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ESCRT (Endosomal Sorting Complex Required for Transport) machinery is essential for acrosomal exocytosis in human sperm[1]

Running Title: ESCRT complexes and acrosomal exocytosis

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ABSTRACT

The sperm acrosome reaction is a unique regulated exocytosis characterized by the secretion of the acrosomal content and the release of hybrid vesicles formed by patches of the outer acrosomal and plasma membranes. In previous reports, we have shown that inward invaginations of the acrosomal membrane delineate ring-shaped membrane microdomains that contact the plasma membrane. We have postulated that the opening and expansion of fusion pores along these rings trigger acrosomal exocytosis. The invaginations of the acrosomal membrane topologically resemble the deformations of the endosomal membrane leading to the assembly of luminal vesicles in multivesicular bodies. In fact, intraacrosomal vesicles are also formed during acrosomal exocytosis. Endosomal Sorting Complex Required for Transport (ESCRT) participates in the organization of membrane microdomains that are invaginated and released as intraluminal vesicles in endosomes. We report here that members of ESCRT I (TSG101), ESCRT III (CHMP4), and the AAA ATPase VPS4 are present in the acrosomal region of the human sperm. Perturbing the function of these factors with antibodies or recombinant proteins inhibited acrosomal exocytosis in permeabilized cells. A similar effect was observed with a dominant negative mutant of VPS4A cross-linked to a cell penetrating peptide in not-permeabilized sperm stimulated with a calcium ionophore. When the function of ESCRT complexes was inhibited, acrosomes showed abnormal deformation of the acrosomal membrane and SNARE proteins that participate in acrosomal exocytosis failed to be stabilized in neurotoxin-resistant complexes. However, the growing of membrane invaginations was not blocked and numerous intraacrosomal vesicles were observed. These observations indicate that ESCRT-mediated processes are essential for acrosomal secretion, implicating these multifunctional complexes in an exocytic event crucial for sperm-egg fusion.

Summary sentence: The Endosomal Sorting Complex Required for Transport (ESCRT) machinery is essential for the secretion of the acrosomal granule in human sperm, implicating these multifunctional complexes in an exocytic event crucial for fertilization.

Keywords: human sperm, acrosome reaction, acrosomal swelling, multivesicular bodies, regulated exocytosis, ESCRT complexes

INTRODUCTION

The acrosome reaction is a regulated secretion with special characteristics that plays a central role in the fertilization process in many species [1]. The human spermatozoon possesses a large and flat granule forming a cap to the anterior part of the nucleus. The plasma membrane covers the extended area of the outer acrosomal membrane facing the cell surface. Both membranes run in parallel for more than 40 μm^2, separated for only about 20 nm. Acrosomal exocytosis involves the opening of a large number of fusion pores, merging both membranes. The expansion of these pores in a particular manner leads to the release of hybrid vesicles, containing patches of plasma and outer acrosomal membranes. Probably the most crucial role for the acrosomal exocytosis is the change in the topology and composition of the limiting membrane of the sperm [2]. The loss of the outer acrosomal and a large region of the plasma membrane exposes the inner acrosomal membrane to the medium and changes the composition of the plasma membrane, especially at the equatorial region. These changes are fundamental for the interaction and fusion of the sperm with the oocyte [1].

If we compare the acrosome reaction with exocytosis in other cells, where comparatively small and round vesicles fuse with the plasma membrane, striking differences are evident. The contact area for a vesicle approaching the plasma membrane is very small compared with the extended surface of interaction of the acrosomal membrane with the cell limiting membrane. The opening and expansion of a fusion pore in round vesicles leads to the incorporation of the vesicle membrane to the plasma membrane. In contrast, in acrosomal exocytosis the fusion and expansion of multiple fusion pores leads to the release of hybrid vesicles and the loss of the outer acrosomal and part of the plasma membrane.

In previous reports we have shown that by inhibiting the fusion pore opening during the acrosome reaction, we stopped the process at a stage were the acrosome was swollen and the flat outer acrosomal membrane presented deep invaginations [3]. We also described the appearance of intraacrosomal vesicles that appear to be formed by the pinching-off of the invaginations. We found areas of close proximity between the outer acrosomal and plasma membranes at the limiting edge of the invaginations. We postulated that the inward invaginations of the outer acrosomal membrane delineate ring-shaped membrane microdomains that contact the plasma membrane. The bent edge of the invaginations, probably enriched in membrane fusion factors, leads to the SNARE-dependent docking of the granule to the plasma membrane. Our aim was to identify and characterize molecular mechanisms implicated in the organization of membrane microdomains at the edge of acrosomal membrane invaginations responsible for interaction with the plasma membrane.

The acrosome is a secretory granule with the characteristics of a “Lysosomal Related Organelle” that originates from the Golgi in combination with the endocytic pathway during spermiogenesis [4;5]. Multivesicular bodies (MVBs) in the endocytic pathway are endosomes containing internal vesicles that are formed by invaginations of the limiting membrane of these organelles that sequester specific lipids and proteins to be targeted for lysosomal degradation or to be released as exosomes upon fusion of these organelles with the plasma membrane [6;7]. Several molecular mechanisms have been implicated in the segregation of membrane microdomains on the endosome limiting membrane and the formation of internal vesicles [8]. ESCRT (Endosomal Sorting Complex Required for Transport) are known players involved in several membrane deformation processes that generate budding structures from the cytoplasmic face of cell membranes (e.g., virus budding and intraluminal vesicle formation) [9]. ESCRTs consist of approximately twenty proteins organized in four complexes (ESCRT 0, I, II and III)

with associated proteins (VPS4, VTA1, ALIX), which are conserved from yeast to mammals. The ESCRT 0 complex has both membrane- and ubiquitin-binding properties, allowing the concentration of ubiquitinated cargo on the endosomal membrane. ESCRT 0 also recruits ESCRT I and II complexes. Recent work suggests that ESCRT I and II are responsible for membrane deformation into buds with sequestered cargo. ESCRT III subsequently promotes vesicle scission and the binding of VPS4, an AAA ATPase required for disassembly and recycling of the ESCRT machinery.

Because of the similitude between the inward invaginations observed in the outer acrosomal membrane and the mechanism of formation of intraluminal vesicles in endosomes, we speculated that ESCRT complexes might be activated during the acrosome reaction. We report here that members of the ESCRT I and III, and the AAA ATPase VPS4, are present in the acrosomal region of the sperm. Perturbing the function of these factors by antibodies or recombinant proteins inhibited acrosomal exocytosis and generated abnormal deformation of the acrosomal membrane. These observations indicate that ESCRT-mediated processes are essential for acrosomal secretion, implicating these proteins in a regulated exocytic event, a new function that has not been described before for these multifunctional complexes.

MATERIALS AND METHODS

Reagents

Pisum sativum lectin labeled with fluorescein isothiocyanate (FITC-PSL), poly-L-lysine, bovine serum albumin (BSA) and 4',6-diamidino-2-phenylindole (DAPI) were from Sigma-Aldrich S.A. (Buenos Aires, Argentina). 1,2-bis-(o-aminophenoxy)-ethane-N,N,N',N'-tetraacetic acid-acetoxymethyl ester (BAPTA-AM) was from Molecular Probes (Invitrogen Argentina). Ni-NTA-agarose was from Qiagen (Tecnolab SA, Buenos Aires, Argentina); DL- dithiothreitol (DTT) and isopropyl β-D-1-thiogalactopyranoside (IPTG) were from ICN (Eurolab SA, Buenos Aires, Argentina). Anti-VPS4A (mouse polyclonal, purified IgG, catalog number ab69415); anti VPS4B (rabbit polyclonal, purified IgG, catalog number ab81416); anti-TSG101 (mouse monoclonal, purified IgG, catalog number ab83) and anti-CHMP4B (rabbit polyclonal, purified IgG, catalog number ab105767) were from Abcam (Cambridge Massachusetts, USA). Anti-VAMP2/Syb2 (mouse monoclonal, clone 69.1, purified IgG) was from Synaptic Systems (Goettingen Germany). Horseradish peroxidase (HRP)-coupled goat anti-mouse; HRP-coupled goat anti-rabbit; Cy3-labeled goat anti-mouse and Cy3-labeled goat anti-rabbit antibodies were from Jackson ImnunoResearch (Sero-immuno Diagnostics, Inc. Tucker, GA, USA). All electron microscopy supplies were from Pelco (Ted Pella INC. California, USA). All other chemical reagents were of analytical grade and were purchased from ICN or Sigma-Aldrich (Buenos Aires, Argentina).

Recombinant proteins

Perfringolysin O (PFO) was purified as previously described [10]. Plasmids pET-16b encoding human VPS4A wild type, VPS4B wild type, VPS4A^{E228Q} and VPS4B^{E235Q} were generously provided for Dr. Monica Bajorek (Department of Biochemistry, University of Utah, Salt Lake City, Utah, USA). The plasmid pET-21b encoding human TSG101 UEV domain was provided for Dr. Eric Freed (Army Medical Research Institute of Infectious Diseases, Frederik, MD, USA). The plasmid pET-23d encoding SNF7/CHMP4 yeast protein was generously provided for Dr. Scott Emr (Weill Institute for Cell and Molecular Biology, Cornell University, Ithaca, NY,

USA). All plasmids encoding His_6-tagged proteins were transformed into *Escherichia coli* strain BL21 (DE3) pLysS. Protein synthesis was induced 2.5-3 hours at 37 °C with 1 mM IPTG. Bacteria were harvested and lysed by sonication and proteins were purified under native conditions by affinity chromatography on Ni-NTA-agarose. Plasmids encoding the light chains of tetanus toxin (TeTx) and botulinum toxin B (BoNT/B) were generously provided by Dr. T. Binz (Medizinische Hochschule Hannover, Hannover, Germany). Neurotoxin light chains were purified as previously described [11]. Protein concentrations were determined by the Bio-Rad protein assay in 96-well microplates. Bovine serum albumin (BSA) was used as a standard, and the results were quantified on a 3550 Microplate Reader (Bio-Rad). After purification, recombinant proteins were divided in 15-50 µl aliquots and stored at –80°C in the elution buffer (250 mM imidazole, 100 mM NaCl, 50 mM Tris-HCl, pH 8).

Preparation of KR_9C-$VPS4A^{E228Q}$ conjugate

Conjugation reactions were performed using a Peptide–Protein Crosslinking Kit (Solulink, San Diego, California, USA), according to the manufacturer's instruction. Briefly, the cell penetrating peptide (CPP) KRRRRRRRRRC (Innovagen AB, Lund, Sweden) was linked to succinimidyl-6-hydrazino-nicotinamide (S-HyNic), and $VPS4A^{E228Q}$ was linked to succinimidyl-4-formylbenzamide (S-4FB), through primary amines. HyNic-modified peptide and 4FB-modified protein (3:1 molar equivalent) were cross-linked by incubating for 3 h at room temperature (RT), in the presence of aniline (100 mM) as a catalyst. The conjugate was further purified using a Sephadex G25 column after the conjugation reaction. Purity was confirmed by SDS-PAGE analysis.

Human sperm sample preparation and acrosome reaction

Human semen samples were obtained from normal healthy donors. The informed consent and protocol for semen handling were approved by the Ethic Committee of the Medical School, Universidad Nacional de Cuyo. Semen was allowed to liquefy for 30–60 min at 37 °C. We used a swim-up protocol to isolate highly motile sperm under capacitating conditions in Human Tubal Fluid media (HTF, as formulated by Irvine Scientific, Santa Ana, CA) supplemented with 0.5% BSA for 1 h at 37 °C in an atmosphere of 5% CO_2/95% air. Sperm concentration was adjusted to $5–10 \times 10^6$ cells/ml before incubating for at least 2 h under capacitating conditions. We have previously reported that sperm incubated under these conditions present a robust progesterone response with a minimal number of spontaneously reacted spermatozoa [12]. Permeabilization was accomplished as described in [10]. Briefly, washed spermatozoa were resuspended in cold phosphate-buffered saline (PBS) containing 25 nM PFO for 15 min at 4 °C. Cells were washed once with PBS and resuspended in ice cold sucrose buffer (250 mM sucrose, 0.5 mM EGTA, 20 mM Hepes-K, pH 7) containing 2 mM dithiothreitol. For acrosome reaction assays, we added inhibitors and stimulants sequentially as indicated in the figures, and incubated for 15-30 min at 37 °C after each addition. Spermatozoa were spotted on Teflon-printed slides, air dried, and fixed/permeabilized in ice cold methanol for 1 min. Acrosomal status was evaluated by staining with FITC-coupled Pisum sativum lectin (FITC–PSL) according to [13]. At least 200 cells were scored using a Nikon Optiphot II microscope (Nikon, Inc., Melville, NY) equipped with epifluorescence optics. Basal (no stimulation, "control") and positive (0.5 mM $CaCl_2$, corresponding to 10 µM free Ca^{2+}, "Ca^{2+}") controls were included in all experiments. Acrosomal exocytosis was calculated by subtracting the number of spontaneously reacted spermatozoa

(ranged 5%–15%) from all values. Experiments with spontaneously reacted cells larger than 15% or response to stimulation lower than 10% were excluded from the analysis.

Electrophoresis and Western blot

Spermatozoa (90 x 10^6) were washed twice in cold PBS, and proteins were extracted and partitioned in Triton X-114 (to improve antibody detection specificity) as described [14;15]. Proteins in the aqueous and detergent phases were precipitated, resuspended in sample buffer (2% SDS, 10% glycerol, 62.5 mM Tris-HCl) and adjusted to 5% β- mercaptoethanol, boiled for 3-5 min, and used immediately or stored at -20°C. When indicated, 50 ng recombinant VPS4A, 100 ng recombinant VPS4B or 100 µg total proteins from an HeLa cell extract were use as standards. Proteins were separated on 10% Tris-tricine-SDS gels and transferred to nitrocellulose membrane (Hybond-ECL, GE Healthcare, Buenos Aires, Argentina). Non-specific reactivity was blocked by incubation for 1 h at RT with 1% skim milk in washing buffer (0.1% Tween 20 in PBS). Blots were incubated with primary antibodies anti-VPS4A, anti-VPS4B, anti-TSG101 or anti-CHMP4B (1 µg/ml), in blocking solution for 1 h at RT. HRP-coupled goat anti-mouse or anti-rabbit were used as secondary antibodies (0.25 µg/ml, 1 h at RT). Excess first and second antibodies were removed by washing five times for 10 min each in washing buffer. Detection was accomplished with an enhanced chemiluminescence system (ECL; Amersham Biosciences) and visualized with a FujiFilm LAS-4000 Scanner (FujiFilm, Tokyo, Japan).

Indirect immunofluorescence

Sperm cells were spotted on poly-L-lysine-covered slides, fixed in 2% paraformaldehyde in PBS for 10 min at RT, and permeabilized in 0.1% Triton X-100 in PBS for 10 min at RT. Cells were incubated in 50 mM glycine–PBS for 30 min at RT and then blocked with 5% BSA in PBS during 1 h. Cells were labeled with 20 µg/ml anti-VPS4A antibody, 14 µg/ml anti-VPS4B antibody, 20 µg/ml anti-TSG101 antibody or 20 µg/ml anti-CHMP4B antibody (overnight at 4°C in 1% BSA-PBS), followed by a Cy3-labeled anti-mouse IgG or anti-rabbit IgG as secondary antibodies (4 µg/ml in 1% BSA) for 1 h at 4°C. Slides were washed with PBS between incubations. Finally, cells were fixed for 1 min in cold methanol and stained with FITC-PSL (50 µg/ml in PBS, 40 min at RT) and washed with distilled water for 20 min at 4°C. Slides were mounted in 1% propyl-gallate, 1 µg/ml DAPI in PBS /50% glycerol. VAMP2/Syb2 immunostaining was performed as described previously [11]. Sperm cells were analyzed by confocal microscopy using an Olympus FluoView TM FV1000 confocal microscope (Olympus, Argentina), with the FV10-ASW (version 01.07.00.16) software.

Transmission electron microscopy

We processed permeabilized human spermatozoa (5–7 × 10^6 cells/ tube) as described for acrosome reaction assays and then the reaction was stopped with 2.5% glutaraldehyde in sucrose buffer. Sperm suspensions were incubated overnight at 4 °C. Fixed sperm samples were washed twice in PBS and post fixed in 1% osmium tetroxide–PBS for 2 h at RT, washed three times in PBS, and dehydrated sequentially with increasing concentrations of ice-cold acetone. Cells were infiltrated in 1:1 acetone:Spurr 2 h or overnight at RT and finally embedded in fresh pure resin overnight at RT. Samples were cured 24 h at 70 °C. Thin sections were cut (60–80 nm) with a diamond knife (Diatome, Washington, DC, USA) on a Leica Ultracut R ultramicrotome, collected on 200-mesh copper grids and stained with saturated uranyl acetate in methanol plus lead citrate. Grids were observed and photographed in a Zeiss 902 electron microscope at 50 kV.

We included negative (not stimulated) and positive (stimulated with 0.5 mM $CaCl_2$ in presence of 20 μM BAPTA-AM) controls in all experiments. The intra-acrosomal vesicles perimeter was measured using Image J software (version 1.32j, National Institutes of Health, USA. http://rsb.info.nih.gov/ij/java).

RESULTS

VPS4A and B are present in the acrosomal region of human sperm

The acrosomal granule swells in preparation for exocytosis. The flat outer acrosomal membrane, visible in not stimulated sperm, undergoes profound deformation after triggering the exocytic process with a calcium ionophore or progesterone. The invaginations of the membrane have the same topology observed in endosomes during the formation of intraluminal vesicles. One of the best-characterized mechanisms of endosomal membrane microdomains organization and sequestration in intraluminal vesicles involves the recruitment of ESCRT complexes. We hypothesized that the same complexes could be involved in the mechanism of membrane deformation in the acrosome. VPS4 is an AAA ATPase that participates in the last step in the formation of internal vesicles disassembling and recycling the ESCRT complexes [9]. Two isoforms are present in the human genome. We detected the presence of VPS4 by Western blot in the aqueous phase after a partition in Triton X-114 using two different antibodies that recognize VPS4A (full-length sequence of the human protein) and VPS4B (residues 1-100 of the human protein). The antibodies labelled bands of the expected molecular mass (49 kDa, Fig. 1A and 1B). When tested with recombinant proteins, the antibodies showed a very weak cross-reactivity, indicating that both isoforms were present in spermatozoa (Supplemental Fig. S1; available online at www.biolreprod.org). When the same antibodies were used for indirect immunofluorescence, a predominantly acrosomal labeling was observed (Fig. 1C and 1D). About 30% of the sperm presented a clear co-localization between VPS4 and the acrosomal marker that was not observed in the absence of the primary antibody (Fig, 1E and 1F). In conclusion, two isoforms of VPS4, which are essential factors in the mechanism of intraluminal vesicle formation in endosomes, are present in the acrosomal region of human sperm.

Both VPS4 isoforms are required for acrosomal exocytosis

The presence of these proteins in the acrosomal region of sperm suggested that they might have a role in acrosomal exocytosis. To test this hypothesis, we assessed the effect of the antibodies in acrosomal exocytosis in permeabilized cells. As shown in Fig. 2A and 2B, both antibodies inhibited secretion in a dose dependent manner (ED50 39 ± 5 nM for anti-VPS4A and ED50 14 ± 3 nM for anti-VPS4B).

A point mutation in the ATPase domain of VPS4A or VPS4B renders the proteins unable to hydrolyze ATP [16;16-18]}[19]. His_6-tagged wild type and mutant version of both isoforms were expressed in bacteria and purified by affinity columns. The mutant recombinant proteins were inhibitory in the exocytosis assay in permeabilized sperm with ED50 50 ± 8 nM for $VPS4A^{E228Q}$ and ED50 34 ± 8 nM for $VPS4B^{E235Q}$ (Fig. 2C). In contrast, the wild type proteins did not affect the assay (Fig. 2D). None of the proteins had an effect in not stimulated sperm (data not shown).

The functional assays with recombinant proteins an antibodies were performed with permeabilized sperm. We have previously reported that a calcium ionophore causes acrosomal swelling in not-permeabilized sperm, indicating that granule deformations are not an artifactual

product of sperm permeabilization [3;20]. To assess whether inhibition of VPS4 function in not-permeabilized cells affects exocytosis, we generated a membrane-permeant version of the VPS4A^{E228Q} mutant. The recombinant protein cross-linked to the KR_9C peptide (an arginine-rich CPP) was added to spermatozoa before challenging with A23187. The protein did not affect cell motility assessed qualitatively under the light microscope, indicating that it was not toxic for the cells (data not shown). However, the protein caused an almost complete block of the acrosomal exocytosis (Fig. 2E). In contrast, VPS4A^{E228Q} without the peptide, which completely blocked secretion in permeabilized sperm (Fig. 2C), did not affect A23187-stimulated exocytosis in not-permeabilided cells (Fig. 2E).

We then tested whether we could compete the inhibitory effect of the mutants with the wild type proteins in permeabilided sperm. As shown in Fig. 2F, 200 nM wild type proteins completely recovered the effect of the mutants. These results rule out the possibility that the inhibitory effect of the mutants was due to a nonspecific effect unrelated to the function of the proteins, and at the same time, indicate that the wild type proteins were fully functional. The fact that both isoforms are present in sperm and that antibodies against individual isoforms can fully block exocytosis suggest that both version of VPS4 are necessary for acrosomal exocytosis. To confirm this possibility, we attempted to rescue the effect of dominant negative VPS4A with wild type VPS4B, and vice versa. Results in Fig. 2F show that the isoforms are not redundant. Reversion of the negative mutants was only achieved by the corresponding wild type isoform. These observations indicate that both VPS4A and VPS4B are necessary for acrosomal exocytosis.

VPS4 is necessary for efficient stabilization of SNARE complexes

We have previously reported that SNAREs are protected in cis complexes resistant to neurotoxins in unstimulated sperm [11]. Cis complexes are disassembled upon stimulation, in a process that requires the PTP1B-dependent activation of NSF [21]. Our data suggest that the SNARE complexes re-assemble in trans and become resistant to TeTx at the contact sites between the acrosomal and plasma membranes. If ESCRT complexes participate in the bending of the acrosomal membrane that originates these contact sites, perturbation of ESCRT-mediated mechanisms may affect the assembly/disassembly cycle of the SNARE complexes. It was then interesting to assess the toxin sensibility of the SNARE complexes at the step where the anti-VPS4 antibody and the dominant negative mutant inhibited exocytosis. In a first approach, toxin sensitivity was assessed by an immunofluorescence assay [11]. The rationale for this assay is that if VAMP2/Syb2 is assembled in cis complexes, it should be resistant to TeTx and BoNT/B, whereas if it is part of trans complexes, it should be resistant to TeTx but sensitive to BoNT/B. The protein should be sensitive to both neurotoxins when not assembled in stable complexes [11]. The number of immunostained cells decreases when VAMP2/Syb2 is proteolyzed by the neurotoxins. The results are shown in Fig. 3A and 3B. When sperm where stimulated in the presence of anti-VPS4B antibody, the dominant negative mutant VPS4A^{E228Q}, or a combination of both mutants (VPS4A^{E228Q} + VPS4B^{E235Q}) VAMP2/Syb2 became sensitive to both neurotoxins, suggesting that perturbing the function of VPS4 does not prevent cis complex disassembly and halts exocytosis when SNAREs are not yet protected in trans complexes.

The observation that we could rescue the dominant negative mutant inhibition by adding wild type protein gave us the possibility of assessing neurotoxin sensitivity by using a functional assay. In this protocol, sperm were stimulated in the presence of VPS4B^{E235Q}. Neurotoxins were added at this stage to assess whether VAMP2/Syb2 had assembled in toxin resistant complexes.

Afterwards, the effect of the mutant was competed with wild type VPS4B. The results in Fig. 3C show that VAMP2/Syb2 was sensitive to both neurotoxins at the step where the dominant negative mutant stopped the process, in agreement with the observation obtained with the immunofluorescence assay.

Other components of the ESCRT complexes are necessary for acrosomal exocytosis

VPS4 may have other functions besides its well-stablished role in ESCRT-mediated processes. It has been shown that it regulates cholesterol trafficking independently of ESCRT III [22]. Hence, to prove that the effect of VPS4A and B on acrosomal exocytosis was due to an ESCRT-dependent mechanism, we searched for the presence and function of other ESCRT proteins. We focused on TSG101 (belonging to ESCRT I complex) and on CHMP4 (belonging to ESCRT III complex). TSG101 was detected in the hydrophilic fraction after a Triton-114 partition by Western blot using an antibody that recognizes aminoacids 167-375 of the human protein (Fig. 4A). CHMP4 was also detected in the hydrophilic fraction after detergent partition by an antibody recognizing the full-length human protein (Fig. 4B). The same antibodies labelled the acrosomal region of spermatozoa by indirect immunofluorescence (Fig. 4C and 4D). About 60%-80% of the sperm presented a clear co-localization between these two proteins and the acrosomal marker (Fig, 4E and 4F).

To assess the role of these proteins on acrosomal exocytosis, we resorted to the same antibodies that recognized the endogenous proteins. Fig. 5A and 5B show that both antibodies abrogated calcium triggered exocytosis in a dose dependent manner (ED50 13 ± 3 nM for anti-TSG101 and ED50 12 ± 3 nM for anti-CHMP4). To confirm the role of TSG101 on acrosomal exocytosis, we included in the assay the ubiquitin binding domain of the human protein (aminoacids 1 to 145). We observed a prominent inhibitory effect with this recombinant protein (Fig. 5C, ED50 33 ± 16 nM). Similarly, when the full length yeast CHMP4 protein (named SNF7), which has a 50% similarity with the human protein [23], was added to the assay, a dose dependent inhibition was observed (Fig. 5D, ED50 29 ± 11 nM). In conclusion, the facts that TSG101 and CHMP4 are present in the acrosomal region of the sperm and that manipulations of the availability of both proteins alter the acrosome reaction strongly suggest that an ESCRT-mediated process is required for the exocytosis of the acrosomal granule.

ESCRT complex manipulation deregulates intraacrosomal vesicle formation

ESCRT is one of the best characterized molecular mechanism for membrane deformation and vesicle budding outward of the cytosolic face of cellular membranes [9]. We then analyzed the morphology of the acrosomal membranes under conditions that affect the ESCRT complexes and that impaired acrosomal exocytosis by transmission electron microscopy. Representative images of sperm heads under these conditions are shown in Fig. 6C to 6F. Images of an unstimulated sperm and a sperm stimulated in the presence of an intraacrosomal calcium chelator -which prevent the efflux of calcium from the acrosome- are shown in Fig. 6A and 6B, respectively. These conditions have been characterized in a previous report and were taken as negative and positive controls for acrosomal swelling [3]. The results indicate that reagents affecting ESCRT-mediated processes did not inhibit acrosomal swelling, defined as a notorious increase in the distance between the inner and outer acrosomal membranes observed in sperm head sections (compare the average distance in Fig. 6A versus this parameter in Fig. 6B to 6F). The percentage of sperm with swollen acrosomes after calcium stimulation was similar to the one observed when exocytosis was blocked by preventing calcium release from intracellular stores (Fig. 6I).

Surprisingly, intraacrosomal vesicles were more frequently observed in swollen acrosomes when VPS4 function was altered by addition of the wild type proteins, the dominant negative mutants or the inactivating antibodies (Fig. 6J). The number of internal vesicles per acrosome was very variable; some acrosomes where profusely vesiculated whereas other had just one vesicle (Fig. 6C, 6D, and 6K). It was interesting to note that the size of the internal vesicles in the presence of wild type VPS4 was reduced (Fig. 6L), suggesting that this protein may be involved in the final pinching off of the budding vesicle, as it has been suggested for other budding processes [24]. Addition of recombinant SNF7/CHMP4 did not affect the percentage of swollen acrosomes (Fig. 6I) or the percentage of swollen acrosomes with internal vesicles (Fig. 6J). However, acrosomes extraordinarily swollen with a large number of internal vesicles where frequently observed (Fig. 6E and 6K). SNF7/CHMP4 did not significatively alter the size of the internal vesicles (Fig. 6L). The most striking feature in the presence of SNF7/CHMP4 was the appearance of acrosomes showing dense material accumulated in deep invaginations of the outer acrosomal membrane and in intraacrosomal vesicles (Fig. 6E, 6G, and 6H). When SNF7/CHMP4 was added in combination with mutant VPS4, the phenotype was very similar to the one observed with SNF7/CHMP4 alone, suggesting that this protein stopped the process at a stage previous to the one affected by mutant VPS4. In conclusion, altering the ESCRT complex function causes abnormal deformation of the acrosomal membrane but does not block the growing of membrane invaginations and the appearance of internal vesicles.

DISCUSSION

One of the striking peculiarities of acrosomal exocytosis is the swelling and deformation of the acrosomal granule leading to the growing of deep invaginations of the outer acrosomal membrane. Swelling is also accompanied by the appearance of internal vesicles with a topology equivalent to the luminal vesicles in MVBs. ESCRT complexes have a well-known function in the sorting of membrane component in internal vesicles of MVBs, a subpopulation of which are eventually released as exosomes [6]. However, these complexes have not been involved in the exocytosis of secretory granules before. By preventing late steps of the membrane fusion process that opens pores connecting the extracellular medium with the acrosomal lumen, we have observed tight apposition of the membranes in the limiting edge of invaginations of the outer acrosomal membrane, which depend on SNAREs and SNARE-associated proteins [3;25;26]. We speculate that these interacting regions define the boundary of the hybrid vesicle that will form upon the opening and expansion of the fusion pores. Furthermore, invaginations can eventually form intraacrosomal vesicles that will be released during the acrosome reaction. These vesicles would be surrounded exclusively by outer acrosomal membrane. Because of topological similitude of outer acrosomal membrane invaginations with the mechanism of MVB formation, we speculated that ESCRT complexes might be involved in acrosomal exocytosis. A recent and very complete proteomic analysis of human sperm reported the presence of a large set of ESCRT proteins, including TSG101, CHMP4 and VPS4A and VPS4B [27]. We confirmed the presence of these proteins by Western blot analysis and, in addition, we showed that the proteins concentrated in the acrosomal region of the sperm suggesting a possible role in the acrosome reaction.

VPS4 is an AAA ATPase that participates in the last step of ESCRT function by disassembling the complexes [9;24]. It was interesting to observe that both VPS4A and B are present in the human sperm, confirming what was reported in the proteomic analysis [27]. A functional assay with permeabilized sperm showed that both isoforms were required for

acrosomal exocytosis. Depletion of any of the endogenous proteins by specific antibodies abrogated exocytosis, and the wild type proteins only rescued exocytosis of their corresponding inhibitory mutant. The two proteins have a strong homology, interact in two hybrid systems, and likely participate in the same event of ESCRT III complex disassembly [28]. However, both isoforms are conserved along evolution and do not fully co-localize in mouse 3T3 cells, suggesting that they may have differential functions [28]. Our results indicate that the two isoforms are not redundant for acrosomal exocytosis. Other processes also require both isoforms; depletion of either VPS4A or B by siRNA interferes with centrosome and spindle maintenance in HeLa cells [29].

Permeabilized sperm have proved to be a reliable model to study the mechanism of membrane fusion during acrosomal exocytosis. However, permeabilized sperm lose ionic and electric gradients across the plasma membrane and the composition of the cytosol surrounding the acrosomal granule is altered. We have previously reported that a calcium ionophore causes acrosomal swelling in not-permeabilized sperm, indicating that granule deformations are not an artifactual product of sperm permeabilization [3;20]. To interfere with the normal function of ESCRT complexes in not-permeabilized cells, we designed a membrane-permeant variant of a dominant negative mutant of VPS4A. We have shown in previous reports that proteins carrying polyarginine peptides are able to cross the sperm plasma membrane. Glutathione S-transferase linked to a polyarginine peptide translocates into the sperm citosol without affecting acrosomal exocytosis; whereas, Rab3A, carrying the same peptide, triggers secretion in not-permeabilized sperm [30]. $VPS4A^{E228Q}$ cross-linked to the KR_9C peptide efficiently blocked A23187-induced exocytosis in alive sperm, supporting the idea that ESCRT complexes are necessary for acrosomal exocytosis.

The function of two other ESCRT proteins, TSG101 and CHMP4, belonging to complex I and III, respectively, were tested on acrosomal exocytosis. Both proteins were required for secretion as addition of inactivating antibodies completely blocked the process. Exocytosis was also inhibited by the ubiquitin binding domain of TSG101. This domain blocks the ESCRT-dependent release of HIV-1 virus in Hela cells [31]. The ubiquitination machinery is active during spermatogenesis and it has a role in acrosome formation [32-34]. The TSG101 domain may block exocytosis by preventing the proper organization of membrane domains containing essential ubiquitinated proteins. The CHMP4 yeast homolog protein (named SNF7) was also inhibitory. It has been shown that an excess of this protein inhibits ESCRT function by altering the stoichiometry of the complexes [35].

The effect of ESCRT manipulation on acrosomal exocytosis was very prominent, indicating that ESCRT complexes are necessary for acrosomal content secretion; notwithstanding, invaginations of the outer acrosomal membrane and formation of internal vesicles were not blocked under these conditions. The process seems to be deregulated, as the number of the intraluminal vesicle was extremely variable when ESCRT function was inhibited. Notice that some intraacrosomal vesicles observed by transmission electron microscopy may be deep membrane invaginations connecting with the acrosomal membrane in a plane not included in the thin section analyzed. In other models, depletion of ESCRT proteins blocks the recruitment of EGF receptors into luminal vesicles [36]. However, internal vesicles are formed in the absence of ESCRT complexes, indicating that other mechanisms of vesicle formation are present in cells [36]. ESCRT seems to be necessary for the organization of membrane microdomains where ubiquitinated proteins are recruited. These complexes may help to organize special membrane microdomains in the bending areas of cellular membranes [8]. This

association likely promotes further bending and eventually the pinch-off of intraluminal vesicles. As a working model, we propose that ESCRT are recruited to the bent area of outer acrosomal membrane invaginations, promoting the organization of microdomains that are necessary for interaction with complementary domains in the plasma membrane (Fig. 7). In the absence of functional ESCRT complexes, stable associations with the plasma membrane are prevented, leaving SNARE proteins unable to be stabilized in trans complexes. However, the acrosomal membrane still forms invaginations and eventually internal vesicles in a deregulated manner. It is worth to mention that MVBs formed in the absence of ESCRT complexes also show a disorganized structure [36]. Additional studies will be required to characterize other molecular players involved in acrosomal swelling and membrane deformation during the acrosome reaction. In brief, our observations indicate that ESCRT-mediated processes are essential for acrosomal secretion, implicating these multifunctional complexes in an exocytic event that is necessary for sperm-egg fusion.

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FIGURE LEGENDS

Figure 1. VPS4 proteins are present in human sperm and localizes to the acrosomal region. **A-B**) Whole sperm homogenates (90 x 10^6 cells) were partitioned in Triton X-114. Proteins from the aqueous (ap) and detergent phases (dp) and purified recombinant proteins (VPS4A, 50 ng; VPS4B, 100 ng) were electrophoresed on 10% Tris-tricine-SDS PAGE, transferred to a nitrocellulose membrane and immunoblotted with an antibody raised against VPS4A (**A**) or VPS4B (**B**). Molecular mass standards are indicated to the left. Black arrows point to the 49 kDa specific VPS4 bands. **C-F**) Sperm were fixed and spotted on glass slides and labeled with anti VPS4A (**C**) or anti VPS4B (**D**). Nuclei were stained with DAPI and acrosomes with PSL-FITC (bar = 5 μm). As control, the same protocol was applied without including the anti-VPS4 antibodies. At least 100 cells were quantified for each condition and the percent of sperm with intact acrosome showing positive VPS4A (**E**) or VPS4B (**F**) acrosomal label was recorded. The bars represent the mean ± SEM of 3-5 independent experiments.

Figure 2. VPS4 is necessary for acrosomal exocytosis in human sperm. **A-D**) Permeabilized sperm were incubated for 15 min at 37ºC with increasing concentrations of antibodies (**A** and **B**), recombinant dominant negative VPS4 mutants (**C**) or 160 nM wild type proteins (**D**). Afterwards, the cells were incubated for 15 min without stimulus (control) or with calcium (10 μM free calcium). **E**) Not-permeabilized sperm were incubated for 30 min at 37ºC with increasing concentrations of VPS4A cross-linked to the CPP peptide KR_9C (circles) or not

modified (triangle). Afterwards, the cells were incubated for 30 min without stimulus (control) or with 10 μM A23187. **F**) Permeabilized spermatozoa were incubated first with VPS4 mutants (60 nM, VPS4A or VPS4B) to compete with the endogenous protein, then, stimulated with calcium to induce secretion, and finally with the wild type proteins (200 nM, VPS4A or VPS4B) to rescue exocytosis (15 min incubation at 37ºC for each step, black bars). As controls (gray bars), some aliquots were incubated without calcium (control), with calcium in the absence of inhibitors (Ca^{2+}), or without adding wild type proteins to rescue secretion. For all panels, acrosomal exocytosis was evaluated using PSL-FITC. The data represent mean ± SEM of 3-4 independent experiments. The means were compared with the control condition (**D**) or with the respective inhibitory condition (**F**) by ANOVA and Dunnett test (*P<0.05; **P<0.01; NS, not significantly different).

Figure 3. VPS4 is necessary for efficient stabilization of SNARE complexes. A) Permeabilized spermatozoa were incubated for 15 min at 37ºC with 130 nM anti VPS4B (anti VPS4B), 200 nM recombinant $VPS4A^{E228Q}$, alone ($VPS4A^{E228Q}$, not shown), or in combination with 200 nM $VPS4B^{E235Q}$ (VPS4Mut); subsequently cells were incubated for 15 min at 37ºC with calcium (10 μM free calcium) to stimulate the exocytic process that should stop at the step requiring VPS4. Afterwards VAMP2/syb2 susceptibility to neurotoxin digestion was tested by adding 300 nM BoNT/B or 300 nM TeTx (15 min at 37ºC). As controls, an aliquot was incubated without any addition and two others with neurotoxins without stimulation. Cells were then fixed and triple stained with the mouse monoclonal anti-VAMP2/syb2 antibody (red, top panels), PSL-FITC (to stain the acrosome; green, central panels), and DAPI (to visualize all cells in the field; blue, bottom panels). Asterisks (*) indicate cells with intact acrosomes lacking VAMP2/syb2 immunostaining (bar = 5 μm). **B**) Quantification of the relative percentage of cells exhibiting VAMP2/syb2 acrosomal staining (normalized to the control) under different experimental conditions for 3 independent experiments (mean ± SEM). The means were compared with the control condition (100%) by Student's t test for single group mean (*P<0.05; **P<0.01). **C**) Permeabilized spermatozoa were incubated for 15 min at 37ºC with 60 nM $VPS4B^{E235Q}$ to compete with the endogenous protein, and then stimulated with calcium for 15 min at 37ºC to initiate secretion. VAMP2/syb2 susceptibility to neurotoxin digestion was tested by adding 300 nM BoNT/B or TeTx at this stage (15 min at 37ºC). Exocytosis was re-initiated by adding 200 nM wild type VPS4B (black bars). As control (gray bars), cells were incubated without stimulus (control) or with calcium (Ca^{2+}), or preincubated with neurotoxins or $VPS4B^{E235Q}$ before stimulation or rescuing with the wild type protein without neurotoxin treatment. Acrosomal exocytosis was evaluated using PSL-FITC. The data in C represent mean ± SEM of 3-4 independent experiments. The means were compared with the condition where the effect of the mutant was competed with the wild type protein without neurotoxin treatment by ANOVA and Dunnett test (**P<0.01).

Figure 4. TSG101 and CHMP4B are both present in the acrosomal region of human sperm. A-B) Whole sperm homogenates (90 x 10^6 cells) were partitioned in Triton X-114. Proteins from the aqueous (ap) and detergent phases (dp) and from a HeLa cell extract were electrophoresed on 10% Tris-tricine-SDS PAGE, transferred to a nitrocellulose membrane and immunoblotted with an antibody raised against TSG101 (**A**) or CHMP4B (**B**). Molecular mass standards are indicated to the left. Black arrows point to the 47 kDa TSG101 and 35 kDa CHMP4B bands. **C-F**) Sperm were fixed and spotted on glass slides and labeled with Cy3-coupled anti TSG101 (**C**)

or anti CHMP4B (**D**). Nuclei were stained with DAPI and acrosomes with PSL-FITC (bar = 5 μm). As control, the same protocol was applied without including the anti-TSG101 or the anti-CHMP4B antibodies. At least 100 cells were quantified for each condition and the percent of sperm with intact acrosome showing positive TSG101 (**E**) or CHMP4 (**F**) acrosomal label was recorded. The bars represent the mean ± range of 2 independent experiments.

Figure 5. TSG101 (ESCRT I) and CHMP4 (ESCRT III) are necessary for acrosomal exocytosis in permeabilized human sperm. A-D. Permeabilized sperm were incubated for 15 min at 37ºC with increasing concentrations of anti-TSG101 antibody (**A**), anti-CHMP4B antibody (**B**), recombinant TSG101-UEV domain (**C**), or recombinant yeast CHMP4 (SNF7, **D**). Afterwards, the cells were incubated for 15 min without stimulus (control) or with calcium (10 μM free calcium). Acrosomal exocytosis was evaluated using PSL-FITC. The data represent mean ± SEM of 2-4 independent experiments.

Figure 6. ESCRT complex manipulation deregulates intraacrosomal vesicle (IAV) formation. Permeabilized spermatozoa were incubated for 15 min at 37ºC with 20 μM BAPTA-AM (**B**, blue bars/circles from **I** to **L**); 200 nM VPS4A wild type + 200 nM VPS4B wild type + 20 μM BAPTA-AM (**C**, brown bars/circles from **I** to **L**); 200 nM $VPS4A^{E228Q}$ + $VPS4B^{E235Q}$ (**D**, green bars/circles from **I** to **L**); 130 nM anti-VPS4A + 130 nM anti-VPS4B (orange bars/circles from **I** to **L**); 200 nM SNF7/CHMP4 (**E**, **G**, **H**, yellow bars/circles from **I** to **L**) or 200 nM SNF7/CHMP4 + 200 nM $VPS4B^{E235Q}$ (**F**, light blue bars/circles from **I** to **L**). Subsequently, the cells were stimulated for 30 min at 37ºC with calcium (10 μM free calcium). As a control, an aliquot was incubated in the absence of inhibitors and calcium (**A**). Samples were fixed and processed for electron microscopy as described in Materials and Methods. N, nucleus; PM, plasma membrane; IAM, inner acrosomal membrane; OAM outer acrosomal membrane; A, acrosome; IAV, intraacrosomal vesicle; arrows, dense material in the cytosolic face of OAM invaginations and in IAV. The percentage of sperm with swollen acrosomes (**I**, notice that the level of swelling in unstimulated sperm is indicated with a horizontal blue line; mean ± SEM), the percentage of swollen acrosomes containing internal vesicles (**J**), the number of internal vesicles per acrosome (**K**), and the perimeter of the internal vesicles (**L**) were quantified for each experimental condition. Bars in **I** and **J** represent mean ± SEM. Circles in **K-L** represent individual values. Values in **I** to **L** were obtained from 4 independent experiments where at least 200 cells were quantified for each condition. NS, not significantly different; *, **, and *** significantly different for $P<0.05$, $P<0.01$, and $P<0.001$, respectively (compared with BAPTA-AM + calcium). Tests used: **I**, ANOVA and Dunnett test; **J**, Chi-square test; **K**, non-parametric multiple comparison (Dunnett test equivalent) to compare means and **F** to compare variances; **L**, non-parametric multiple comparison (Dunnett test equivalent).

Figure 7. Working model for ESCRT complexes function in acrosomal exocytosis. In resting sperm, SNAREs are assembled in cis complexes. Upon stimulation, these complexes are disassembled and organized in ESCRT-dependent membrane microdomains at the edge of the outer acrosomal membrane (OAM) invaginations of swollen acrosomes. Some invaginations can pinch off inside the acrosome forming intraacrosomal vesicles. In other instances, SNARE rings at the edge of invaginations contact SNAREs at the plasma membrane and trigger membrane fusion in these circular regions. The expansion of the fusion pores causes the release of hybrid vesicles leaving large holes connecting the acrosomal lumen with the external medium. In the

absence of functional ESCRT, acrosomal swelling still occurs and the OAM forms invaginations in a deregulated manner, but SNAREs in this membrane are unable to form productive trans complexes with SNAREs at the plasma membrane and exocytosis is inhibited.

Figure 1. VPS4 proteins are present in human sperm and localizes to the acrosomal region.

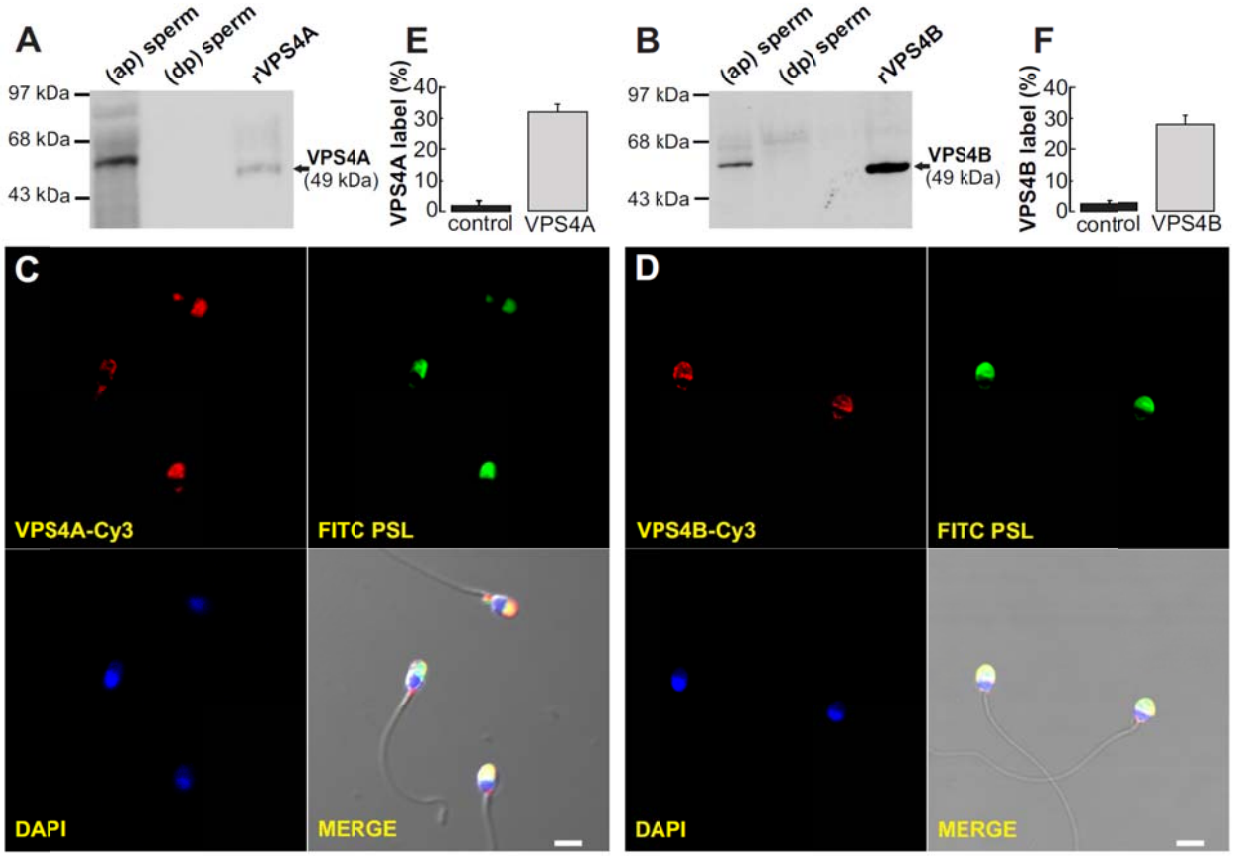

Figure 2. VPS4 is necessary for acrosomal exocytosis in human sperm.

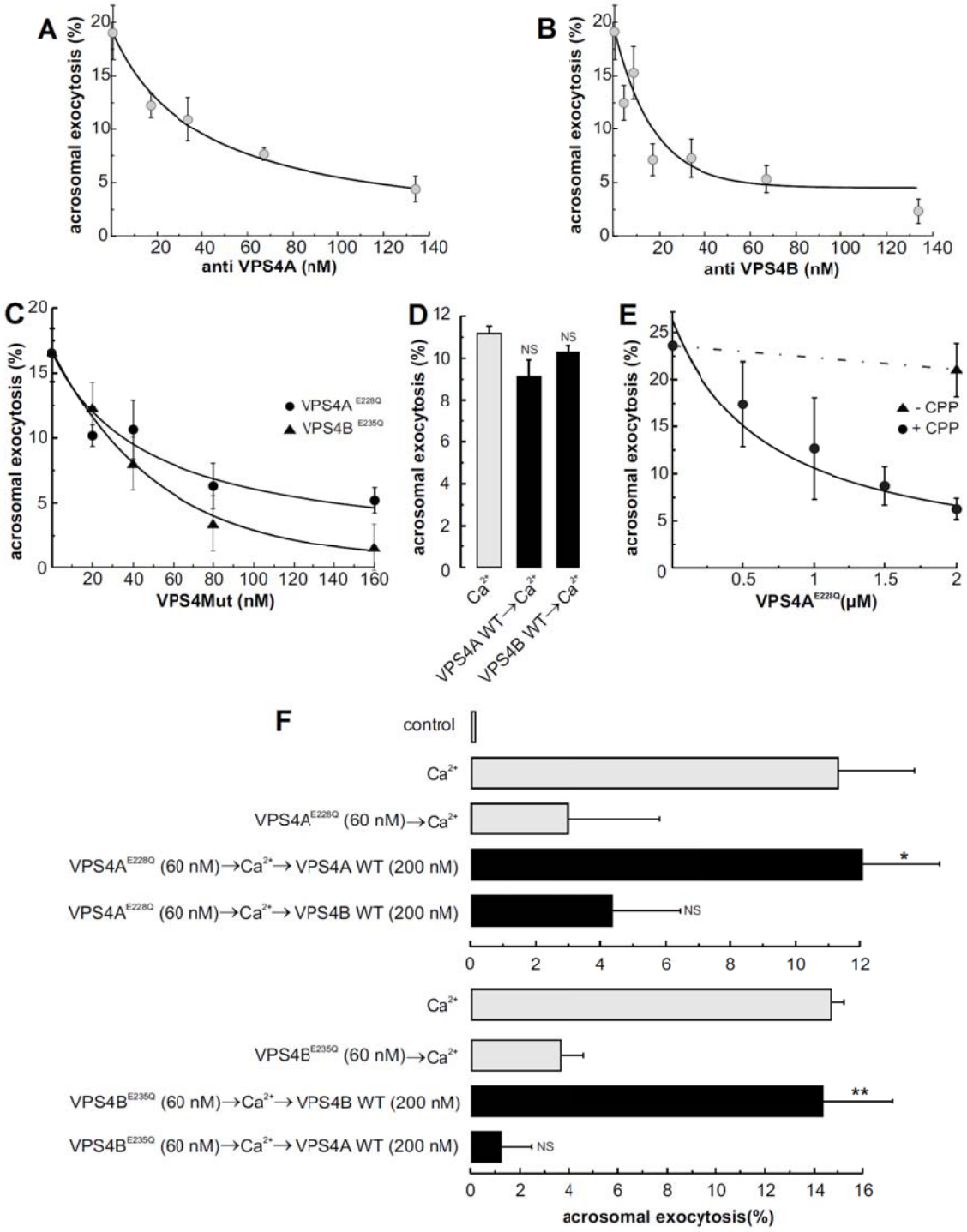

Figure 3. VPS4 is necessary for efficient stabilization of SNARE complexes.

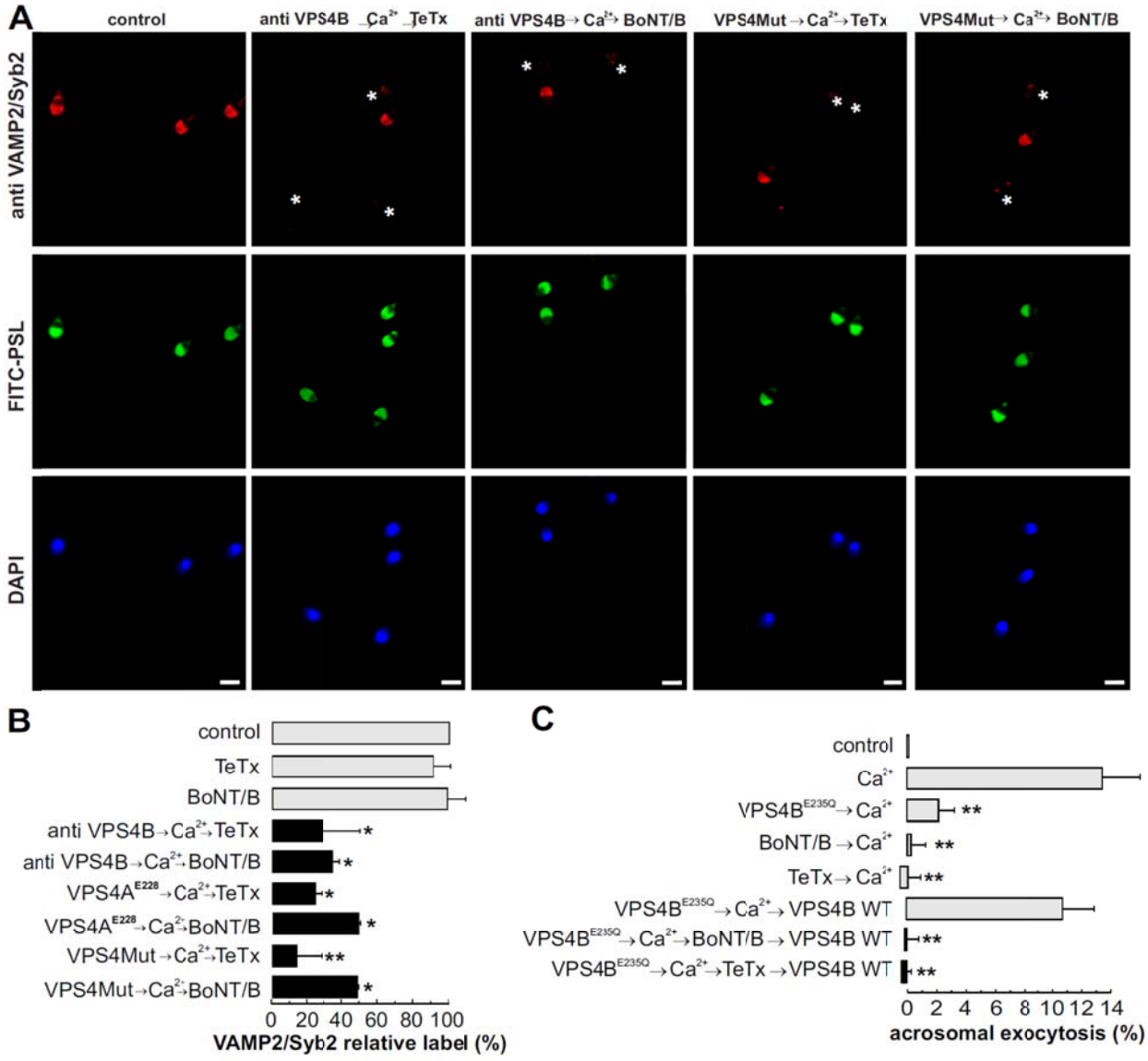

Figure 4. TSG101 and CHMP4B are both present in the acrosomal region of human sperm.

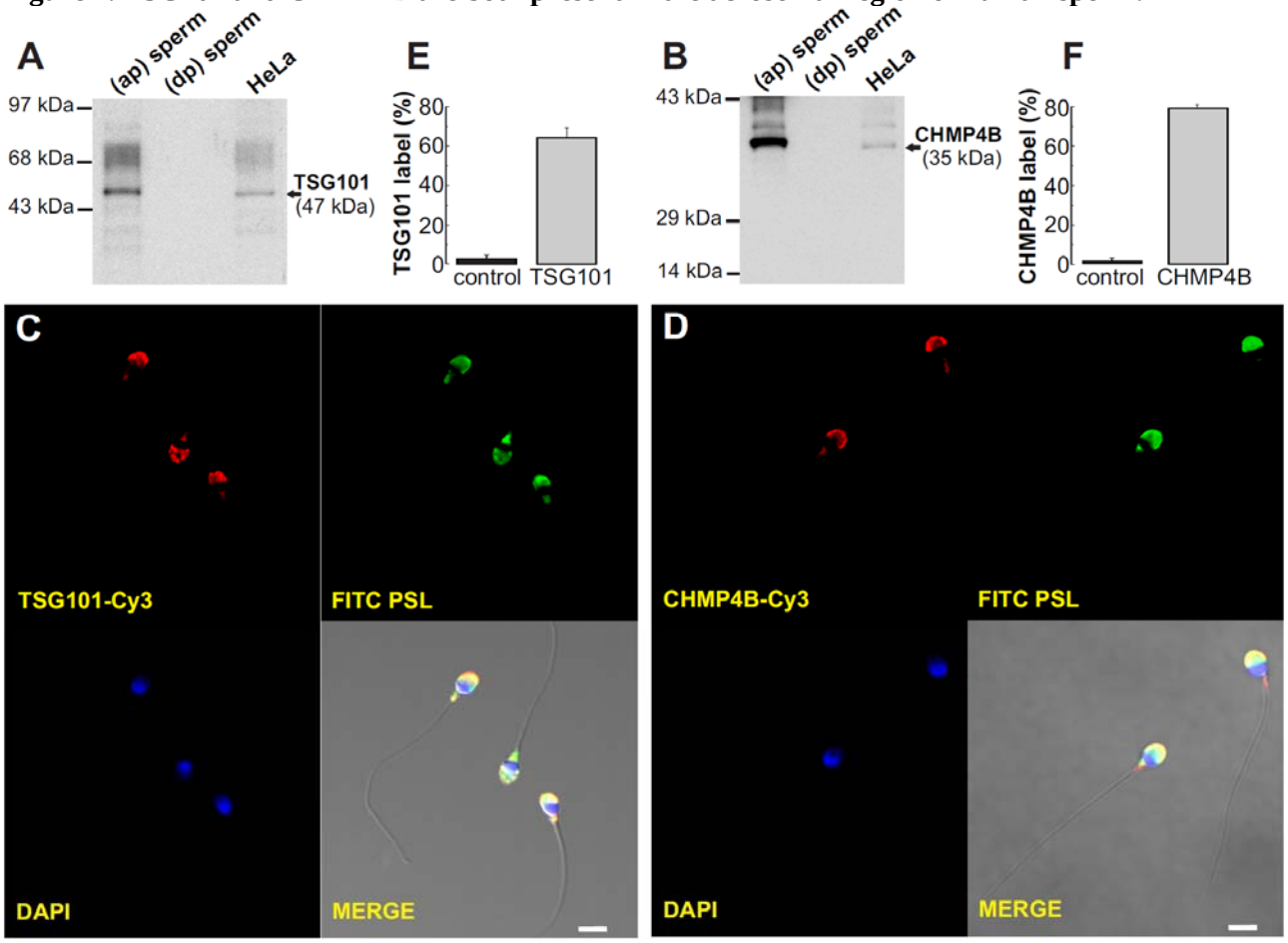

Figure 5. TSG101 (ESCRT I) and CHMP4 (ESCRT III) are necessary for acrosomal exocytosis in permeabilized human sperm.

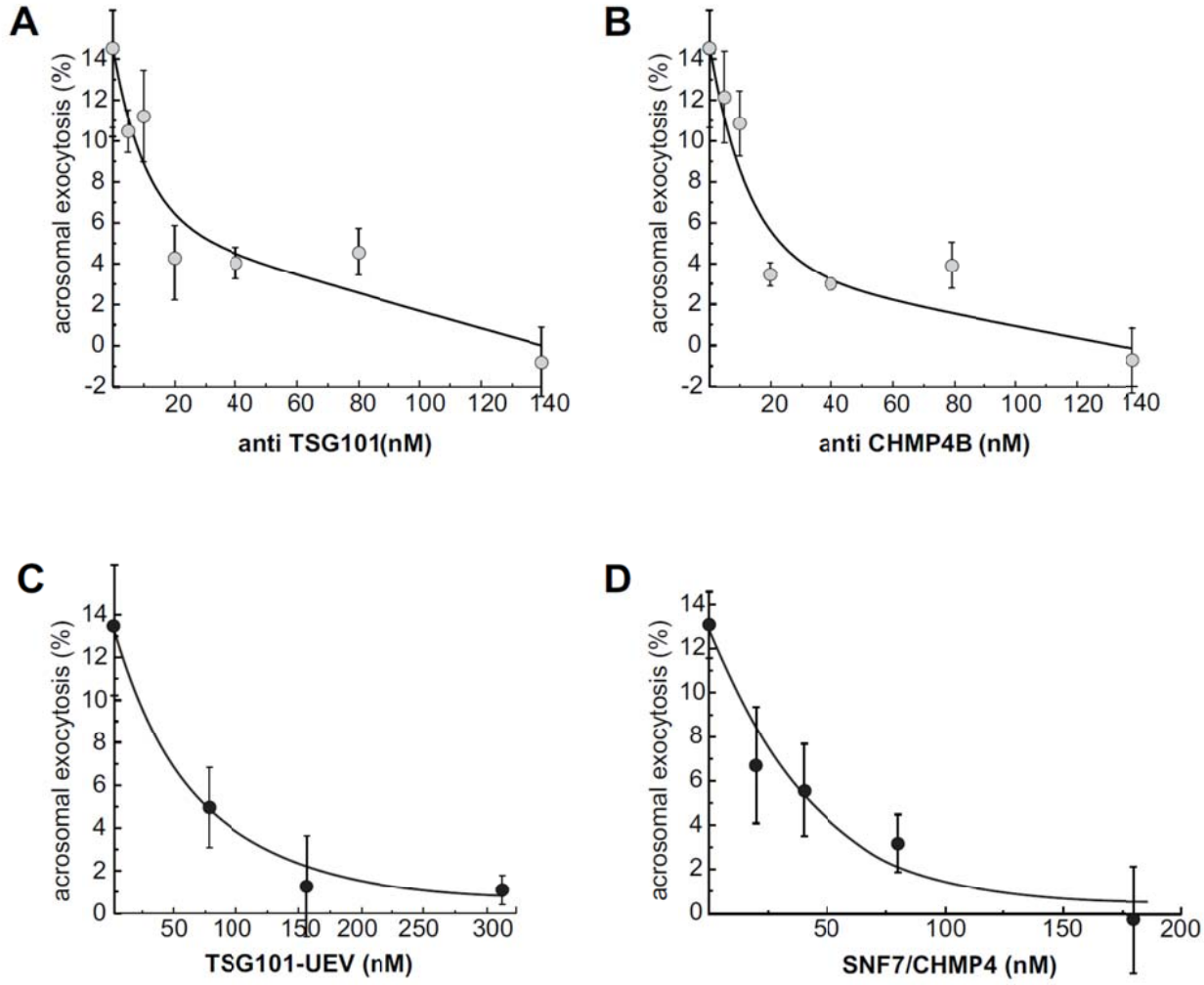

Figure 6. ESCRT complex manipulation deregulates intraacrosomal vesicle (IAV) formation.

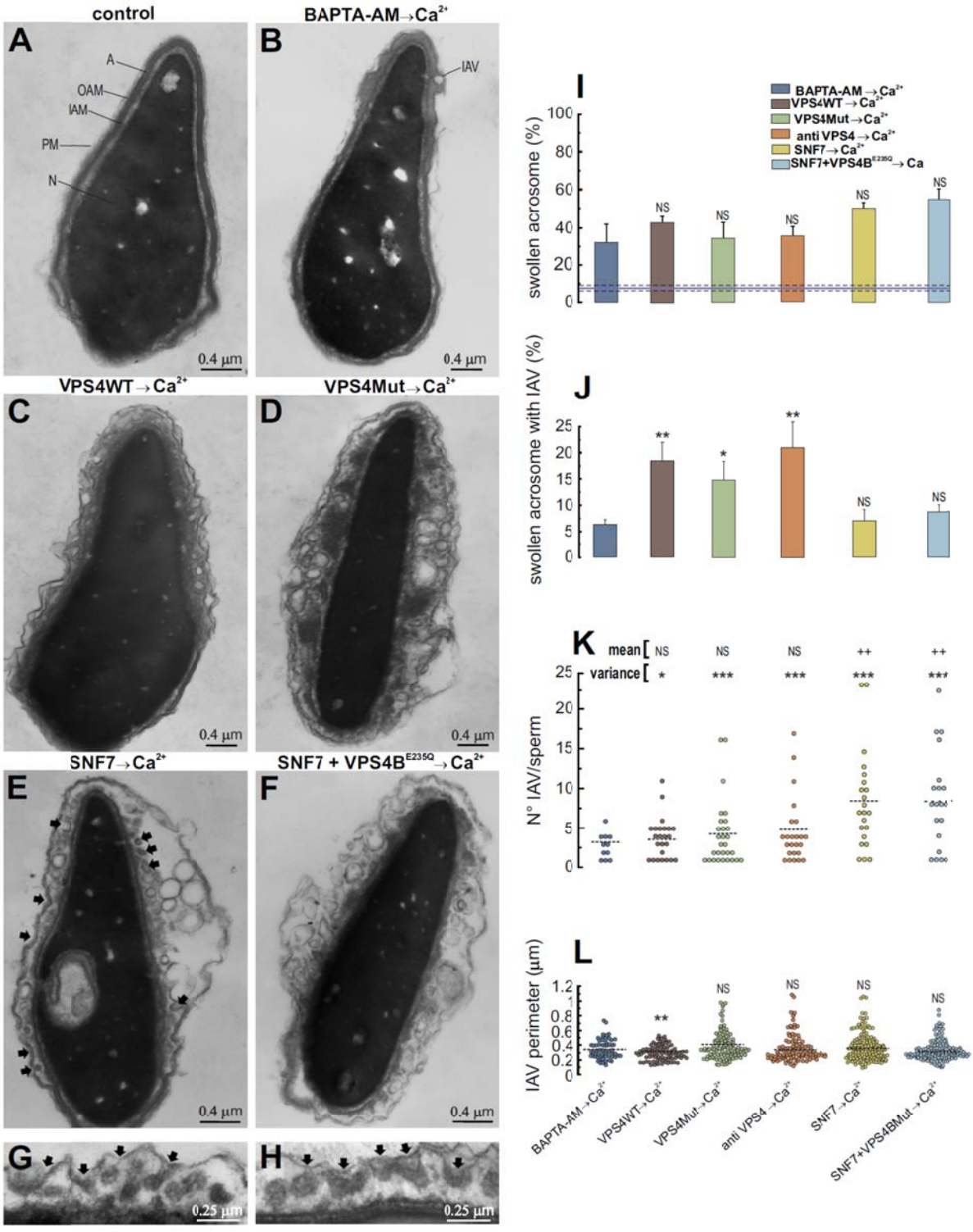

Figure 7. Working model for ESCRT complexes function in acrosomal exocytosis.