scientific reports

OPEN

Sortilin knock-down alters the expression and distribution of cathepsin D and prosaposin and up-regulates the cation-dependent mannose-6-phosphate receptor in rat epididymal cells

Andrea Carolina Aguilera[1,2], Natalia Leiva[1,2], Pablo Ariel Alvarez[2], Georgina Pulcini[3], Laura Lucía Pereyra[3], Carlos Ramón Morales[4], Miguel Ángel Sosa[2,3] & Lorena Carvelli[2,3✉]

The selective transport to lysosomes can be mediated by either mannose-6-phosphate receptors (CD-MPR and CI-MPR) or sortilin. In mammalian epididymis, some lysosomal proteins are secreted into the lumen through unknown mechanisms. To investigate the underlying mechanisms of lysosomal protein transport in epididymal cells we studied the expression and distribution of cathepsin D (CatD) and prosaposin (PSAP) in a sortilin knocked down RCE-1 epididymal cell line (RCE-1 KD) in comparison with non-transfected RCE-1 cells. In RCE-1 cells, CatD was found in the perinuclear zone and co-localize with sortilin, whereas in RCE-1 KD cells, the expression, distribution and processing of the enzyme were altered. In turn, PSAP accumulated intracellularly upon sortilin knock-down and redistributed from LAMP-1-positive compartment to a perinuclear location, remaining co-localized with CatD. Interestingly, the sortilin knock-down induced CD-MPR overexpression and a redistribution of the receptor from the perinuclear zone to a dispersed cytoplasmic location, accompanied by an increased co-localization with CatD. The increase in CD-MPR could result from a compensatory response for the proper delivery of CatD to lysosomes in epididymal cells. The intracellular pathway taken by lysosomal proteins could be an approach for addressing further studies to understand the mechanism of exocytosis and therefore the role of these proteins in the epididymis.

The mammalian epididymis plays a crucial role in the development of the sperm fertilizing capacity, providing a proper environment for gamete maturation[1]. The composition of the epididymal luminal fluid varies among mammalian species and organ regions (caput, corpus and cauda)[2,3]. Caput lining cells secrete 70–80% of the total epididymal proteins[4,5], which play a critical role in sperm maturation. Among these proteins, there are several enzymes that participate in the remodeling of the sperm surface as a stage in the maturation of gametes[6–8]. Some of these enzymes are lysosomal proteins, which are synthesized by the epididymal epithelium and secreted into the organ lumen[9,10]. The secretion mechanism of these enzymes is still poorly understood, and their participation in sperm maturation is not fully known.

The lysosomal protease cathepsin D (CatD) has been proposed as a key enzyme in the remodeling of the sperm plasma membrane and improvement of its fertilizing capacity[11–13]. Other authors have suggested the participation of CatD in the processing of other lysosomal proteins such as prosaposin (PSAP)[14]. Secreted CatD and PSAP have been shown to be acquired by the gametes in the epididymal duct, suggesting a role of these proteins

[1]CONICET, Facultad de Ciencias Médicas, Universidad Nacional de Cuyo, M5500 Mendoza, Argentina. [2]Facultad de Ciencias Exactas y Naturales, Universidad Nacional de Cuyo, M5500 Mendoza, Argentina. [3]IHEM-CONICET, Facultad de Ciencias Médicas, Universidad Nacional de Cuyo, M5500 Mendoza, Argentina. [4]Faculty of Medicine, McGill University, Montreal, QC H3A0C7, Canada. ✉email: fcarvelli@mendoza-conicet.gob.ar

in sperm maturation[11–13]. As in other organs of the male reproductive tract, the epididymis secretory activity has been found to be hormone dependent. Previous reports have shown that androgen deprivation induces changes in the expression, intracellular distribution, and secretion, of both CatD and PSAP in rat epididymis[9,10,15,16]. However, the mechanisms that regulate the secretion of these proteins are not fully understood.

In most eukaryotic cells, lysosomal enzymes are selectively transported to the lysosomes via specific membrane mannose-6-phosphate receptors (MPRs). The MPRs recognize mannose-6-phosphate (M6P)-bearing enzymes to target them selectively from the trans-Golgi network (TGN) to the lysosomes[17–21]. Two forms of MPRs have been described so far; the cation-dependent (CD-MPR) and the cation-independent (CI-MPR)[17–22], which coexist in most eukaryotic cells. Both types of MPRs are mostly located in endosomes, TGN, and the plasma membrane[23]. In some cell types, the CD-MPR present in the plasma membrane has been proposed to play a role in the exocytosis of M6P-bearing ligands[24–26]. Particularly, in breast cancer cells, the transport and secretion of CatD is driven by a CD-MPR delivery route, which is regulated by estrogen[27]. Although CatD is widely known to be a ligand for MPRs, other alternative routes have been described for this enzyme[28]. Experimental evidence indicates that CatD can also be transported by the membrane receptor sortilin, through its interaction with PSAP, the natural ligand of this receptor[29–31]. Studying the role of sortilin in the intracellular transport of PSAP-CatD complexes[29] might be key to elucidate whether this receptor participates in the CatD secretion by the epididymal cells[16].

Sortilin is a member of the vacuolar protein sorting 10 protein family (Vps10p) expressed in several mammalian cell types as well such as neurons, hepatocytes, and macrophages[32]. Many authors suggest that an altered expression or function of sortilin leads to the development of several diseases[33]. The highest density of sortilin is found in the TGN, where it is involved in the trafficking of multiple ligands to either endosome/lysosome compartments or to the secretory pathway. One tenth of this receptor is present at the plasma membrane, playing a role in either the internalization of ligands or the intracellular signaling[33]. Sortilin is involved in the trafficking of lysosomal proteins such as, acid sphingomyelinase, GM2AP, PSAP, cathepsin H and less frequently, CatD[30]. In mice, the sortilin gene inactivation did not result in any perceptible lysosomal pathology and the endogenous and exogenous PSAP continued to be targeted to lysosomes, suggesting the existence of an alternative receptor complementing the sorting role of sortilin[34].

The purpose of this study is to understand the mechanism involved in the transport of lysosomal proteins in the epididymis and therefore to infer its role in the conditioning of the proper luminal environment for sperm maturation. To this end, epididymal cells from rat caput (RCE-1)[35] were knocked down for sortilin to evaluate the expression and distribution of the lysosomal proteins CatD and PSAP, as well as CD-MPR. Our results suggest that the transport of lysosomal proteins by CD-MPR and sortilin in rat epididymal cells is accomplished through a concerted mechanism of both receptors and provide new insights to understand the processes associated with sperm maturation carried out in the epididymis.

Materials and methods

Reagents and antibodies. The rabbit anti-CD-MPR antiserum was gently provided by Dr. Annette Hille-Rehfeld (Georg-August Universität, Göttingen, Germany). The goat anti-CatD antiserum was purchased from Santa Cruz Biotechnology (sc-6487 Dallas, TX, USA). The polyclonal rabbit anti-PSAP antiserum was produced by Dr. Carlos R. Morales (McGill University, Montreal, Canada). The mouse anti-sortilin antibody was purchased from BD Biosciences (cat. 612100, New Jersey, US). The mouse anti-LAMP-1 antiserum was from Invitrogen (MA1-164, Burlington, ON, Canada). The anti-rabbit conjugated to Alexa 488 (Cat # A-21206), anti-mouse conjugated to Alexa 488 (Cat # A-21202), anti-mouse conjugated to Alexa 594 (Cat # A-21203) and anti-goat conjugated to Alexa 594 (Cat # A-11058) antibodies were from Invitrogen (Burlington, ON, Canada). The HRP-conjugated anti-goat IgG antiserum was obtained from Merck Millipore, (cat.401515, Calbiochem ® Burlington, MA, USA), the HRP-conjugated anti-rabbit IgG was purchased from Sigma (A9169, St. Louis, MO, US) and the HRP-conjugated anti-mouse IgG (whole molecule) was purchased from Sigma (A9044, St. Louis, MO, US). Nitrocellulose (NC) membranes (0.45 μm pore size) were purchased from GE Healthcare (Cat# 10600003, Germany). The chemiluminescent reagent was prepared with 1.25 mM luminol (Cat# A8511, Sigma-Aldrich, St Louis, MO, US), and 198 μM p-coumaric acid (Cat #722812, Sigma-Aldrich, St Louis, MO, US) in 100 mM Tris–HCl (pH 8.5)[36]. The transfection reagent used was PolyFect ® Transfection Reagent (Cat# 301105, Qiagen, Mississauga, ON, Canada). All other chemicals and reagents were purchased from Sigma-Aldrich (St. Louis MO, USA) unless otherwise specified.

Cell culture. The caput epididymal cell line RCE-1 was gently provided by Dr. Daniel G. Cyr (Université du Québec, Laval, Canada)[35]. Cells were cultured in plates coated with extracellular matrix (EM, Cat# E1270, Sigma-Aldrich, St Louis, MO, US) diluted 1:10 in Dulbecco's modified Eagle medium (DMEM)/Ham nutrient mixture F12 supplemented with 2 mM L-glutamine, 10 μg/mL insulin, 10 μg/mL transferrin, 80 ng/mL hydrocortisone, 10 ng/mL epidermal growth factor, 10 ng/mL cAMP, 50 IU/50 μg/mL penicillin–streptomycin (Gibco, MA, US) and with 10% fetal bovine serum (Gibco, MA, US) at 33 °C in a humidified chamber with 5% CO_2.

Transfection. The DNA plasmid pSilencer™ 3.1-H1 neo (Ambion, Austin, TX) that contained a subcloned sortilin siRNA template oligonucleotide (AAGGTGGTGTTAACAGCAGAG) was used in this study[37]. Cells were transfected following the manufacturer's instructions. Briefly, DNA and Polyfect transfection reagent were pre-incubated for 10 min at room temperature to allow complex formation. Afterwards, cells were washed once with sterile phosphate-buffered saline (PBS) and supplemented DMEM culture medium was added with the transfection reagents and incubated for 24–48 h. The transfected clones (RCE-1 KD cells) were selected with

500 µg/mL geneticin (Cat # 11811031, Gibco, MA, US). Cells were mock-transfected with an empty pSilencer TM 3.1-H1 neo-vector as control (RCE-1 M cells)[30].

Immunoblotting. Cells were lysed and homogenized with lysis buffer (PBS containing 1% Nonidet P-40 and 1 mM PMSF) followed by sonication. Proteins (40 µg) from homogenates were resuspended in Laemmli's buffer[38], boiled for 5 min and subjected to 10% SDS-PAGE. The inmunoblotting procedure was carried out according to Aguilera et al.,[39]. Membranes were incubated overnight at 4 °C with either; anti-CatD (1:1000), anti-PSAP (1:300), anti-sortilin (1:1000), or anti-CD-MPR (1:250) antisera, all diluted with 1% BSA (bovine serum albumin) in PBS-T (PBS containing 0,1% Tween 20). After that, membranes were washed three times with PBS-T and incubated with the corresponding HRP-conjugated secondary antibody for 2 h. Specific bands were detected by enhanced chemiluminescence and quantified by densitometric scanning using an ImageQuant LAS 4000 Luminescent Image Analyzer (Fujifilm Life Sciences, NY, US). Band intensities were quantified by densitometry using the ImageJ software (Image Processing and Analysis in Java; National Institutes of Health, Bethesda, MD, USA). As loading control, nitrocellulose membranes were stained with Ponceau S (Cat# P3504, Sigma-Aldrich, St Louis, MO, US). From the Ponceau S staining three bands were taken as references and quantified individually by densitometry with ImageJ. Then, the bands of each protein under study (Sortilin, CD-MPR, PSAP or CatD) were normalized against the mean of the ponceau selected bands.

Indirect immunofluorescence and confocal microscopy. For IFI studies, cells were cultured on 12 mm glass coverslips under the experimental conditions described above. When a 50% confluence was reached, cells were washed once with PBS and fixed with 2% paraformaldehyde for 20 min. Afterwards, cells were permeabilized with 0.1% saponin for 15 min, washed three times with PBS and blocked with 5% horse serum for 30 min. Cells were then incubated overnight at 4 °C with either; anti-CatD (1:200), anti-PSAP (1:1000), anti-sortilin (1:50), anti-CD-MPR (1:250) or anti-LAMP-1 (1:200) antibody, all diluted in PBS containing 5% horse serum (PBS-HS). Cells were washed three times with PBS and incubated with the corresponding fluorochrome-conjugated secondary antibodies diluted in PBS-HS for 90 min. Cell nuclei were stained with 0.007 mg/mL Hoechst 33342, and coverslips were mounted using Mowiol 4–88 mounting solution. The immunofluorescent staining was analyzed under an Olympus FV 1000 confocal microscope and images were acquired using the FV 10-ASW 1.7 software (Olympus, Japan). The images acquisition was set at 20 ms/Pixel (sampling speed) and 3 times integration count (Kalman = 3).

Image and quantitative co-localization analysis. The image analysis and quantification were carried out using ImageJ software from 40 to 70 cells for each cell line. The co-localization degree of proteins was analysed by the Manders correlation coefficient (MCC)[27]. MCC-M1 and MCC-M2 were used to determine the amount of each protein that co-localized with other protein in the cells. The MCC values ranged from 0 to 1, indicating no co-localization or complete co-localization, respectively. Proteins were considered to co-localize when the values of the coefficients were above 0.5. The analysis was done using the JACoP plugin (Just Another Co-localization Plugin; NIH)[40] of the ImageJ software.

Statistics. Data were analysed by the Tukey–Kramer multiple comparisons test. The level of significance was set at $p < 0.05$. At least three independent experiments were performed in each case.

Results

Sortilin is expressed in epididymal cells and co-localizes with cathepsin D.
Sortilin was found to be highly expressed in the epididymal RCE-1 cell line, displaying a perinuclear and cytoplasmic punctate location (Fig. 1A). In addition, a high co-localization of sortilin with the lysosomal protease CatD was observed (Fig. 1A). These results suggest that sortilin could participate in sorting and delivery of CatD to lysosomes in principal epididymal cells.

Based on these findings, it was of interest to determine if the sortilin knock-down could impact on the expression and distribution of the lysosomal proteins CatD and PSAP. For this purpose, we have established a stable sortilin-silencing RCE-1 epididymal cell line (RCE-1 KD) by transfection with the pSilencer™ sortilin siRNA plasmid. The sortilin depletion was confirmed by immunoblotting and immunofluorescence staining in selected transfected clones (Supplementary Fig. 1A,B). With this silencing method, a decrease of about 85.2 ± 0.2% in the expression of sortilin was attained.

Sortilin knock-down (KD) alters distribution and processing of cathepsin D.
The sortilin knock-down induced a decrease of intracellular CatD (Fig. 1C) and a slight redistribution of CatD from the perinuclear to a cytoplasmic dispersed location (Fig. 1B). In turn, CatD displayed lower co-localization with the lysosomal protein LAMP-1 (Fig. 1B), indicating that the enzyme could be detoured from the route to lysosomes. This finding was consistent with the decreased cleavage of pro-CatD (52 kDa) to a processed form of 48 kDa, as observed by immunoblotting (Fig. 1C).

The sortilin knock-down (KD) alters the expression and intracellular distribution of prosaposin in RCE-1 cells.
Since the lysosomal protein PSAP has been described as a counterpart for the transport and processing of pro-CatD, we also studied the effect of sortilin knock-down on PSAP expression and distribution in epididymal cells. The basal expression of PSAP in epididymal cells is low, but it was significantly increased upon sortilin knock-down (Fig. 2A,B). Furthermore, PSAP redistribution from LAMP-1 positive compartments

A)
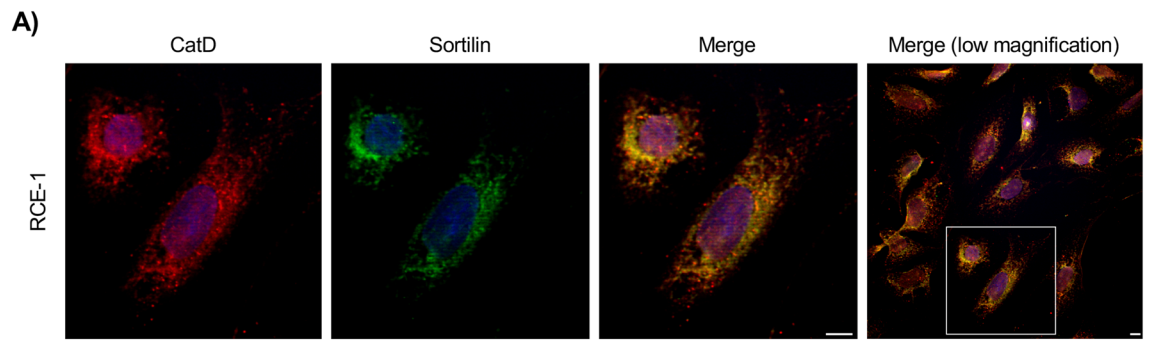
	MCC-M1	MCC-M2
RCE-1	0,976 ± 0,052	0,931 ± 0,043

B)
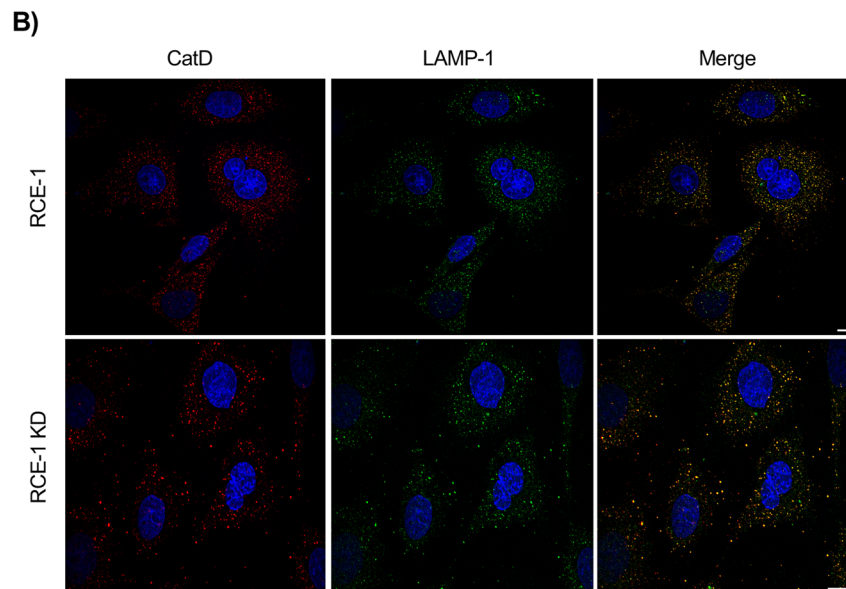
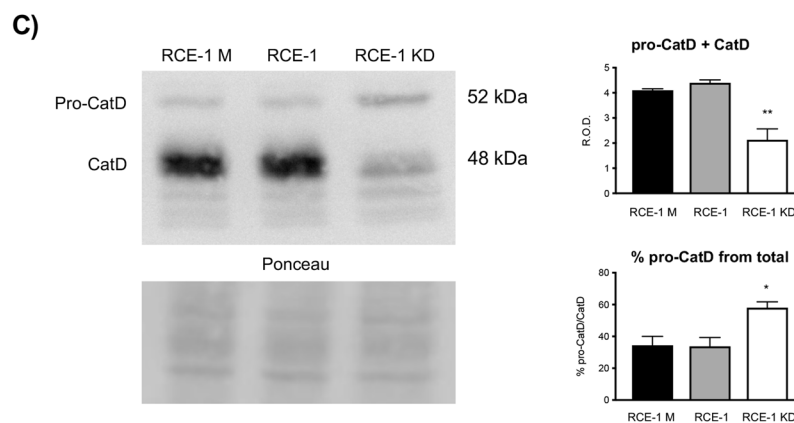|	MCC-M1	MCC-M2
RCE-1	0,90 ± 0,024	0,73 ± 0,025
RCE-1 KD	0,766 ± 0,03*	0,67 ±0,06

C)

◄**Figure 1.** Effect of sortilin depletion on the distribution and processing of Cathepsin D in RCE-1 cells. (**A**) Immunofluorescence staining of sortilin and CatD in the RCE1 cells. Confocal representative images and quantification of co-localization of sortilin and CatD (MCC-M1); CatD and sortilin (MCC-M2). The shown cells were taken from the framed area of the lower magnification image (right merge). (**B**) Representative immunofluorescence staining of CatD and LAMP-1 in RCE1 and RCE-1 KD cells. Quantification of co-localization of LAMP-1 and CatD (MCC-M1); CatD and LAMP-1 (MCC-M2). Values are expressed as the means of Manders colocalization coefficients 1 and 2 (MCC-M1 and MCC-M2, respectively) ± SEM. (*) significant difference from RCE-1 (p < 0.01). Scale bars = 10 μm. (**C**) Representative immunoblot of CatD in RCE-1 M (mock-depleted), RCE-1 and RCE-1 KD cells. Bands intensities of pro-CatD (52 kDa) and CatD (48 kDa) were quantified separately. Bars represent the means of the total CatD (pro-CatD + CatD), as relative optical densities (R.O.D.) ± SEM (upper histogram) and the means of pro-CatD percentage in each sample ± SEM (lower histogram) from four independent experiments. (*) and (**) significant differences (p < 0.01 and p < 0.05, respectively). Ponceau S staining were used as loading control. The full-lenght immunoblot is presented in Supplementary Fig. 2.

to a perinuclear vesicular location was also observed in the sortilin knock-down (Fig. 2B). As for the distribution of CatD, it was found that the enzyme mostly co-localizes with PSAP both in control cells (RCE-1) and in RCE-1 KD cells (Fig. 3).

The sortilin knock-down (KD) induces changes in the CD-MPR. In most cell types, CatD is commonly delivered to lysosomes via mannose-6-phosphate receptors (MPRs). Additionally, in some cellular models, the PSAP/sortilin-mediated pathway has been proposed to participate in CatD trafficking; however, it is not known whether both are alternative or redundant pathways for the enzyme. Therefore, we evaluated whether the sortilin silencing affects the state and distribution of the cation-dependent mannose-6-phosphate receptor (CD-MPR) in epididymal cells. By immunoblotting, we observed that the CD-MPR expression is significantly increased when the sortilin is knocked down in the RCE-1 cells (Fig. 4A). In addition, the CD-MPR was redistributed from a perinuclear location in the control cells to a cytoplasmic dispersed distribution in the RCE-1 KD cells (Fig. 4B). This phenomenon was accompanied by a significant increased co-localization of CD-MPR with CatD in the sortilin knocked down cells (Fig. 4B).

Discussion

Mammalian epididymal cells have a highly developed lysosomal apparatus and maintain an adequate luminal environment for sperm maturation through the secretion of lysosomal proteins and other molecules[41–43].

As in other cell types, the lysosome functionality depends on proper acidification and correct delivery of soluble lysosomal proteins to lysosomes[44,45]. In turn, the delivery of soluble lysosomal proteins to lysosomes is ensured primarily by mannose-6-phosphate receptors (MPRs). However, when the MPR-mediated pathway is non-functional, as it occurs in I-cell disease (Mucolipidosis type II), lysosomal proteins, such as CatD, can be transported by the sortilin receptor[30]. Sortilin was initially described as the neurotensin receptor-3, but its name was changed due to its involvement in the traffic of several proteins between intracellular compartments and/or to the cell surface.

PSAP is a lysosomal protein described as a natural ligand for sortilin and is selectively transported to lysosomes to be processed into saposins (A, B, C and D), which are required for the hydrolysis of glycosphingolipids[34,46]. It is also known that PSAP has a high affinity for CatD and that both may be transported to lysosomes via sortilin[30]. Moreover, it has been described that PSAP is involved in the processing of pro-CatD in acidic compartments[47].

Although most mammalian cells release about 5–20% of soluble lysosomal proteins, part of which is recaptured via receptor-mediated endocytosis[48], some cell types secrete higher amounts of lysosomal proteins into the extracellular compartment[9,10,41,49]. However, the mechanism of exocytosis as well as the function of these proteins in an extra lysosomal environment remain poorly understood.

One of the paradigms of lysosomal protein secretion occurs in the mammalian epididymis, whose epithelium synthesizes and releases abundant lysosomal proteins into the organ lumen[9,10,16,41,43]. Among them, CatD has been detected in the rat epididymal lumen, which is secreted as the 52 kDa precursor pro-CatD[10] probably under androgen control. PSAP has also been found in epididymal fluid[50] forming complexes with pro-CatD[16]. So far, the presence of these proteins in the epididymal fluid has been poorly explained and the mechanism of their release into the lumen is still unknown.

It could be possible that the mechanism of protein secretion is contingent to the intracellular transport. Thus, a correlation between the expression and distribution of receptors and lysosomal proteins could provide clues about the mode of secretion. In this regard, considerable levels of expression of both MPRs have been detected in rat[10,15] and bovine epididymis[43]. In turn, sortilin has also been detected in rat epididymis (Supplementary Fig. 5) and efferent ducts[34].

Although it has been reported that CatD is mainly transported via MPR in most cell types[51,52], in this study we observed high co-localization levels between sortilin and CatD in the epididymal cell line RCE-1 (Fig. 1). This finding poses the question whether sortilin has a role in the trafficking of this enzyme along with PSAP in epididymal cells. To test this hypothesis, we have established the RCE-1 cell line, which has been depleted of sortilin (named RCE-1 KD cells) by an iRNA gene silencing method. After sortilin knockdown, a significant increase of PSAP expression was detected by immunoblotting. As observed in other sortilin-depleted cells[37], PSAP redistributed from late endosomes and lysosomes to the perinuclear region in the epididymal RCE-1 KD cells, suggesting that PSAP accumulates in a compartment that prevents the processing to mature saposins.
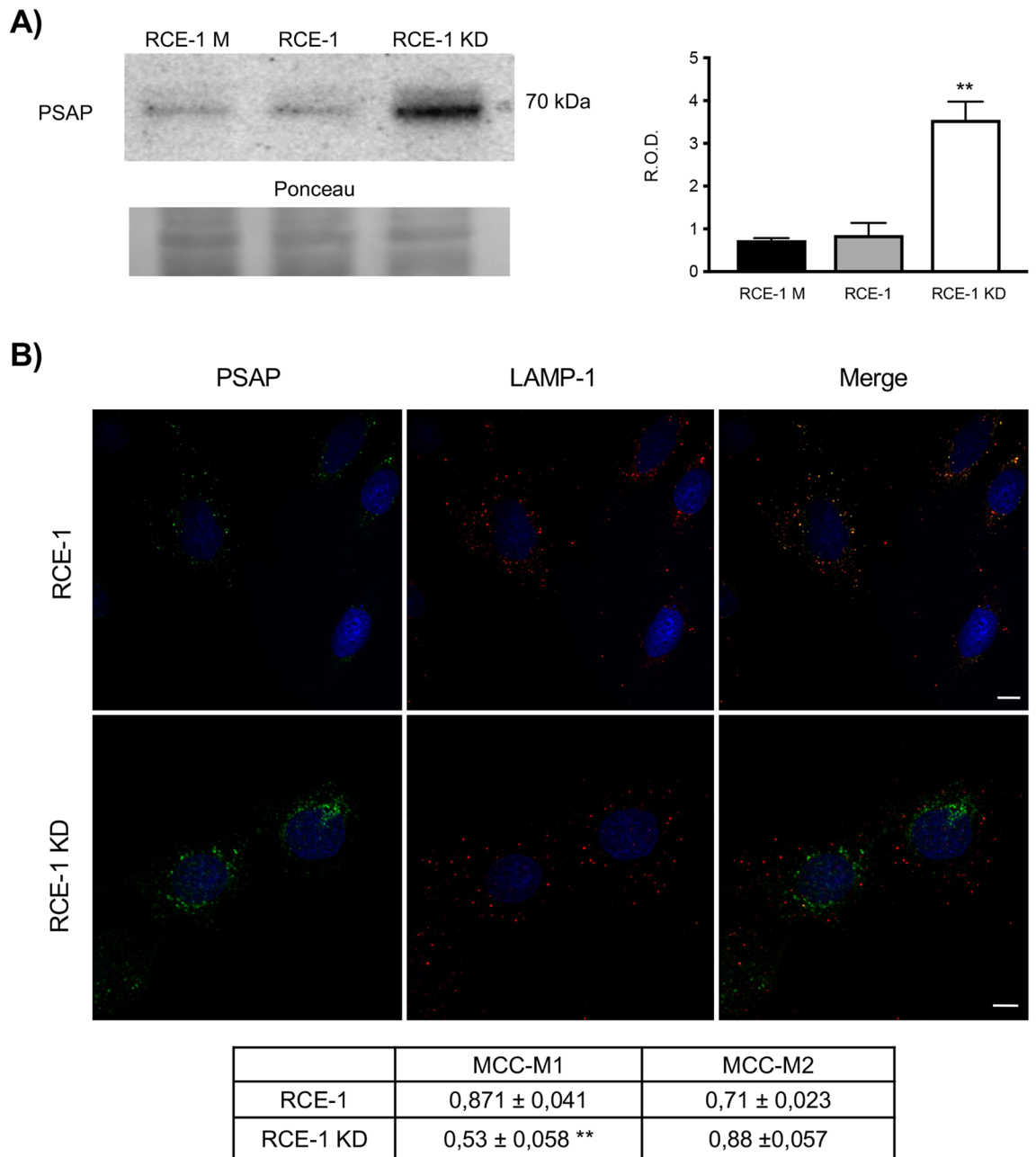
Figure 2. Effect of sortilin depletion on the expression and distribution of PSAP in RCE-1 cells. (**A**) Representative immunoblot of PSAP in RCE-1 M (mock-depleted), RCE-1 and RCE-1 KD cells and the corresponding quantification of bands. Bars represent the means of relative optical density (R.O.D.) ± SEM from three independent experiments. (**) significant difference from control and RCE-1 ($p < 0.05$). Ponceau S staining were used as loading control. The full-lenght immunoblot is presented in Supplementary Fig. 3. (**B**) Representative immunofluorescence staining of PSAP and LAMP-1 in RCE-1 and RCE-1 KD cells. Quantification of co-localization of PSAP and LAMP-1 (MCC-M1); LAMP-1 and PSAP (MCC-M2). Values are expressed as the means of Manders colocalization coefficients 1 and 2 (MCC-M1 and MCC-M2, respectively) ± SEM. (**) significant difference from RCE-1 ($p < 0.05$). Scale bars = 10 μm.

The redistribution of PSAP in RCE-1 KD cells was accompanied by CatD, with a concomitant decrease of this protease in LAMP-1 positive compartments.

Furthermore, our results showed that part of the perinuclearly accumulated PSAP co-localizes with CatD, suggesting that both proteins could be mostly complexed as reported in other cell types[29,47,53,54], although, in this case, in a non-lysosomal compartment. Given that the processing of CatD occurs in lysosomes and that is impaired in RCE-1 KD cells, we propose that this enzyme mostly reaches the lysosomal compartment via a sortilin-PSAP mediated pathway. In turn, the intracellular accumulation of PSAP could be the result of a decreased activity of CatD, as it occurs in neural progenitor cells[55].
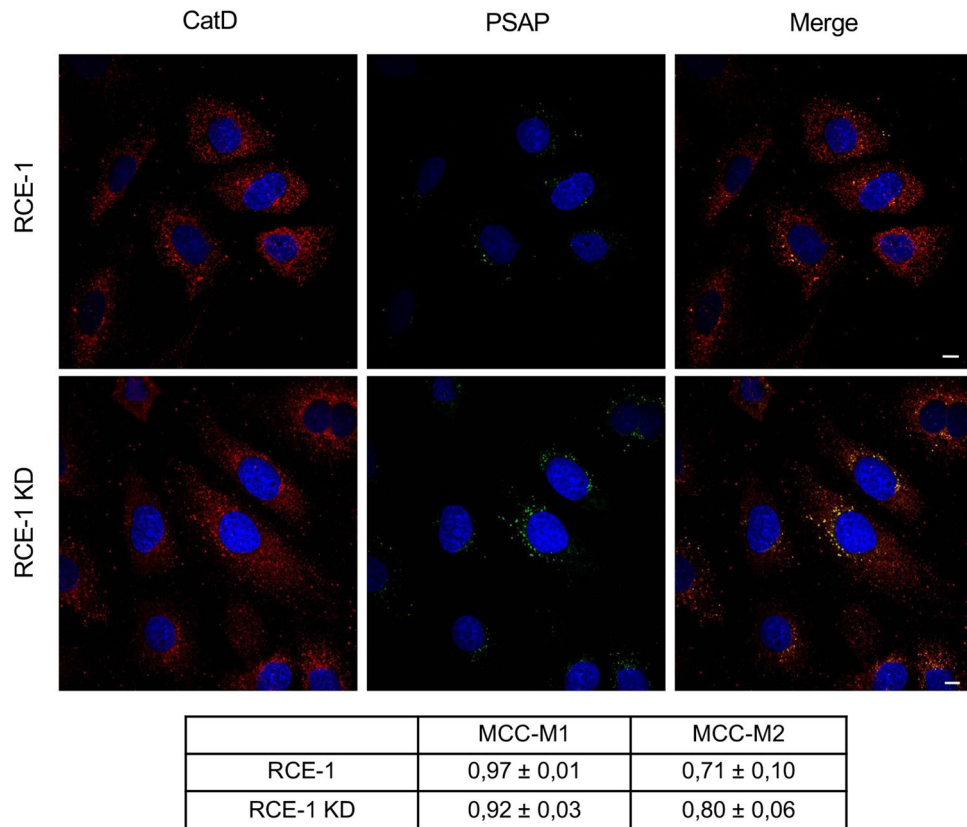
	MCC-M1	MCC-M2
RCE-1	0,97 ± 0,01	0,71 ± 0,10
RCE-1 KD	0,92 ± 0,03	0,80 ± 0,06

Figure 3. Distribution of PSAP and CatD in RCE-1 and RCE-1 KD cells. Representative immunofluorescence staining of PSAP and CatD in RCE-1 and RCE-1 KD cells. Quantification of co-localization of PSAP and CatD (MCC-M1); CatD and PSAP (MCC-M2). Values are expressed as the means of Manders colocalization coefficients 1 and 2 (MCC-M1 and MCC-M2, respectively) ± SEM. Scale bars = 10 μm.

It is worth mentioning that sortilin deficiency also induced a decrease of CatD levels (Fig. 1), which could be related to either an increased secretion of the enzyme because of its missorting or that sortilin somehow regulates the expression of CatD. Further studies (e.g. qPCR) are needed to confirm or rule out one these hypotheses. These results would suggest that sortilin participates, either directly or indirectly, in the transport of CatD and PSAP to lysosomes in principal cells of the epididymis.

Studies performed in another cell model, in which a dominant-negative sortilin construct and small interfering RNA (siRNA) was used, suggested that sortilin functions as an alternative sorting receptor to the MPR for CatD[30]. Interestingly, the sortilin depletion induced a significant increase in the CD-MPR expression in the epididymal cells, indicating that both receptors could regulate each other and that the observed increase in CD-MPR results from a compensatory mechanism for the accurate delivery of proteins to lysosomes. A similar reciprocal regulation has been demonstrated in other cell type[31]. In this regard, the sorting of sortilin is specifically upregulated in type II mucolipidosis to compensate the loss of MPR-dependent targeting of hydrolases. In addition to the increased expression of CD-MPR, we observed that this receptor is redistributed from a classic perinuclear location to a scattered and punctuated cytoplasmic location, accompanied by an increased co-localization with CatD. This suggests that CD-MPR is an alternative receptor to sortilin for the transport of CatD to lysosomes in epididymal cells.

The results obtained in this investigation would support the hypothesis that in RCE-1 cells CatD is transported to lysosomes by both the CD-MPR and/or the sortilin/PSAP pathways, and that CatD requires the presence of PSAP to be processed to the 48 kDa form[47] in epididymal cells. In conclusion, these results have shown for the first time a compensatory response of CD-MPR to sortilin depletion and provided new insights into the mechanism of lysosomal protein transport in epididymal cells.
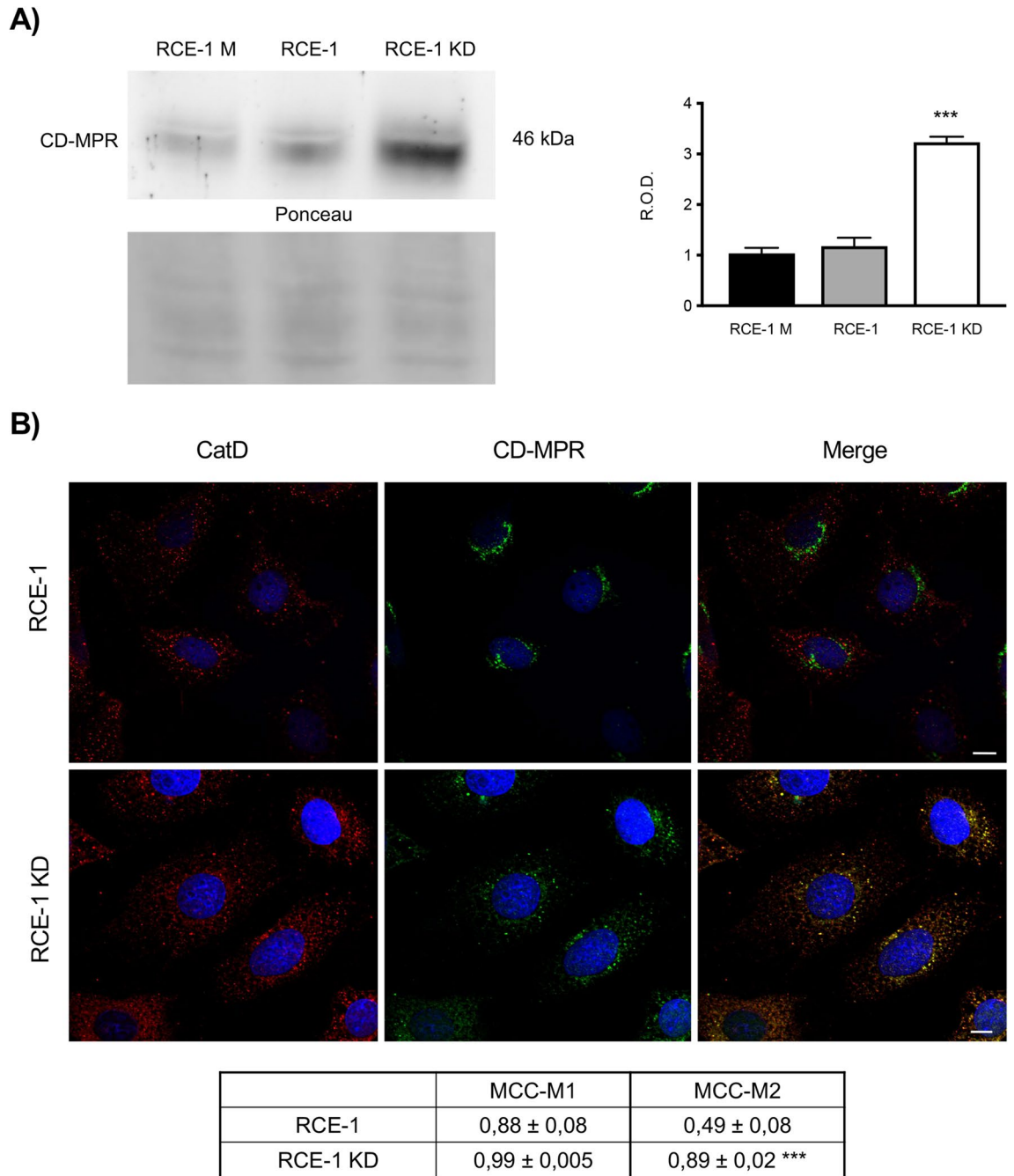
Figure 4. Expression and distribution of CD-MPR in RCE-1 and RCE-1 KD cells. (**A**) Representative immunoblot of CD-MPR in RCE-1 M (mock-depleted), RCE-1 and RCE-1 KD cells with their band intensity quantification. Bars represent the means of relative optical density (R.O.D.) ± SEM from three independent experiments. (***) significant difference from RCE-1 M and RCE-1 (p < 0.001). Ponceau S staining were used as loading control. The full-lenght immunoblot is presented in Supplementary Fig. 4. (**B**) Representative immunofluorescence staining of CD-MPR and CatD in RCE-1 and RCE-1 KD cells. Quantification of co-localization of CDMPR and CatD (MCC-M1); CatD and CD-MPR (MCC-M2). Values are expressed as the means of Manders colocalization coefficients 1 and 2 (MCC-M1 and MCC-M2, respectively) ± SEM. (***) significant difference from RCE-1 cells (p < 0.01). Scale bars = 10 μm.

Data availability

The full-length images of the original immunoblot membranes are available online in Supplementary Information. All data that support the findings of this study are available from the corresponding author on reasonable request.

References

1. Dacheux, J. L. *et al.* The contribution of proteomics to understanding epididymal maturation of mammalian spermatozoa. *Syst. Biol. Reprod. Med.* **58**, 197–210 (2012).
2. Dacheux, J. L. *et al.* Mammalian epididymal proteome. *Mol. Cell. Endocrinol.* **306**, 45–50 (2009).
3. Belleannée, C. *et al.* Identification of luminal and secreted proteins in bull epididymis. *J. Proteomics* **74**, 59–78 (2011).
4. Cornwall, G. A. New insights into epididymal biology and function. *Hum. Reprod. Update* **15**, 213–227 (2009).
5. Zhou, W., Sipilä, P., De Iuliis, G. N., Dun, M. D. & Nixon, B. Analysis of epididymal protein synthesis and secretion. *J. Vis. Exp.* **2018**, 138 (2018).
6. Kohane, A. C., Gonzalez Echeverria, F. M. C., Pineiro, L. & Blaquier, J. A. Interaction of proteins of epididymal origin with spermatozoa. *Biol. Reprod.* **23**, 737–742 (1980).
7. Lan, Z. J., Labus, J. C. & Hinton, B. T. Regulation of gamma-glutamyl transpeptidase catalytic activity and protein level in the initial segment of the rat epididymis by testicular factors: role of basic fibroblast growth factor. *Biol. Reprod.* **58**, 197–206 (1998).
8. Turner, T. T., Miller, D. W. & Avery, E. A. Protein synthesis and secretion by the rat caput epididymidis in vivo: Influence of the luminal microenvironment. *Biol. Reprod.* **52**, 1012–1019 (1995).
9. Belmonte, S. A., Romano, P., Sartor, T. & Sosa, M. A. Compartmentalization of lysosomal enzymes in cauda epididymis of normal and castrated rats. *Arch. Androl.* **48**, 193–201 (2002).
10. Carvelli, L. F., Bannoud, N., Aguilera, C. A., Morales, C. R. & Sosa, M. A. Castration induces changes in the cation-dependent mannose-6-phosphate receptor in rat epididymis: Possible implications in secretion of lysosomal enzymes. *J. Cell Biochem.* **110**, 1101–1110 (2010).
11. Saewu, A. *et al.* Cathepsin D in human reproductive tissues: Cellular localization in testis and epididymis and surface distribution in different sperm conditions. *J. Androl.* **33**, 726–734 (2012).
12. Moura, A. A., Chapman, D. A., Koc, H. & Killian, G. J. Proteins of the cauda epididymal fluid associated with fertility of mature dairy bulls. *J. Androl.* **27**, 534–541 (2006).
13. Dandekar, S. & Harikumar, P. Seminal profiles of lysosomal enzymes in normal and infertile men. *J. Postgrad. Med.* **43**, 33 (1997).
14. Hiraiwa, M. *et al.* Lysosomal proteolysis of prosaposin, the precursor of saposins (sphingolipid activator proteins): Its mechanism and inhibition by ganglioside. *Arch. Biochem. Biophys.* **341**, 17–24 (1997).
15. Carvelli, L. *et al.* Testosterone influences the expression and distribution of the cation-dependent mannose-6-phosphate receptor in rat epididymis: Implications in the distribution of enzymes. *Andrologia* **46**, 224–230 (2014).
16. Carvelli, L. *et al.* Castration causes an increase in lysosomal size and upregulation of cathepsin D expression in principal cells along with increased secretion of procathepsin D and prosaposin oligomers in adult rat epididymis. *PLoS ONE* **16**, e0250454 (2021).
17. Hille-Rehfeld, A. Mannose 6-phosphate receptors in sorting and transport of lysosomal enzymes. *Biochim. Biophys. Acta* **1241**, 177–194 (1995).
18. Gary Sahagian, G., Distler, J. J. & Jourdian, G. W. Membrane receptor for phosphomannosyl residues. *Methods Enzymol.* **83**, 392–396 (1982).
19. von Figura, K. & Hasilik, A. Lysosomal enzymes and their receptors. *Annu. Rev. Biochem.* **55**, 167–193 (1986).
20. von Figura, K. Molecular recognition and targeting of lysosomal proteins. *Curr. Opin. Cell Biol.* **3**, 642–646 (1991).
21. Munier-Lehmann, H., Mauxion, F. & Hoflack, B. Function of the two mannose 6-phosphate receptors in lysosomal enzyme transport. *Biochem. Soc. Trans.* **24**, 133–136 (1996).
22. Sleat, D. E. & Lobel, P. Ligand binding specificities of the two mannose 6-phosphate receptors. *J. Biol. Chem.* **272**, 731–738 (1997).
23. Gartung, C., Braulke, T., Hasilik, A. & von Figura, K. Internalization of blocking antibodies against mannose-6-phosphate specific receptors. *EMBO J.* **4**, 1725–1730 (1985).
24. Chao, H. H. J., Waheed, A., Pohlmann, R., Hille, A. & Von Figura, K. Mannose 6-phosphate receptor dependent secretion of lysosomal enzymes. *EMBO J.* **9**, 3507–3513 (1990).
25. Stein, M., Zijderhand-Bleekemolen, J. E., Geuze, H., Hasilik, A. & von Figura, K. Mr 46,000 mannose 6-phosphate specific receptor: Its role in targeting of lysosomal enzymes. *EMBO J.* **6**, 2677–2681 (1987).
26. Ma, Z., Grubb, J. H. & Sly, W. S. Cloning, sequencing, and functional characterization of the murine 46-kDa mannose 6-phosphate receptor. *J. Biol. Chem.* **266**, 10589–10595 (1991).
27. Bannoud, N. *et al.* Cation-dependent mannose-6-phosphate receptor expression and distribution are influenced by estradiol in MCF-7 breast cancer cells. *PLoS ONE* **13**, e0201844 (2018).
28. Capony, F. *et al.* Specific mannose-6-phosphate receptor-independent sorting of pro-cathepsin D in breast cancer cells. *Exp. Cell Res.* **215**, 154–163 (1994).
29. Zhu, Y. & Conner, G. E. Intermolecular association of lysosomal protein precursors during biosynthesis. *J. Biol. Chem.* **269**, 3846–3851 (1994).
30. Canuel, M., Korkidakis, A., Konnyu, K. & Morales, C. R. Sortilin mediates the lysosomal targeting of cathepsins D and H. *Biochem. Biophys. Res. Commun.* **373**, 292–297 (2008).
31. Barnes, J. W. *et al.* Upregulation of sortilin, a lysosomal sorting receptor, corresponds with reduced bioavailability of latent TGFβ in mucolipidosis II cells. *Biomolecules* **10**, 670 (2020).
32. Glerup, S., Nykjaer, A. & Vaegter, C. B. Sortilins in neurotrophic factor signaling. *Handb. Exp. Pharmacol.* **220**, 165–189 (2014).
33. Ouyang, S., Jia, B., Xie, W., Yang, J. & Lv, Y. Mechanism underlying the regulation of sortilin expression and its trafficking function. *J. Cell. Physiol.* **235**, 8958–8971 (2020).
34. Zeng, J., Racicott, J. & Morales, C. R. The inactivation of the sortilin gene leads to a partial disruption of prosaposin trafficking to the lysosomes. *Exp. Cell Res.* **315**, 3112–3124 (2009).
35. Dufresne, J., St-Pierre, N., Viger, R. S., Hermo, L. & Cyr, D. G. Characterization of a novel rat epididymal cell line to study epididymal function. *Endocrinology* **146**, 4710–4720 (2005).
36. Mruk, D. D. & Cheng, C. Y. The myotubularin family of lipid phosphatases in disease and in spermatogenesis. *Biochem. J.* **433**, 253–262 (2011).
37. Lefrancois, S., Canuel, M., Zeng, J. & Morales, C. R. Inactivation of sortilin (a novel lysosomal sorting receptor) by dominant negative competition and RNA interference. *Biol. Proced. Online* **7**, 17–25 (2005).
38. Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685 (1970).
39. Aguilera, A. C., Boschin, V., Carvelli, L., Cavicchia, J. C. & Sosa, M. A. Glycosidases interact selectively with mannose-6-phosphate receptors of bull spermatozoa. *J. Cell. Biochem.* **117**, 2464–2472 (2016).
40. Bolte, S. & Cordelières, F. P. A guided tour into subcellular colocalization analysis in light microscopy. *J. Microsc.* **224**, 213–232 (2006).
41. Mayorga, L. S. & Bertini, F. The origin of some acid hydrolases of the fluid of the rat cauda epididymidis. *J. Androl.* **6**, 243–245 (1985).
42. Tulsiani, D. R., Orgebin-Crist, M. C. & Skudlarek, M. D. Role of luminal fluid glycosyltransferases and glycosidases in the modification of rat sperm plasma membrane glycoproteins during epididymal maturation. *J. Reprod. Fertil. Suppl.* **53**, 85–97 (1998).

43. Aguilera, A. C. *et al.* Changes in lysosomal enzymes and mannose-6-phosphate receptors related to sexual maturation in bull epididymis. *Reprod. Fertil. Dev.* **28**, 1608–1617 (2015).
44. Yang, C. & Wang, X. Lysosome biogenesis: Regulation and functions. *J. Cell Biol.* **220**, 2001 (2021).
45. Bajaj, L. *et al.* Lysosome biogenesis in health and disease. *J. Neurochem.* **148**, 573–589 (2019).
46. Hassan, A. J., Zeng, J., Ni, X. & Morales, C. R. The trafficking of prosaposin (SGP-1) and GM2AP to the lysosomes of TM4 Sertoli cells is mediated by sortilin and monomeric adaptor proteins. *Mol. Reprod. Dev.* **68**, 476–483 (2004).
47. Gopalakrishnan, M. M. *et al.* Purified recombinant human prosaposin forms oligomers that bind procathepsin D and affect its autoactivation. *Biochem. J.* **383**, 507–515 (2004).
48. Braulke, T. & Bonifacino, J. S. Sorting of lysosomal proteins. *Biochim. Biophys. Acta Mol. Cell Res.* **1793**, 605–614 (2009).
49. Lacombe, J., Karsenty, G. & Ferron, M. Regulation of lysosome biogenesis and functions in osteoclasts. *Cell Cycle* **12**, 2744 (2013).
50. Sylvester, S. R., Morales, C., Oko, R. & Griswold, M. D. Sulfated glycoprotein-1 (saposin precursor) in the reproductive tract of the male rat. *Biol. Reprod.* **41**, 941–948 (1989).
51. Munier-Lehmann, H., Mauxion, F., Bauer, U., Lobel, P. & Hoflack, B. Re-expression of the mannose 6-phosphate receptors in receptor-deficient fibroblasts: Complementary function of the two mannose 6-phosphate receptors in lysosomal enzyme targeting. *J. Biol. Chem.* **271**, 15166–15174 (1996).
52. Zaidi, N., Maurer, A., Nieke, S. & Kalbacher, H. Cathepsin D: A cellular roadmap. *Biochem. Biophys. Res. Commun.* **376**, 5–9 (2008).
53. Laurent-Matha, V. *et al.* Procathepsin D interacts with prosaposin in cancer cells but its internalization is not mediated by LDL receptor-related protein. *Exp. Cell Res.* **277**, 210–219 (2002).
54. Carvelli, L., Libin, Y. & Morales, C. R. Prosaposin: A protein with differential sorting and multiple functions. *Histol. Histopathol.* **30**, 647–660 (2015).
55. Salvioli, R. *et al.* The secretion and maturation of prosaposin and procathepsin D are blocked in embryonic neural progenitor cells. *Biochim. Biophys. Acta Mol. Cell Res.* **1783**, 1480–1489 (2008).

Author contributions

L.C., C.R.M. and M.A.S. conceived the central idea and designed the experiments. A.C.A., N.L., P.A.A., G.P., and L.L.P. performed the experiments. L.C., A.C.A. and M.A.S. analyzed the data. L.C., C.R.M., A.C.A. and M.A.S. wrote the manuscript. The manuscript was reviewed and approved by all authors.

Funding

Competing interests

The authors declare no competing interests.

Additional information

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-023-29157-z.

Correspondence and requests for materials should be addressed to L.C.

Reprints and permissions information is available at www.nature.com/reprints.

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.