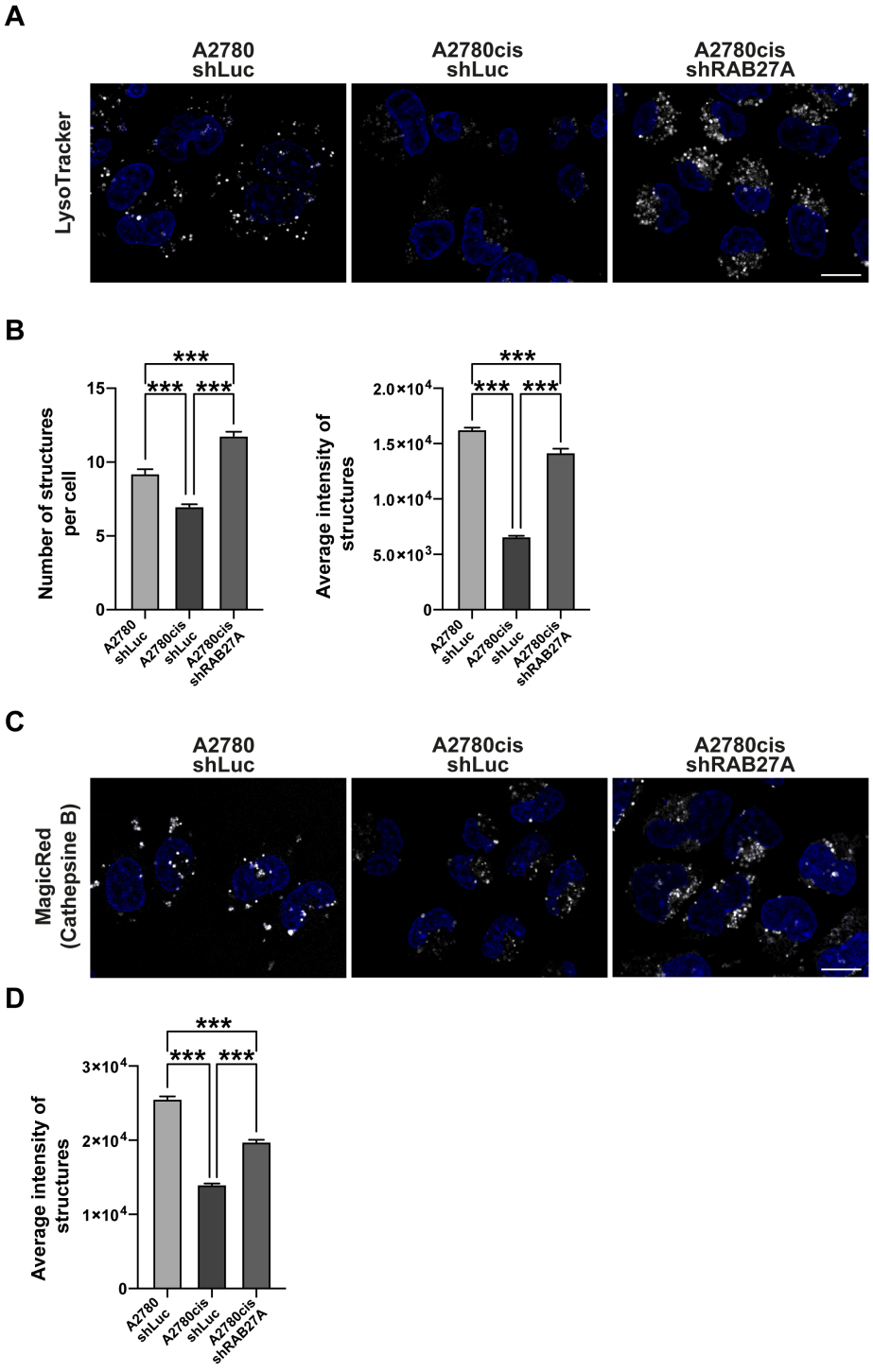

A
A2780 shLuc
A2780cis shLuc
A2780cis shRAB27A
LysoTracker
B
Number of structures per cell
15
10
5
0
***
***
***
A2780 shLuc
A2780cis shLuc
A2780cis shRAB27A
Average intensity of structures
2.0×10^4
1.5×10^4
1.0×10^4
5.0×10^3
0
***
***
***
A2780 shLuc
A2780cis shLuc
A2780cis shRAB27A
C
A2780 shLuc
A2780cis shLuc
A2780cis shRAB27A
MagicRed (Cathepsine B)
D
Average intensity of structures
3×10^4
2×10^4
1×10^4
0
***
***
***
A2780 shLuc
A2780cis shLuc
A2780cis shRAB27A

Figure 7. RAB27A KD induces a recovery of the lysosomal acidity and function in CDDP-resistant OvCa cells. **(A)** Analysis of LysoTracker from A2780 shLuc, A2780cis shLuc, and A2780cis shRAB27A cells; bar 10 μm. **(B)** The number and average intensity analysis of LysoTracker from (A), with SEM error bar; ***P<0.001; parametric unpaired t-Test. **(C)** Analysis of MagicRed probe from A2780 shLuc, A2780cis shLuc, and A2780cis shRAB27A cells; bar 10 μm. **(D)** The average intensity analysis of MagicRed from (C); with SEM error bar; ***P<0.001; parametric unpaired t-Test.

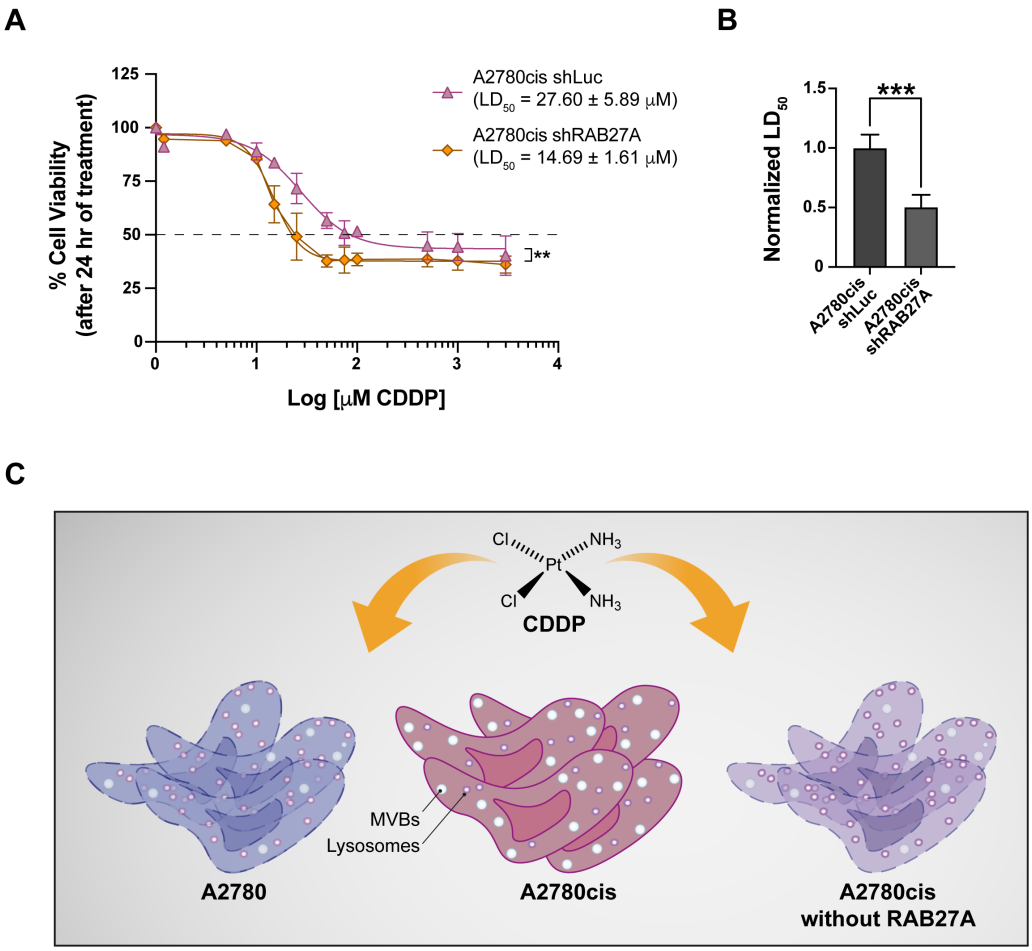
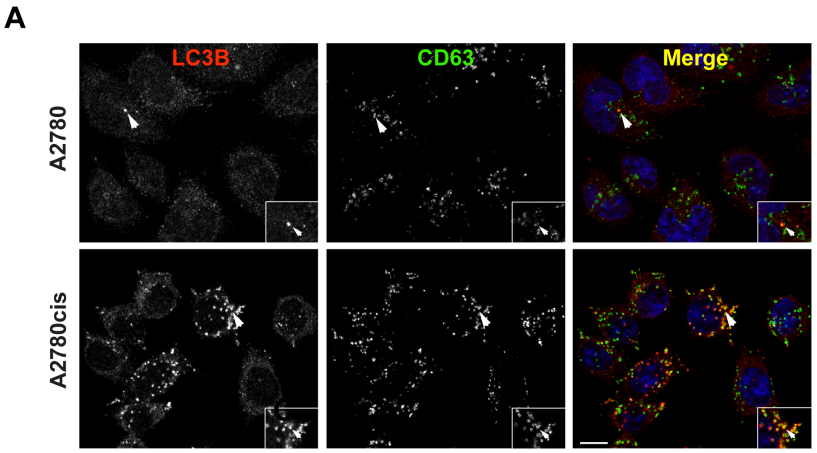

A
B
C
% Cell Viability
(after 24 hr of treatment)
Log [μM CDDP]
A2780cis shLuc
(LD50 = 27.60 ± 5.89 μM)
A2780cis shRAB27A
(LD50 = 14.69 ± 1.61 μM)
**
Normalized LD50
***
A2780cis shLuc
A2780cis shRAB27A
CDDP
MVBs
Lysosomes
A2780
A2780cis
A2780cis without RAB27A

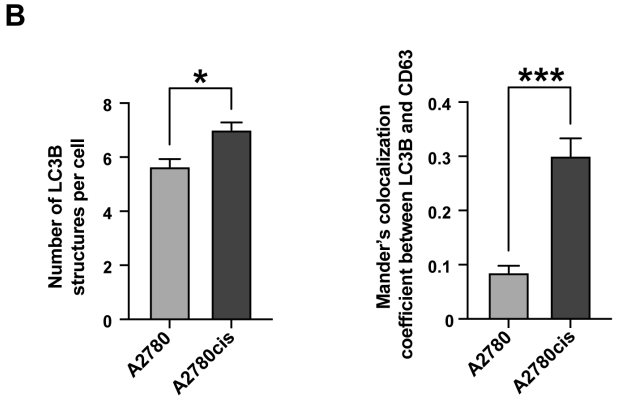
Figure 8. The interruption of exosome secretion promotes chemosensitivity to cisplatin in CDDP-resistant OvCa cells. **(A)** Cell viability percentage curves obtained from the average of biological replicates of A2780cis shLuc and A2780cis shRAB27A treated with increasing doses of CDDP; indicating the comparative analysis of curves by two-way ANOVA, **P<0.01; and the general Lethal $Dosis_{50}$ (LD_{50}) to CDDP for each cell. **(B)** Comparative analysis of LD_{50} to CDDP of 2780cis shLuc and A2780cis shRAB27A obtained from each biological replicate, with SD error bar; ***P<0.001; nonparametric paired t-Test. **(C)** The A2780cis resistant cells had less lysosome in number and activity but more MVBs concerning A2780 sensitive cells secreted in response to CDDP. The absence of RAB27A in A2780cis promoted an increase in lysosome number and activity but a reduction in MVBs (such as the phenotype of A2780 sensitive cells), promoting chemosensitivity to CDDP.

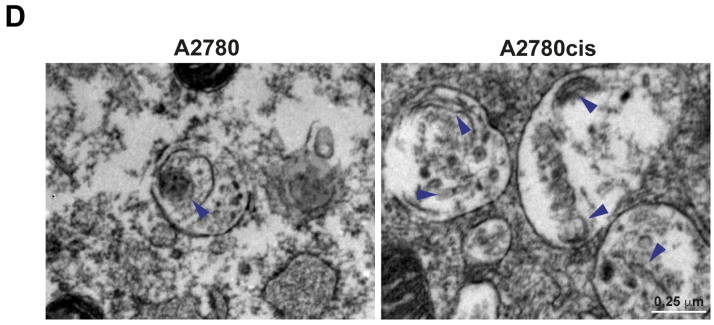
CDDP (Fig. 8A). In addition, a reduction in LD_{50} from 27.60 ± 5.89 μM for A2780cis/shLuc to 14.69 ± 1.61 μM in A2780cis/shRAB27 cells was observed (Fig 8A). Quantification of the LD_{50} in both cell lines confirmed the LD_{50} decreased by half with the reduction of RAB27A (Fig. 8B). These findings confirm that RAB27A silencing is a potent strategy to revert CDDP chemoresistance in OvCa A2780cis resistant cells switching to a phenotype of OvCa A2780 sensitive cells (Fig. 8C).

5.6 TARGETING AUTOPHAGOSOMES AND AMPHISOMES AS AN ALTERNATIVE STRATEGY TO CONTROL RAB27A/RAB7 RATIO IN CDDP-RESISTANT A2780CIS OVCA CELLS

MVBs are dynamic organelles that can constantly fuse with autophagosomes leading to the formation of hybrid organelles called amphisomes. Importantly, amphisomes can also facilitate exosome secretion, a pathway directly implicated in the chemoresistance of OvCa cells to CDDP. Recent findings have suggested that lysosomal impairment triggers the formation of amphisomes promoting exosome secretion (Peng et al., 2021). In agreement with this idea, it has been reported that reduction in autophagosome biogenesis by the transient silencing of BECLIN1, a protein known to mediate autophagosomal biogenesis, causes chemosensitization of A2780cis cells to CDDP (Bao et al., 2015). However, the mechanism that could explain the chemosensitization phenotype by the absence of BECLIN-1 is unknown. As our findings showed that A2780cis have an increased number of CD63-positive MVBs we asked whether these secretory MVBs could in part correspond to amphisomes. To evaluate this, we studied the colocalization of CD63-positive punctate structures with LC3B, a well-known marker of autophagosomes in A2780cis and A2780 cells by immunofluorescence confocal microscopy (Klionsky, Abdel-Aziz, et al., 2021; Tanida et al., 2004). Interestingly, we found a significant increase in the number of LC3B punctate structures in A2780cis cells compared to A2780 cells (Fig. 9A, B), that is in agreement with previous findings (Bao et al., 2015) indicates that CDDP-resistant cells have an increase in autophagosomal biogenesis. Moreover, we found a significant increase in the colocalization of CD63 and LC3B positive punctate


A
LC3B
CD63
Merge
A2780
A2780cis


B
*
Number of LC3B structures per cell
8
6
4
2
0
A2780
A2780cis
***
Mander's colocalization coefficient between LC3B and CD63
0.4
0.3
0.2
0.1
0
A2780
A2780cis


D
A2780
A2780cis
0.25 µm

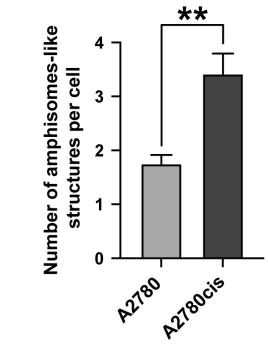

E
**
Number of amphisomes-like structures per cell
4
3
2
1
0
A2780
A2780cis

Figure 9. Increased number of amphisome-like structures in CDDP-resistant OvCa cells. **(A)** Immunofluorescence detection of LC3B and CD63 structures in A2780 and A2780cis cells, bar 10 μm; arrowhead indicates colocalization spots. **(B)** The number LC3B structures from (A), with SEM error bar; *P<0.05; parametric unpaired t-Test. **(C)** Mander´s colocalization coefficient analysis between LC3B and CD63 from (A), with SEM error bar; ***P<0.001; Non-parametric unpaired t-Test. **(D)** Ultrastructural analysis of amphisome-like structures from Transmission Electron Microscopy of A2780 and A2780cis; arrowhead indicates the characteristic autophagosome membrane-derived inside of MVBs. **(E)** Quantification of amphisome-like structures per cell in A2780 and A2780cis from (D), with SEM error bar; **P<0.01; parametric unpaired t-Test.

structures in A2780cis cells compared to A2780 cells (Fig. 9A, C), a result that was confirmed at an ultrastructural level by TEM (Fig. 9D, E) showing characteristic amphisome-like structures with the presence of ILVs and autophagic structures as single membranes (indicated by arrowhead in Fig. 9C) (Peng et al., 2021). All these findings demonstrate CDDP-resistant OvCa A2780 cells contain CD63-positive hybrid amphisomes. We investigated the effect of autophagosomal inhibition in A2780cis cells further. For this, we performed transient silencing of FIP200, a crucial member of the ULK1/2-protein complex implicated in the initiation of autophagosomal formation (Hara et al., 2008; Nishimura et al., 2017). A2780cis cells were transiently transfected with either a non-target siRNA (Ctrl) or a specific siRNA against the coding region of FIP200 to induce KD of FIP200 during 72 h. First, we confirmed the efficient silencing of FIP200 by western blot analysis (Fig. 10A, B). Next, we tested the functional effect of FIP200 KD analyzing levels of SQSTM1/p62 and lipidated LC3B (LC3-II) versus non-lipidated LC3B (LC3-I) (Fig. 10C). As expected, by western blot analysis we observed that silencing of FIP200 caused an enhancement in the SQSTM1/p62 (Fig. 10D) and an inhibition in the levels of LC3B-II and the ratio LC3-II/LC3-I (Fig. 10E). Together confirming silencing of FIP200 causes an efficient inhibition of autophagosome biogenesis in A2780cis.

Finally, considering the role of autophagosomes in amphisomes, we investigated the impact of autophagosome inhibition in the levels of RAB27A and RAB7 proteins by western blot in A2780cis cells. Surprisingly, we found FIP200 silencing levels cause significant changes in these two GTPases, observing a decrease in RAB27A and an increase in RAB7 (Fig. 11A, B). Moreover, and similar to our previous findings with RAB27A silencing , we found that FIP200 silencing causes a significant reduction in the number and average intensity of CD63 punctate structures in A2780cis cells, compared to control cells (Fig. 11C, D), strongly suggesting inhibition of autophagosomes have a positive impact in the endo-lysosomal pathway.

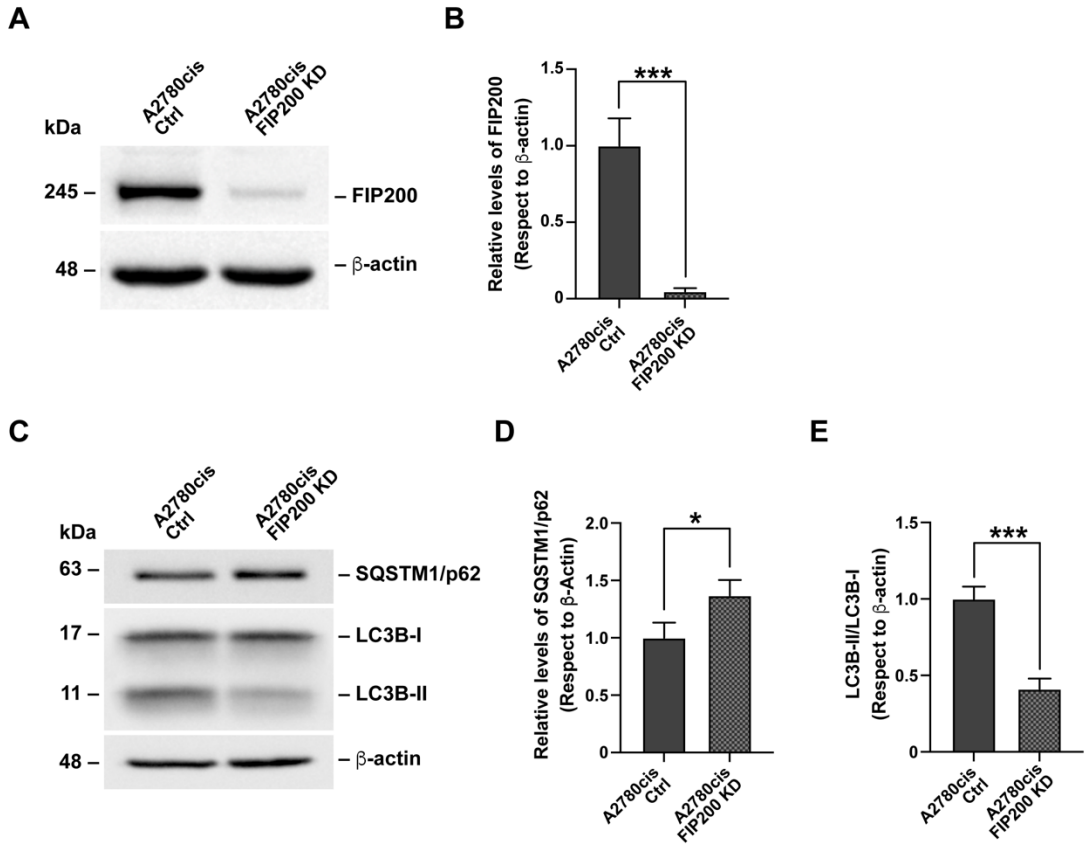


Figure 10. The FIP200 silencing reduces autophagy in CDDP-resistant OvCa cells. **(A)** Western blot of endogenous proteins levels of FIP200 and β-actin from A2780cis cells transfected with siRNA Non-Target (A2780cis Ctrl) or siRNA for FIP200 (A2780cis FIP200 KD). **(B)** Densitometric quantification of Western blot indicated in (A), with SD error bar; ***P<0.001; nonparametric paired t-Test. **(C)** Western blot of endogenous proteins levels of SQSTM1/p62, LC3B and β-actin from A2780cis cells transfected with siRNA Non-Target (A2780cis Ctrl) or siRNA for FIP200 (A2780cis FIP200 KD). **(D)** Densitometric quantification of Western blot indicated in (C), with SD error bar; *P<0.05; nonparametric paired t-Test. **(C)** LC3B conversion analysis from (C) represented as LC3B-II/LC3B-I reasons, with SD error bar; ***P<0.001; nonparametric paired t-Test.

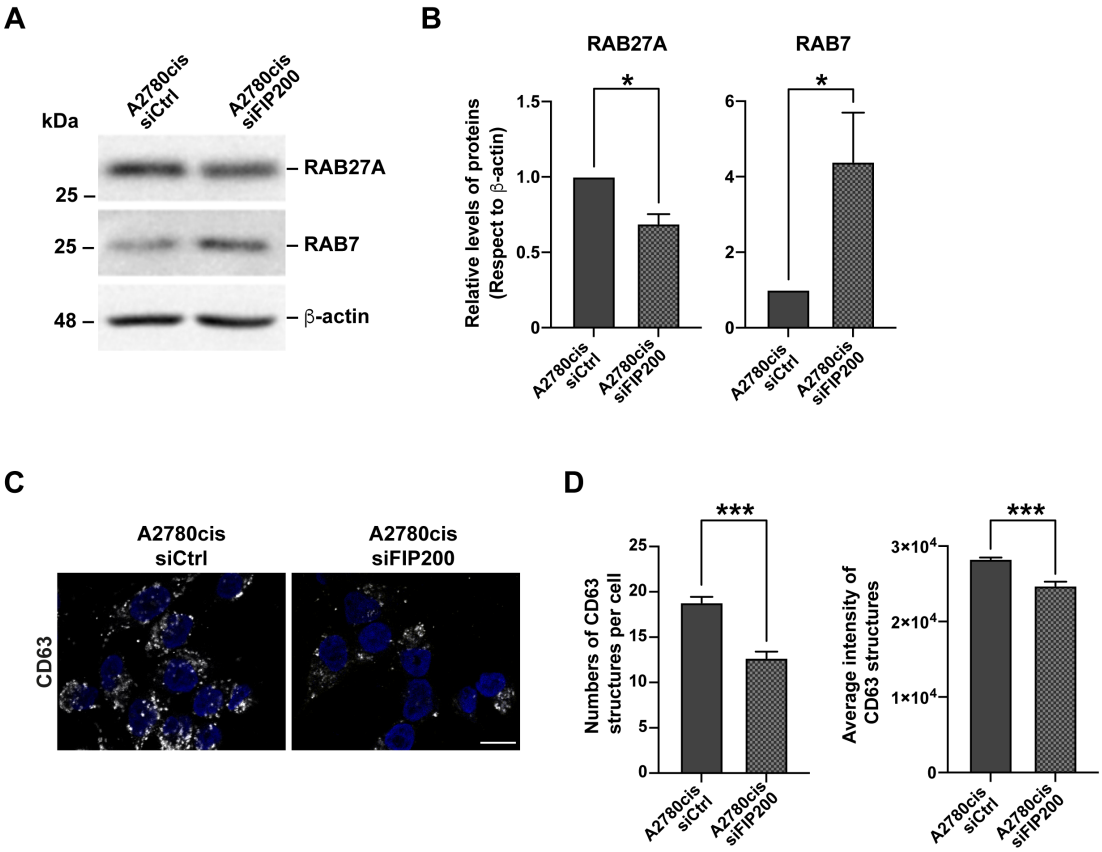


Figure 11. FIP200 silencing promotes the endo-lysosomal pathway in CDDP-resistant OvCa cells. **(A)** Western blot of endogenous proteins levels of RAB27A, RAB7, and β-actin from A2780cis cells transfected with siRNA Non-Target (A2780cis Ctrl) or siRNA for FIP200 (A2780cis FIP200 KD). **(B)** Densitometric quantification of Western blot indicated in (C), with SD error bar; *P<0.05; nonparametric paired t-Test. **(C)** Immunofluorescence detection of CD63 structures in A2780cis cells transfected with siRNA Non-Target (A2780cis Ctrl) or siRNA for FIP200 (A2780cis FIP200 KD); bar 10 μm. **(D)** The number and average intensity of structures analysis of CD63 from (E), with SEM error bar; ***P<0.001; parametric unpaired t-Test.

6 DISCUSSION

Exosomes are currently a hot spot of cancer research due their participation in several processes which support tumor progression such as the acquisition of chemoresistance. Although it is known that exosomes promote cancer development, and that secretion might be exacerbated in response to adaptive cellular processes contributing to chemoresistance. Thus, the development of new intervention strategies is currently an urgent global challenge. Exosomes have been identified as key players in the acquisition of chemoresistance to CDDP, particularly in ovarian cancer (Nakamura et al., 2019; Safaei et al., 2005), as a high percentage of patients develop resistance to this drug during treatment (Galluzzi et al., 2012; D. W. Shen et al., 2012). Therefore, understanding the molecular and cellular mechanisms related to the role of exosomes in this context may provide the discovery of new strategies of intervention oriented at attacking the progression of aggressive ovarian tumors acquiring chemoresistance to CDDP, resulting in a direct impact on the survival rate of patients.

Exosomal secretion can be modulated at a molecular level by proteins that mediates the biogenesis of MVBs/ILVs and/or by controlling critical steps in the trafficking and fusion of these organelles with the PM (Steinbichler et al., 2019; Van Niel et al., 2018). Despite evidence indicating an increased secretion of exosomes in response to CDDP (Bandari et al., 2020; Mosquera-Heredia et al., 2021; Steinbichler et al., 2019; S. Yu et al., 2015), it still remains unexplored whether CDDP-resistant OvCa could be correlated with changes in the proteins that regulate MVBs/ILVs cellular biology. In this PhD thesis we revealed that the A2780cis CDDP-resistant model of OvCa displayed an increased number of MVBs/ILVs in comparison to the A2780 CDDP-sensitive model of OvCa (Fig. 1A, B, C, D). These findings agree with the results that A2780cis CDDP-resistant cells express high levels of ESCRTs proteins in comparison to A2780 CDDP-sensitive cells (Fig. 1E, F). To complete/further enhance knowledge on the molecular machinery implicated in MVBs/ILVs biogenesis, future studies are

needed to assess the role of the ESCRT-independent pathways and their contribution to the chemoresistance of A2780cis CDDP-resistant OvCa cells.

Besides proteins related to biogenesis, RAB GTPases also plays a crucial role in the control of MVBs/ILVs (Zerial & McBride, 2001). Between all RABs known to participate as regulators in the trafficking and fusion of MVBs with the PM to achieve exosome secretion, we discovered that A2780cis CDDP-resistant cells have high levels of RAB27A expression, compared to A2780 CDDP-sensitive cells (Fig. 2A, B, C). In this regard, it is possible that during the acquisition of resistance to CDDP, tumoral cells adapt activating the machinery related to MVBs/ILVs biogenesis and secretion through the overexpression of ESCRT machinery components and RAB27A. It is known that mutation in the DNA (Fodale et al., 2011; Wallace, 2012) and non-mutational epigenetic reprogramming gene expression (through non-coding RNA (ncRNA), DNA methylation, and histone modification) change the gene expression of the cell affecting cellular adaptative processes (Chapman-Rothe et al., 2013; Hanahan, 2022; Xie et al., 2021). In fact, A2780cis CDDP-resistant cells have different levels of DNA methylation (Chan et al., 2021; Strathdee et al., 1999; Zeller et al., 2012). It is necessary to evaluate the existence of DNA mutation and non-mutational epigenetic reprogramming in the genes evaluated in this thesis that could explain the differential expression observed in A2780cis CDDP-resistant cells.

Recently, it has been reported that the transcription factor EGR1, which controls RAB27A expression, is overexpressed in A2780cis cells (Ma et al., 2020; Rouillard et al., 2016). In addition, another transcription program involved in the expression of RAB27A is the canonical NF-kB pathway. CDDP activates NF-kB increasing the expression of RAB27A through RelA-p60, a mechanism that has been involved in CDDP chemoresistance in bladder cancer (Kan et al., 2020; Liu et al., 2017). One aspect to be explored in future studies to explain the increased levels in the machinery needed for MVBs/ILVs biogenesis and secretion of exosomes is the contribution of genetic/epigenetic changes. Another possible explanation for the upregulation of RAB27A could be related with a mechanism independent of the gene expression regulation, connected with the function of

KIBRA and the ubiquitin-proteasome system (Song et al., 2019). This recent study proposed that KIBRA-RAB27A interaction prevents RAB27A proteasomal degradation, giving the possibility that an upregulation of KIBRA could explain the increase in the RAB27A protein levels. However, we tested levels of KIBRA, observing no changes between A2780 and A2780cis cells (data not shown). It is plausible that multifactorial events could contribute in a synergic manner to the increase in RAB27A, something to be investigated in a future project.

Previous studies suggested that the C13 CDDP-resistant model of OvCa cells secreted more exosomes than its sensitive counterpart cellular model (Safaei et al., 2005). According to our findings, we expected a higher basal secretion of exosomes in A2780cis CDDP-resistant cells compared to A2780 CDDP-sensitive cells. However, we did not observe any difference in the basal secretion of exosomes when we compared both cell lines, observing only an increase in the secretion of exosomes when A2780cis CDDP-resistant cells were treated with CDDP (Fig. 2E). An explanation for this finding could be related to the ability of CDDP to induce post-translational modifications (PTMs) in RAB27A or in its effectors, and therefore causing the activation of RAB27A to promote exosome secretion. Different PTMs regulate the localization and function of different RABs including phosphorylation, AMPylation, palmitoylation, ubiquitination, among others (Homma et al., 2021; Shinde & Maddika, 2018).

The mechanisms that determine the fine regulation between MVBs on route to the PM and/or lysosomes are poorly understood (Huotari & Helenius, 2011; Van Niel et al., 2018). However, its common origin must be critical in the final function and trafficking of these organelles. One hypothesis in the field is that these two trafficking pathways are regulated by a fine balance between lysosomal degradative function and the secretory capacity of cells through exosomes (Eitan et al., 2016). Our findings demonstrate that tumoral CDDP-resistant OvCa cells have a poor content of lysosomes, and that the remanent lysosomes present are not even acidic enough to be functional (Figs. 3 and 4). In agreement with previous findings (Kalayda et al., 2008; Safaei et al., 2005) we propose that this feature promotes the biogenesis of exosomes for secretion in response to CDDP

(Fig. 2E). Additionally, a dysfunctional endo-lysosomal pathway in A2780cis CDDP-resistant cells may be related with the downregulation on RAB7 (Fig. 4D, E). Lysosomal dysfunction could also be related to alterations in the delivery of hydrolases or proteins needed for pH maintenance, something that could be determined at the levels of the trans-Golgi network (TGN) (Saftig & Klumperman, 2009), a crucial sorting station for lysosomal maintenance (Trivedi et al., 2020; Yang & Wang, 2021). Why is RAB7 downregulated? In 2021 Peng et al. proposed that RAB7 expression is negatively regulated by IKKβ, promoting lysosomal dysfunction and exosome secretion (Peng et al., 2021). However, whether this mechanism plays a role in A2780cis remains unknown.

One mechanism that contributes to CDDP chemoresistance is the inefficient entry of this compound within tumoral cells. This is regulated at different levels including a decrease uptake of CDDP, an increase drug efflux, and mechanisms involved in drug inactivation (Norouzi-Barough et al., 2018; Stordal et al., 2012; Tchounwou et al., 2021; Tong et al., 2019). In this line, it has been recently suggested that lysosomes have a drug sequestering function, diminishing the availability of the drugs (among them, CDDP) within the cells (Galluzzi et al., 2014; Geisslinger et al., 2020; Zhai & El Hiani, 2020; Zhitomirsky & Assaraf, 2016). Based on our findings in A2780cis it is possible that accumulative sequestration of CDDP within lysosomes could be responsible for the lysosomal function observed contributing to the activation of adaptive responses necessary for the acquisition of chemoresistance. We propose that the RAB27A-dependent exosome secretion pathway is a crucial pathway activated under lysosomal dysfunction and is a critical feature for CDDP chemoresistance (Fig. 2). This can be explained by the activation of cellular plasticity mechanisms, a concept considered as a new hallmark of cancer (Hanahan, 2022) that includes the repression of genes associated with original phenotype observed in A2780 cells and the activation of genes associated with the new cell phenotype found in A2780cis cells (Merrell & Stanger, 2016).

A key question to be answered is whether the exosomes are mediators of CDDP chemoresistance in OvCa cells (Cossart & Helenius, 2014; Elkin et al., 2016; Kiss

& Botos, 2009; Mayor et al., 2014). A proposed function for exosomes is related with the expulsion/efflux of CDDP. Recently, Petruzzelli et. al described that the copper transporter ATP7B is redistributed to MVBs structures after exposure to CDDP in IGROV-CP20 CDDP-resistant OvCa cells and may contribute to the loading of CDDP into MVBs/ILVs for its secretion through exosomes (Petruzzelli et al., 2022). This agrees with the phenotype in C13 CDDP-resistant cells (Safaei et al., 2005). We demonstrated A2780cis CDDP-resistant cells have increased levels of components of the ESCRT-dependent pathway and of RAB27A, all key proteins implicated in exosome secretion (Fig. 1E, F and Fig 2A, B). Because ESCRTs mediate other relevant processes related with the maintenance of cellular homeostasis (Vietri et al., 2020), instead we investigated the effect of RAB27A through silencing in A2780cis CDDP-chemoresistance. As demonstrated in previous studies (Blanc & Vidal, 2018; Bobrie et al., 2012; H. Huang et al., 2021; Ostrowski et al., 2010; Salimu et al., 2017), we interrupted the exosome secretion by stable RAB27A KD in A2780cis CDDP-resistant cells. This model gave us a good tool to evaluate the role of exosomal secretion in chemoresistance to CDDP (Fig. 5A, B). Interestingly, together with the recovery of the lysosomal function we observed that RAB27A silencing promoted an increase in C63/Cathepsin D colocalization, strongly suggesting the potentiation of the entire endo-lysosomal pathway (Fig. 5C, D). Interestingly, this phenotype was accompanied by the upregulation of RAB7 levels (Fig 6A, B), suggesting a closed regulatory mechanism between these two GTPases, an aspect that has been poorly explored. Importantly, by affecting the capacity of cells in terms of exosome secretion revert chemosensitivity of tumoral OvCa cells, opening an attractive alternative for drug discovery. Moreover, because chemosensitivity is also correlated with an increment in lysosomal function it becomes interesting to investigate whether a stimulus that promotes lysosomal biogenesis could have a chemosensitivity effect on tumoral OvCa cells in the future.

One interesting system that regulates lysosomal biogenesis and function is the Coordinated Lysosomal Expression And Regulation (CLEAR) pathway that operates through the gene Transcription Factor EB (TFEB). Several lysosomal

proteins such as LAMP1 and RAB7 are regulated by this system (Palmieri et al., 2011). This antecedent opens the possibility that RAB27A silencing and/or blockage of exosomal secretion could activate TFEB, explaining the increase in LAMP1, Cathepsin D, and RAB7 in CDDP resistant OvCa cells, something to be explored in the future. Our findings could also be relevant in the context of neurodegenerative diseases. RAB27A silencing could be a good strategy to promote lysosome biogenesis such as in Alzheimer's, Parkinson's, and Niemann Pick Disease Type C, diseases that are characterized by lysosomal function impairment and enhanced exosome secretion (Adams et al., 2021; Eitan et al., 2016; Guix et al., 2021; D. Huang et al., 2022; Miranda et al., 2018; Ortega et al., 2019; Strauss et al., 2010; van de Vlekkert et al., 2019; Villarroya-Beltri et al., 2016; J. Zhang et al., 2021).

In recent years, several compounds have been proposed as blockers of the exosome pathway in terms of their biogenesis and/or secretion (Moloudizargari et al., 2018; Huarui Zhang et al., 2020). Particularly, a few of them have been developed as potential specific inhibitors of RAB27A activity (Huarui Zhang et al., 2020). Validating these compounds in the future could provide excellent strategies to be tested in the context of chemoresistance to CDDP in OvCa cellular models as well as in neurodegenerative diseases.

Surprisingly, CDDP is not able to induce secretion of exosomes in A2780 CDDP-sensitive cells (Samuel et al., 2018). In our findings that phenotype could be explained by the high levels of RAB7 and/or low levels of RAB27A, GTPase ratio that could be indicative of an active endo-lysosomal pathway. An opposite scenario is observed in A2780 CDDP-resistant cells offering an excellent intervention strategy . In summary, we propose that the ratio between RAB7 and RAB27A is key in determining the balance between either an active endo-lysosome degradative pathway or a high cellular capacity for exosomal secretion as in agreement with previous findings (Eitan et al., 2016).

Two isoforms of RAB27 have been described (Ostrowski et al., 2010). In some cell types, RAB27A and RAB27B can have redundant or cooperative functions (Mitsunori Fukuda, 2013). In this study we have characterized the role of RAB27A.

For the existence of two isoforms, it is necessary to investigate whether RAB27B is expressed or not in A2780 CDDP-resistant cells, mostly because it is not ubiquitously expressed (Mitsunori Fukuda, 2013; Van Niel et al., 2018). Further studies could explore how to determine the effect of RAB27B in A2780 CDDP-resistant cells.

In our characterization, we found that CDDP-resistant OvCa cells have higher levels of LAMP2 compared to sensitive cells. The antibody tested detected the three isoforms described, LAMP2A, LAMP2B, and LAMP2C (Fig. 3A, B). LAMP2A is a key protein in chaperone-mediated autophagy (CMA) (Tekirdag & Cuervo, 2018). In fact, CMA is known to contribute to resistance to CDDP in esophageal squamous cell carcinoma (Cao et al., 2021). Similar to LAMP2A, LAMP2C mediates the degradation of RNA and DNA by promoting their entry into lysosomes (Hase et al., 2015). Contrary to LAMP2A and LAMP2C, LAMP2B is found enriched in the limiting membrane of EVs (Hung & Leonard, 2015; Z. Li et al., 2020; Liang et al., 2020), postulating that it plays a role in the delivery of specific cargos into exosomes and in the biogenesis of exosomes (T. Yamamoto et al., 2022). Thus, it is possible that the increased levels of LAMP2 in CDDP-resistant cells could correspond to LAMP2B, adding another protein implicated in MVBs/ILVs biogenesis. The role of LAMP2B in chemoresistance of OvCa cells to CDDP should be further investigated.

Our findings also demonstrated that CDDP-resistant cells have an increased number in autophagosomes and amphisomes structures compared to sensitive cells. In this regard, the contribution of autophagy to cancer biology depends on the tumor type, disease stage, and host factors. In bone and colorectal cancers, autophagy plays a role in chemoresistance (Klionsky, Petroni, et al., 2021). Currently, several compounds that stimulate or interrupt autophagy have been investigated in different cancer models (Bhat et al., 2018; Nam, 2021; Whitmarsh-Everiss & Laraia, 2021). The status of lysosomes is essential for the autophagy process (Z. Zhang et al., 2021). Our findings show that lysosomal impairment in A2780cis cells correlated with an increase of autophagosomes, probably due to an inefficient fusion with lysosomes which has been shown to be affected by

disturbances in acidification (A. Yamamoto et al., 1998; Z. Zhang et al., 2021). In many examples, accumulation of autophagosomes compromised cell viability (Button et al., 2017; Kawai et al., 2007; A. Yamamoto et al., 1998). In our model, we did not observe cell viability changes under basal conditions. Importantly, we observed an accumulation of amphisomes observed by the colocalization of CD63 and LC3B punctate structures. Due to the role of amphisome in secretion (Y. Da Chen et al., 2017; Ganesan & Cai, 2021; Peng et al., 2021; Salimi et al., 2020), in this thesis we propose that this hybrid organelle is likely to play a role in the secretion of exosomes by a mechanism dependent on RAB27A (Y. Da Chen et al., 2017), and is a crucial player in CDDP-chemoresistance in OvCa cells. In this line, it is known that drugs that trigger lysosomal damage can promote EVs secretion with autophagy markers (Sagini et al., 2021; Xu et al., 2022). Thus, amphisomes are likely to play an unexpected role in chemoresistance to CDDP, promoting the secretion of exosomes in response to this drug (Kan et al., 2020; Peng et al., 2021), probably alleviating the cytotoxicity generated by lysosomal dysfunction and autophagy flux-impairment.

Autophagosomes are known to mediate secretion of toxic cellular constituents (Button et al., 2017), mediating pathogenic roles beyond neurodegenerative diseases, including cancer. Interestingly, BECLIN-1 silencing expression that shuts-down biogenesis of autophagosomes in A2780cis CDDP-resistant cells increases sensibility to CDDP (Bao et al., 2015). In addition to our findings, these antecedents, together with a recent publication (Peng et al., 2021), allow to postulate amphisomes as a key platform for chemoresistance to CDDP in OvCa cells, and as key regulators in the capacity of cells to secrete exosomes, especially when lysosomal function is impaired. The RAB11A has been suggested to play a role in amphisome secretion (Y. Da Chen et al., 2017; Ganesan & Cai, 2021; Szatmári & Sass, 2014), however we did not observe any changes in the levels of this RAB between A2780 CDDP-sensitive and A2780cis CDDP-Resistant cells (Fig. 2A, B). Another protein to be investigated in CDDP-resistant OvCa is RAB8 (Y. Da Chen et al., 2017; Ganesan & Cai, 2021), due to

its recent involvement in CDDP-chemoresistance (D.-W. Shen & Gottesman, 2012).

In this regard, it is important to investigate the role of amphisomes by affecting the biogenesis of autophagosomes through specific strategies. Our findings with the FIP200 silencing go in that direction adding an important piece to the puzzle related with the importance of the ratio of the two GTPases, RAB27A/RAB7. How do amphisomes control the ratio of RAB27A/RAB7 in order to promote secretion? We propose that CDDP must activate PTMs in these GTPases to trigger efficient adaptive processes with impact in the trafficking and fusion of MVBs with the PM. Taken together, efficient blockage of autophagosomes biogenesis by CRISPR or FIP200 silencing together with specific autophagy inhibitors should be investigated in OvCa chemotherapy approaches. Using these strategies would be critical to investigate the status of the lysosomal activity. Finally, as stable RAB27A silencing promotes lysosome biogenesis and chemosensitivity to CDDP, it is possible that CDDP cytotoxicity is directly related to the status of lysosomal organelles.

7 CONCLUSIONS AND PERSPECTIVES

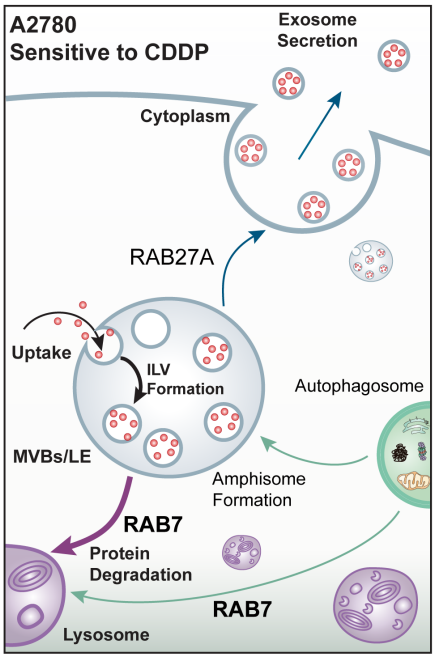
The results presented in this thesis can be summarized as follows:

i. A2780cis CDDP-resistant OvCa cells have an increased machinery for MVBs/ILVs biogenesis and exosome secretion.
ii. A2780cis CDDP-resistant OvCa cells show a reduction in lysosomes as well as in the RAB7 levels.
iii. A2780cis CDDP-resistant OvCa cells have an increased number of amphisome-like structures.
iv. RAB27A silencing expression reverts the phenotype of A2780cis CDDP-resistant OvCa cells promoting chemosensitivity to CDDP.
v. Reduction in autophagosomes and amphisomes by FIP200 silencing impact the ratio RAB27A/RAB7

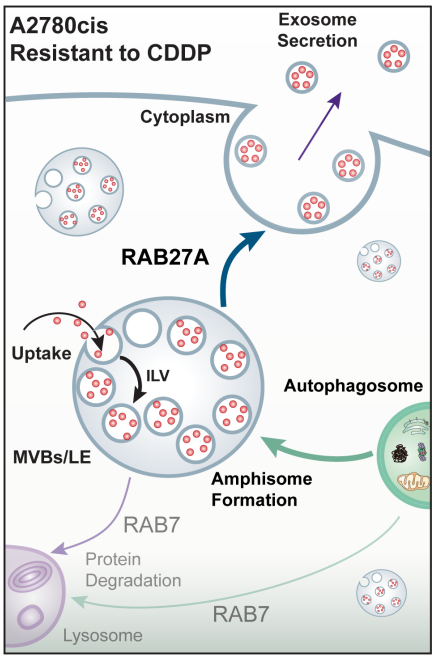
In perspective from the results presented in this thesis we propose:

- The development of two new strategies against CDDP-chemoresistance based on RAB27A or FIP200 silencing.
- To study whether RAB27A or FIP200 silencing could promote lysosomal biogenesis in other cellular models.
- To study amphisomes function inhibition as a target for the treatment of CDDP-chemoresistance in OvCa.

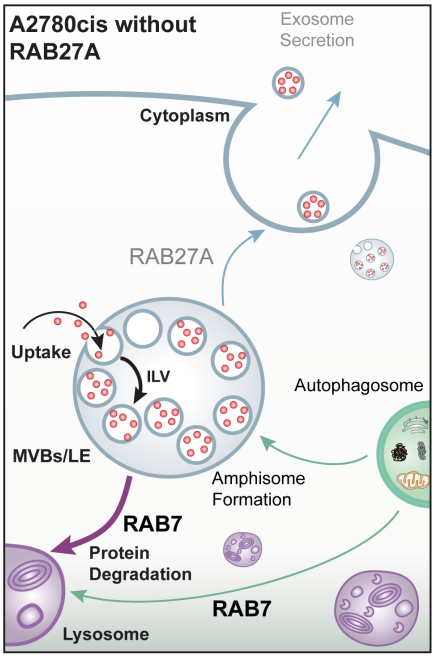
A


A2780
Sensitive to CDDP
Exosome
Secretion
Cytoplasm
RAB27A
Uptake
ILV
Formation
Autophagosome
MVBs/LE
Amphisome
Formation
RAB7
Protein
Degradation
RAB7
Lysosome

B


A2780cis
Resistant to CDDP
Exosome
Secretion
Cytoplasm
RAB27A
Uptake
ILV
Autophagosome
MVBs/LE
Amphisome
Formation
RAB7
Protein
Degradation
RAB7
Lysosome

C


A2780cis without
RAB27A
Exosome
Secretion
Cytoplasm
RAB27A
Uptake
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Autophagosome
MVBs/LE
Amphisome
Formation
RAB7
Protein
Degradation
RAB7
Lysosome

D

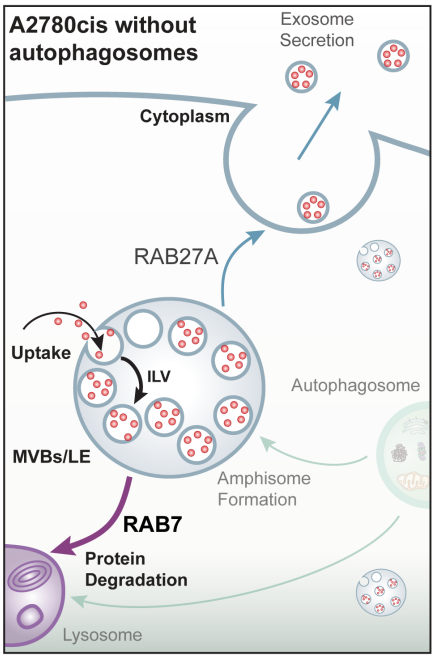
A2780cis without
autophagosomes
Exosome
Secretion
Cytoplasm
RAB27A
Uptake
ILV
Autophagosome
MVBs/LE
Amphisome
Formation
RAB7
Protein
Degradation
Lysosome

Figure 12: Schematic representation of our main findings. **(A) (B)** In contrast to A2780 OvCa CDDP-sensitive cells, A2780cis CDDP-resistant cells have an increased machinery for MVBs/ILVs biogenesis and exosome secretion, a diminished lysosomal function, and a reduction in RAB7 levels. Additionally, CDDP-resistant cells show an increase in the number of amphisome-like structures. **(C)** Silencing of RAB27A-dependent exosome secretion in A2780cis CDDP-resistant OvCa cells reverts the phenotype like A2780 CDDP-sensitive OvCa cells promoting chemosensitivity to CDDP **(D)** Silencing of FIP200-dependent autophagosome/amphisome function in A2780cis CDDP-resistant OvCa cells increased the RAB27A/RAB7 ratio reverting to a phenotype like A2780 CDDP-sensitive OvCa cells.

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9 PUBLICATIONS

Cerda-Troncoso C. Arias-Muñoz E., Cavieres VA, Gaete-Ramírez B., Álvarez-Astudillo F., Varas-Godoy M, and Burgos PV. **RAB27A-dependent exosome secretion in response to lysosomal degradative dysfunction mediates chemoresistance to cisplatin in ovarian cancer**. *(Manuscript in preparation)*.

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