Chapter 12

Small GTPases in Acrosomal Exocytosis

Matias A. Bustos, Ornella Lucchesi, Maria C. Ruete, Luis S. Mayorga, and Claudia N. Tomes

Abstract

Regulated exocytosis employs a conserved molecular machinery in all secretory cells. Soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) and Rab superfamilies are members of this machinery. Rab proteins are small GTPases that organize membrane microdomains on organelles by recruiting specific effectors that strongly influence the movement, fusion and fission dynamics of intracellular compartments. Rab3 and Rab27 are the prevalent exocytotic isoforms. Many events occur in mammalian spermatozoa before they can fertilize the egg, one of them is the acrosome reaction (AR), a type of regulated exocytosis. The AR relies on the same fusion machinery as all other cell types, which includes members of the exocytotic SNARE and Rab superfamilies. Here, we describe in depth two protocols designed to determine the activation status of small G proteins. One of them also serves to determine the subcellular localization of active Rabs, something not achievable with other methods. By means of these techniques, we have reported that Rab27 and Rab3 act sequentially and are organized in a RabGEF cascade during the AR. Although we developed them to scrutinize the exocytosis of the acrosome in human sperm, the protocols can potentially be extended to study other Ras-related proteins in virtually any cellular model.

Key words Acrosome reaction, Exocytosis, Rab27, Rab3, Sperm

1 Introduction

Secretion of vesicles is orchestrated by a complex molecular machinery conserved among all cells and organisms [1, 2]. The proteinaceous machinery involved in this process includes members of SNARE and Rab superfamilies and their interacting and regulatory proteins [2–5]. During regulated exocytosis, secretory Rabs play an essential role recruiting tethering and docking factors required for membrane recognition and fusion [6]. These small GTPases act as molecular "on/off" switches cycling between inactive (GDP-bound) and active (GTP-bound) states [3, 6]. Two kinds of proteins regulate the GDP-GTP cycling, guanine nucleotide exchange factors (GEFs), and GTPase-activating proteins (GAPs). These proteins control not only the activity of small GTPases but also their association/dissociation to/from membranes, both critical

Guangpu Li (ed.), *Rab GTPases: Methods and Protocols*, Methods in Molecular Biology, vol. 1298,
DOI 10.1007/978-1-4939-2569-8_12,

for the proper functioning of Rab proteins. GEF stimulates the exchange of GDP for GTP, generating the activated form of Rab. Rab-GTP interacts with effector proteins responsible for tethering/docking of compartments that are going to fuse [3, 7]. Once Rab has exerted its function, GAP enhances the hydrolysis of the bound GTP to GDP, inactivating Rab. Subsequently, Rab-GDP dissociates from membranes and remains in a cytosolic pool complexed with a GDP dissociation inhibitor (GDI [3, 8]).

Regulated exocytosis consists of multiple steps that lead to the fusion of secretory vesicles with the plasma membrane in response to stimuli. The AR is a calcium-triggered exocytotic process fundamental for fertilization. In this event, sperm's single secretory granule or acrosome fuses with the plasma membrane and releases its contents to the extracellular medium. Release takes place after a complex signaling pathway, invoked by an increase in intracellular calcium, activates the fusion machinery [9–18]. The AR constitutes a straightforward model for regulated exocytosis because there are no interferences due to endocytosis or other types of intracellular transport. Sperm neither transcribe nor translate, so overexpression and silencing RNA technologies to study the role of proteins in exocytosis are not applicable to these cells. To overcome these limitations, we have set up two strategies: (1) a controlled plasma membrane permeabilization protocol with streptolysin O (SLO) or perfringolysin O (PFO) and (2) the delivery of permeable proteins into living cells [19–21].

Rab3A/B/C/D, Rab27A/B, Rab26, and Rab37 are present in a variety of secretory vesicles and modulate their release [8]. We focus here on the roles of Rab3 and Rab27 in the AR. We describe an assay that pulls down active Rabs from whole cell detergent extracts based on their interaction with immobilized protein cassettes (activity probes) that bind specifically Rabs-GTP. Thanks to this assay, we learnt that Rabs are activated in human sperm in response to exocytosis stimuli [22–24, 26]. This assay provides information about the activation state, but tells nothing about the subcellular localization or the number of cells with active Rabs. To address these issues, we developed a technique based on protein–protein interaction and indirect immunofluorescence principles (referred to as far-immunofluorescence [23]). The method consists of overlying sperm smeared on coverslips with the same GST-tagged activity probes used in pull-down assays and later visualizing the binding sites with an anti-GST antibody. The protocol is applicable to permeabilized [23, 25] and non-permeabilized [24] sperm and allowed us to determine that Rabs were activated in the acrosomal region of subpopulations of sperm challenged to undergo the AR. Thanks to this protocol, we also made the unexpected observation that introduction of recombinant Rab27A-GTP into sperm was sufficient to activate endogenous Rab3. We will devote the last part of this chapter to describe a protocol that allowed us to explain these findings and demonstrate that Rab3 and Rab27 act

through a RabGEF cascade during the AR, in which active Rab27A recruits, directly or indirectly, a Rab3A GEF activity [23].

The protocols that we describe here were applied to study Rabs in human sperm exocytosis but they could be extended to scrutinize different biological phenomena in other sperm species or cells types. Moreover, they can be used to analyze other Ras-related proteins. For instance, we applied pull-down [22] and far-immunofluorescence [25] to study the role of Rap during human sperm AR.

2 Materials

2.1 Reagents

Use ultrapure (electrical resistivity 18 MΩ cm at 25 °C) water to dilute all reagents. Utilize analytical grade reagents and follow all of your Institution's disposal regulations when discarding waste materials.

2.1.1 SLO Stock Solutions (*See* Note 1)

1. Resuspend lyophilized SLO in 20 mM HEPES/K, pH 7, 20 % glycerol, and 0.01 % bovine serum albumin (BSA) to 25,000 UI/ml, aliquot and store at −80 °C. Avoid repeated freezing/thawing.
2. Prepare an SLO 300 UI/ml stock solution (*see* **Note 2**) by diluting 6 µl of 25,000 UI/ml SLO in 500 µl phosphate buffer saline (PBS, 2.5.3) containing 20 % glycerol and 0.01 % BSA. Aliquot and store at −20 °C if you will use it within a month; otherwise store at −80 °C.

2.2 Plasmids

1. *His_6-Rab3A*. The cDNA encoding a membrane-permeant version of human Rab3A bearing a Q81L point mutation and subcloned into pQE80L plasmid [20] was generously provided by C. López (University of Cuyo, Mendoza, Argentina).
2. *His_6-Rab27A*. The cDNA encoding human Rab27A was subcloned into pET28 plasmid by GenScript Inc. (Piscataway, NJ, USA).
3. *GST-Rab27A*. The cDNA encoding human Rab27A in pGEX-6p was a kind gift from D. Munafó (Scripps Research Institute, La Jolla, CA, USA).
4. *GST-RIM-RBD*. The cDNA encoding the Rab3-binding domain of rat RIM 1α (amino acids 11–398; RIM-RBD) [27] in pGEX-KG was generously provided by R. Regazzi (University of Lausanne, Lausanne, Switzerland).
5. *GST-Slac2-b-SHD*. The cDNA encoding the Rab27-GTP-binding domain of Slac2-b (Synaptotagmin-like protein homology lacking C2 domains-b; amino acids 1–79) [28] in pGEX-2T was a kind gift from R. Shirakawa (Kyoto University, Kyoto, Japan).
6. *GST*. The cDNA encoding GST was from an empty pGEX-2T vector.

2.3 Recombinant Proteins

2.3.1 Expression and Purification of GST-Fused Proteins

1. Competent *E. coli* BL21 were transformed with plasmids carrying the cDNAs encoding GST or GST-fused proteins following standard procedures.
2. Induce expression of GST and GST-Slac2-b-SHD with 0.5 mM isopropyl-β-D-thio-galactoside (IPTG) for 3 h at 37 °C; GST-RIM-RBD with 0.5 mM IPTG, overnight at 22 °C; and GST-Rab27A with 1 mM IPTG, 3 h at 37 °C.
3. Purify GST-fused proteins under native conditions on glutathione-Sepharose beads following standard procedures. The purification buffers contain 100 mM Tris–HCl, pH 7.4, 120 mM NaCl (lysis and washing buffer) and 100 mM Tris–HCl, pH 7.4, 120 mM NaCl, 20 mM reduced glutathione (elution buffer). For pull-down assays, bacterial lysates (containing 5–10 μg/ml GST-Slac2-b-SHD or GST-RIM-RBD) are frozen at −80 °C until use.

2.3.2 Expression and Purification of His_6-Tagged Proteins

1. Transform the cDNAs encoding His_6-Rab27A and His_6-Rab3A into *E. coli* BLR(DE3) made chemically competent.
2. Induce expression with 0.5 mM IPTG for 3 h at 37 °C for His_6-Rab27A and overnight at 22 °C for His_6-Rab3A.
3. Purify His_6-tagged proteins under native conditions on Ni^{2+}-NTA-agarose beads following standard protocols. The purification buffers contain 20 mM Tris–HCl, pH 7.4, 300 mM NaCl, 8–10 mM imidazole (lysis buffer), 20 mM imidazole (washing buffer), and 250 mM imidazole (elution buffer).

2.4 Antibodies

1. Rabbit polyclonal anti-Rab27 and anti-α-tubulin (affinity purified with the immunogens) antibodies and mouse monoclonal anti-Rab3A (clone 42.2, IgG affinity-purified on protein A-Sepharose, subtype IgG2b) were from Synaptic Systems (Goettingen, Germany).
2. Rabbit polyclonal anti-GST antibody (purified IgG) was from EMD Millipore (Billerica, MA, USA).
3. HRP-conjugated goat anti-rabbit and Cy™3-conjugated donkey anti-rabbit IgGs (H+L) were from Jackson ImmunoResearch (West Grove, PA, USA).
4. HRP-conjugated goat anti-mouse IgG (H+L) was from Kirkegaard & Perry Laboratories Inc. (Gaithersburg, MD, USA).

2.5 Buffers

1. *Human Tubal Fluid medium* (*HTF*). Dissolve reagents (97.8 mM NaCl, 4.69 mM KCl, 0.20 mM $MgSO_4$, 0.37 mM KH_2PO_4, 2.04 mM $CaCl_2$, 25 mM $NaHCO_3$, 2.7 mM D-glucose, 0.33 mM Na pyruvate, 21.4 mM Na lactate, 0.06 g/l penicillin, 0.05 g/l streptomycin, and 0.01 g/l phenol red) in water at room temperature with constant stirring and bring to final volume (*see* **Note 3**). Before using, place an aliquot in a CO_2 incubator overnight to buffer; the media will turn

reddish-orange (*see* **Note 4**). HTF medium is also commercially available.

2. *HTF/BSA medium*. Supplement HTF medium, previously buffered overnight in 5 % CO_2/95 % air incubator, with 0.5 mg/ml BSA.
3. *Phosphate buffer saline* (*PBS*). Dissolve reagents (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and 1.8 mM KH_2PO_4) in water at room temperature with constant stirring. Adjust pH to 7.4 with 1 N NaOH and bring to final volume. Filter-sterilize and store at 4 °C. PBS is commercially available in a variety of presentations.
4. *HB-EGTA* (*permeabilization buffer*). Dissolve reagents (20 mM HEPES free acid, 250 mM sucrose, and 0.5 mM EGTA) in water at room temperature with constant stirring. Adjust pH to 7 with 1 N KOH and bring to final volume. Filter-sterilize and store at 4 °C for up to a month.
5. *Pull-down buffer*. Dilute reagents (50 mM Tris–HCl, pH 7.4, 200 mM NaCl, 2.5 mM $MgCl_2$, 1 % (v/v) Igepal, 10 % (v/v) glycerol) in water from stock solutions by vortexing vigorously at room temperature. Adjust pH to 7.4, bring to final volume, and store at 4 °C. Add protease inhibitors (2 mM AEBSF, 0.3 μM aprotinin, 116 μM bestatin, 14 μM E-64, 1 μM leupeptin, 1 mM EDTA, and 1 mM PMSF) right before using.
6. *Binding buffer*. Dilute reagents (20 mM HEPES/K, pH 7.4, 150 mM K acetate 15 mM $MgCl_2$, 0.05 % (v/v) Tween 20, 5 μM GTP) in water from stock solutions at room temperature by vortexing. Adjust pH to 7.4, bring to final volume, and store at 4 °C until used. Add protease inhibitors (2 mM AEBSF, 0.3 μM aprotinin, 116 μM bestatin, 14 μM E-64, 1 μM leupeptin, 1 mM EDTA, and 1 mM PMSF) right before using.
7. *Lysis buffer*. Dilute reagents (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 5 mM $MgCl_2$, 1 % (v/v) Triton X-100 (TX-100), 10 % (v/v) glycerol) in water from stock solutions at room temperature by vortexing. Adjust pH to 7.4, bring to final volume, and store at 4 °C until used. Add protease inhibitors (2 mM AEBSF, 0.3 μM aprotinin, 116 μM bestatin, 14 μM E-64, 1 μM leupeptin, 1 mM EDTA, and 1 mM PMSF) right before using.

3 Methods

3.1 Human Sperm Samples

3.1.1 Sample Collection

1. Volunteer healthy donors, with at least 48 h of abstinence, should collect semen samples in containers (*see* **Note 5**).
2. Place the specimen container at 37 °C in an incubator until the semen liquefies (30 min to 1 h).
3. Assess semen quality after liquefaction (*see* **Note 6**).

3.1.2 Swim-Up (See Note 7*) and Capacitation (See* Note 8*)*

1. Place 800 μl of HTF/BSA medium in 5 ml polypropylene tubes (12 × 75 mm).
2. Layer carefully 300 μl of liquefied semen under the medium at the bottom of the tube (*see* **Note 9**).
3. Place tubes at a 45° angle, in order to increase the area of the semen-medium interface and incubate for 1 h at 37 °C in a 5 % CO_2/95 % air incubator.
4. Return the tubes gently to the upright position and carefully remove the uppermost 600–700 μl of medium without disturbing the interface. This fraction is enriched in highly motile sperm.
5. Mix well and assess sperm concentration in a Makler Counting Chamber (SEFI Medical Instruments LTD. Distributed by Irvine Scientific, CA, USA; *see* **Note 10**).
6. Dilute sperm to 10×10^6 cells/ml in HTF/BSA medium.
7. Incubate the sperm suspensions in HTF/BSA medium for at least 3 h at 37 °C in a 5 % CO_2/95 % air incubator to promote capacitation.

3.1.3 SLO-Permeabilization

1. Wash capacitated sperm twice with one volume each of ice-cold PBS.
2. Resuspend the pellet in one volume of ice-cold PBS by pipetting gently.
3. Count and adjust sperm concentration to $7–10 \times 10^6$/ml.
4. Add SLO (0.4 to 4 U/ml) and incubate for 15 min at 4 °C (s*ee* **Note 11**).
5. Wash once with one volume of ice-cold PBS to remove unbound toxin.
6. Resuspend the pellet in one volume of ice-cold HB-EGTA containing 2 mM dithiotreitol (DTT, *see* **Note 12**) by pipetting gently.

3.2 In Vitro Solid Phase Isoprenylation and Activation of Rab Proteins

Native Rab proteins are geranylgeranylated, presumably to localize to membranes where they participate in signal transduction networks. These proteins bind magnesium ions and guanine nucleotides (GDP in their inactive state or GTP in their active state) to execute their functions. Because bacteria do not isoprenylate, we geranylgeranylate Rabs expressed in *E. coli* and load them with guanine nucleotides in vitro to make them functional.

3.2.1 Geranylgeranylation Mixture

To prepare 2× isoprenylation mixture (*see* **Note 13**):

1. Dilute reagents (40 mM HEPES/K, pH 7.5, 2 μg/μl mouse brain cytosol, 160 μM geranylgeranyl pyrophosphate (GGPP), 400 μM GDP, 2 mM DTT, 2 mg/ml BSA, 6 mM $MgCl_2$, 4 mM AEBSF, 0.6 μM aprotinin, 260 mM bestatin, 232 μM

bestatin, 28 μM E-64, 2 μM leupeptin and 2 mM EDTA) in water from stock solutions.

2. Keep mixture on ice until use.

3.2.2 Immobilization of Recombinant Rabs

1. Incubate 1 ml bacterial lysates (containing GST/His_6-Rab proteins) with 100 μl of glutathione-Sepharose beads (GST) or 100 μl Ni^{2+}-NTA-agarose beads (His_6) for 1 h a 4 °C.
2. Wash the beads three times (1 ml each) with GST/His_6-washing buffer.
3. Quantify Rabs bound to beads and estimate their purity by boiling a small aliquot of the immobilized material in sample buffer and analyzing by SDS-PAGE.
4. Stain gels with Coommassie brilliant blue R-250 following standard procedures.

3.2.3 Solid Phase Isoprenylation Protocol

1. Incubate 100 μl of 10–20 μM immobilized Rab (as described under Immobilization of Recombinant Rabs) with 100 μl of 2× geranylgeranylation mixture for 2 h at 37 °C with constant rocking.
2. Add four volumes of GST/His_6-washing buffer and incubate during 30 min at 4 °C with constant rocking (*see* **Note 14**).
3. Wash the beads three times (1 ml each) with GST/His_6-washing buffer.
4. Elute geranylgeranylated Rabs with 50 μl GST/His_6-elution buffer.

3.2.4 Loading Rabs with Guanine Nucleotides

To prepare 50 μl of loaded-Rabs (10 μM final concentration):

1. Incubate 10 μM purified geranylgeranylated Rab (as described under Solid Phase Isoprenylation Protocol) with 5 μl activation buffer (50 mM HEPES/K, pH 7.2, 5 mM $MgCl_2$, 0.03 % Igepal or Nonidet-P40, 1 mM DTT and 25 mM EDTA) and 125 μM guanosine 5′-(β-thio)-diphosphate (GDP-β-S), guanosine 5′-(γ-thio) triphosphate (GTP-γ-S) or GDP in a final volume of 50 μl for 1 h at 37 °C (*see* **Note 15**).
2. Prepare a Sephadex G25 (pre-hydrated in PBS) column in a 200 μl tip.
3. Wash the mini column three times (one volume each) with 0.1 % BSA in PBS by centrifugation at 1,100 × *g* for 2 min at room temperature.
4. Run 50 μl of nucleotide-bound Rab through the Sephadex G25 column (*see* **Note 16**).
5. Recover the protein in the void volume by centrifugation at 1,100 × *g* for 2 min at room temperature.
6. Add $MgCl_2$ to 1 mM final concentration (*see* **Note 17**).
7. Aliquot and store at –20 °C for up to a month (*see* **Note 18**).

3.3 Far-Immunofluorescence: Description of a New Method to Detect the Localization of Active Rabs

Cells subjected to different experimental conditions are smeared on coverslips, fixed, permeabilized, and overlaid with activity probes. These are protein cassettes derived from effectors that contain their cognate Rab-GTP binding domains (e.g. GST-RIM-RBD). Because cassettes are GST-tagged (GST) their binding to endogenous targets (e.g. Rab3) is revealed by conventional indirect immunofluorescence with anti-GST (anti-GST) antibodies followed by fluorescently labeled secondary antibodies (anti-rabbit-Cy3; Fig. 1).

3.3.1 Far-Immunofluorescence Protocol

1. Wash 12 mm round coverslips with 96 % ethanol.
2. Flame coverslips and place them on a piece of parafilm, secured in the lid of a 24-well plate.
3. Add 80 µl of poly-L-lysine (dilute 1 µl of stock solution 0.1 % w/v in 20 µl of water) to each coverslip and incubate for 30 min at room temperature in a moisturized chamber (*see* **Note 19**).
4. Attach cell suspensions (treated as detailed in Subheading 3.3.2–3.3.4, *see below*) to poly-L-lysine coated coverslips for 30 min at room temperature in a moisturized chamber (Fig. 1).
5. Store cells in 100 mM glycine in PBS overnight at 4 °C in a moisturized chamber (*see* **Note 20**).
6. Permeabilize the plasma membrane with 0.1 % Triton X-100 (TX-100) for 10 min at room temperature.
7. Wash cells three times, 6 min each at room temperature, with 1 ml of PBS-0.1 % polyvinylpyrrolidone (PBS-PVP; average MW = 40,000).
8. Block non-specific reactivity with 50 µl of 5 % BSA in PBS-PVP for 1 h at 37 °C.
9. Overlay with 50 µl of 140 nM affinity-purified GST, GST-RIM-RBD, or GST-Slac2-b-SHD in 5 % BSA in PBS-PVP and incubate for 1 h at 37 °C (Fig. 1).
10. Wash cells three times, 6 min each at room temperature, with 1 ml PBS-PVP.
11. Add 25 µl of 210 nM (31.5 µg/ml) anti-GST in 3 % BSA in PBS-PVP and incubate for 1 h at 37 °C (Fig. 1).
12. Wash cells twice, 10 min each at room temperature, with 1 ml PBS-PVP.
13. Add 50 µl of 11 nM (1.67 µg/ml) Cy3-conjugated goat anti-rabbit IgG in 1 % BSA in PBS-PVP and incubate for 1 h at room temperature protected from light (Fig. 1).
14. Wash cells six times, 6 min each at room temperature, with 1 ml PBS-PVP.

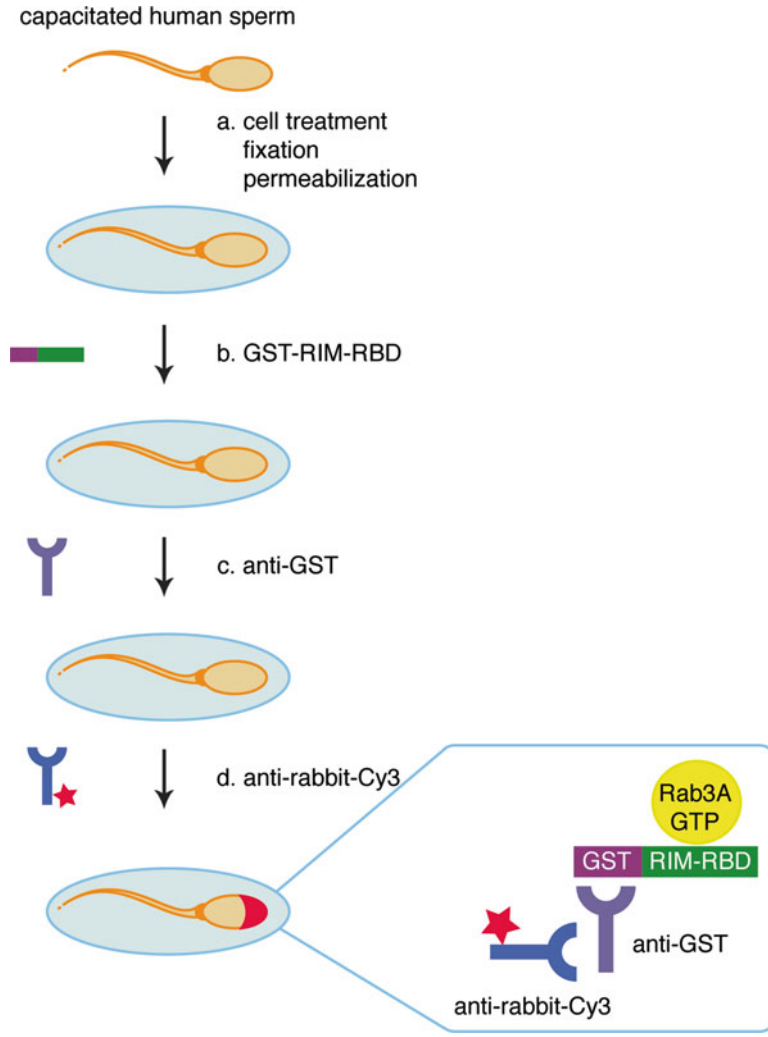

Fig. 1 Schematic representation of the far-immunofluorescence protocol developed to detect GTP-bound Rab proteins. Cells are fixed, immobilized on poly-L-lysine coated coverslips and sperm membranes are permeabilized with TX-100 (**A**). Attached sperm are overlaid with protein domains that bind Rab proteins in their GTP state (shown here is GST-RIM-RBD cassette that interacts with Rab3-GTP, **B**). Sperm are incubated with an anti-GST antibody to detect the cassette bound to endogenous Rab3-GTP (**C**). Slides are exposed to a secondary Cy3-conjugated antibody (**D**) to detect anti-GST in the anti-GST/GST-RIM-RBD/Rab3-GTP complex (*inset*). Finally, cells are mounted and the percentage of sperm with acrosomal Rab3-GTP is scored by fluorescence microscopy. *Inset*: GST-RIM-RBD domain (*green*/*purple*) bound to Rab3-GTP (*yellow*) is recognized by the primary anti-GST antibody (*violet*), which is detected by a secondary Cy3-conjugated (*red star*) anti-rabbit antibody (*blue*)

15. Treat cells with ice-cold methanol for 20 s to permeabilize the acrosomal membrane.
16. Add 80 µl of 25 µg/ml fluorescein-isothiocyanate-coupled *Pisum sativum* agglutinin (FITC-PSA, *see* **Note 21**) to each coverslip and incubate for 40 min in the dark.
17. Wash cells three times, 6 min each at room temperature, with 1 ml PBS-PVP.
18. Mount the coverslips (with the cells attached) in a drop of 5 µl of 1 % propyl-gallate/50 % glycerol in PBS containing 2 µM Hoechst 33342 (*see* **Note 22**) placed on a slide.
19. Seal the edge of coverslips with clear nail polish and allow to dry.
20. Store at –20 °C in the dark until examination (*see* **Note 23**).

3.3.2 Sperm Treatment 1 for Standardization of Activity Probes Binding to Endogenous Active Rabs in Permeabilized Cells (Fig. 2a–d)

1. Add 50 µl (350,000–500,000 cells) of capacitated, SLO-permeabilized sperm in HB-EGTA to each tube.
2. Incubate with 100 µM 2-aminoethoxydiphenylborate (2-APB, *see* **Note 24**) and 5 mM EDTA/1 mM $MgCl_2$ (*see* **Note 25**) for 10 min at 37 °C.
3. Load cells with 40 µM GDP-β-S or GTP-γ-S during 10 min at 37 °C (Fig. 2a–d).
4. Incubate with 15 mM $MgCl_2$ during 5 min at 37 °C (*see* **Note 17**).
5. Incubate with 50 µl of 4 % paraformaldehyde during 15 min at room temperature to fix cell suspensions.
6. Wash fixed cells by centrifugation at 12,600 × *g* for 2 min at room temperature.
7. Resuspend the pellet in 100 µl 100 mM glycine in PBS by gentle mixing and continue as described under Far-Immunofluorescence Protocol.

Fig. 2 (continued) but in only 6 % of sperm treated with GDP-β-S (**d**). Scale bars: 5 µm. (**e**) SLO-permeabilized human sperm treated as described under Far-Immunofluorescence Protocol (3.3.3) are overlaid with GST-Slac2-b-SHD to detect active Rab27 (**e**, *gray bars*) or GST-RIM-RBD to detect active Rab3 (**e**, *black bars*) and counted to score the percentage of cells immunodecorated in the acrosomal region by the anti-GST antibodies. The population of active Rabs increases upon incubation with GTP plus $CaCl_2$ compared with controls incubated with GDP (***$P < 0.001$). Note that by itself, GTP is unable to increase the number of cells depicting active Rabs compared with those treated with GDP (ns, non-statistically significant). Recombinant Rab27A augments the number of cells depicting endogenous Rab3A-GTP in the acrosomal region (ns, with respect to GTP/$CaCl_2$; ***$P < 0.001$, compared with GDP). The converse is not true; recombinant Rab3A does not influence the number of cells with active Rab27 in the head (ns compared with controls loaded with GDP). The Tukey–Kramer post hoc test was used for pairwise comparisons. The data represent mean ± SEM of at least three independent experiments [23]

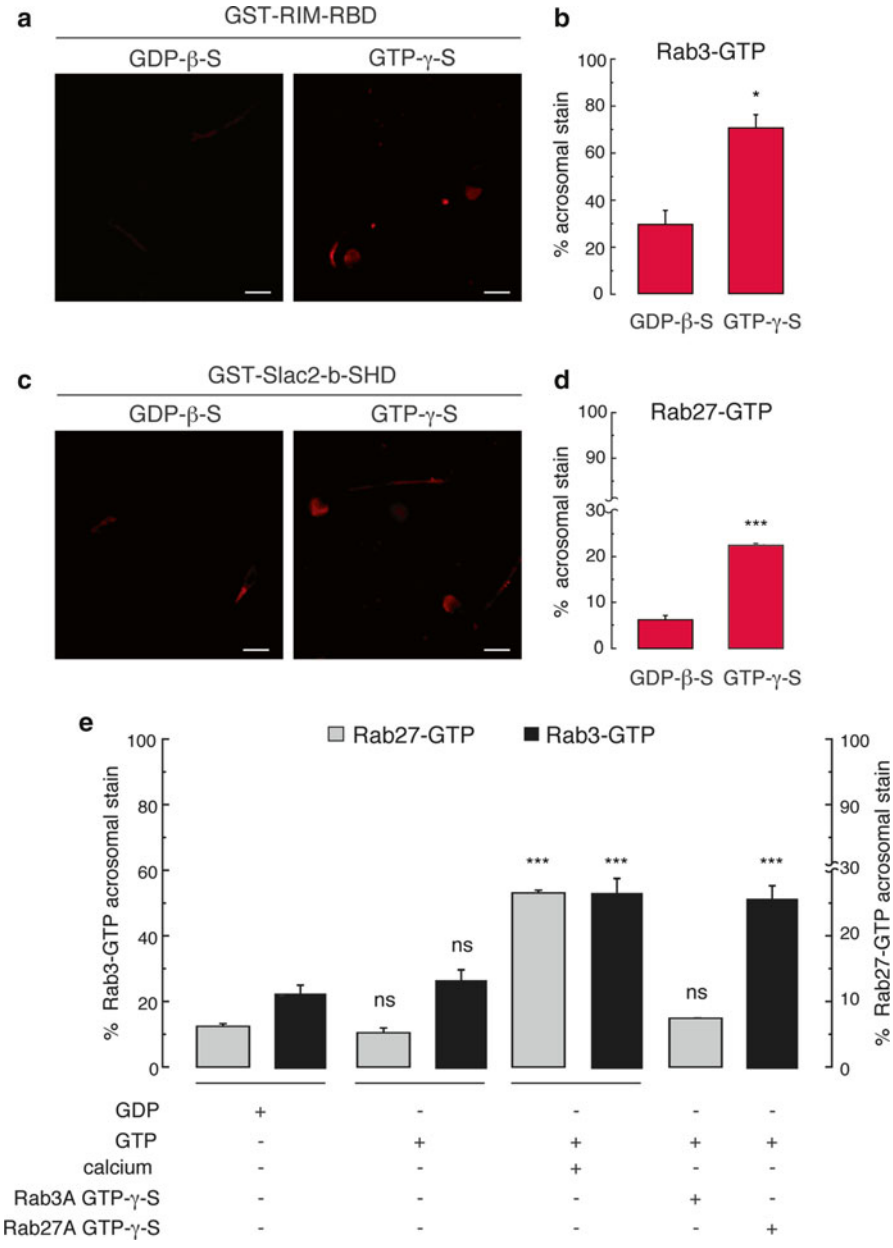

Fig. 2 Detection of nucleotide-binding status of endogenous Rab3 and Rab27. Capacitated, SLO-permeabilized sperm are treated as described under Far-immunofluorescence protocol. Cells are stained with an anti-GST antibody as readout for the activity probes that detect active Rab3 (**a**) and Rab27 (**c**). Quantifications (mean ± SEM of at least three independent experiments) of cells immunostained in the acrosomal region by the anti-GST antibodies are shown to the *right* (**b**, **d**). The activity probe GST-RIM-RBD binds to endogenous Rab3 in 70 % of the cells loaded with GTP-γ-S, whereas only 29 % of sperm treated with GDP-β-S give a detectable signal (**b**). Slac2-b-SHD binds the acrosomal region in 23 % of sperm treated with GTP-γ-S,

3.3.3 Sperm Treatment 2 for Analysis of Rab Activation in Response to Exogenous Stimuli (Fig. 2e)

1. Add 50 μl (350,000–500,000 cells) of capacitated, SLO-permeabilized sperm in HB-EGTA to each tube.
2. Incubate with 100 μM 2-APB (*see* **Note 24**) and 5 mM EDTA/1 mM $MgCl_2$ (*see* **Note 25**) for 10 min at 37 °C.
3. Load cells with 40 μM GDP during 10 min at 37 °C (*see* **Note 26**).
4. Incubate with 15 mM $MgCl_2$ during 5 min at 37 °C (*see* **Note 17**).
5. Incubate the cells with: (1) 200 μM GTP, (2) 200 μM GTP plus 0.5 mM $CaCl_2$ (10 μM free calcium, *see* **Note 27**), (3) 200 μM GTP plus 300 nM geranylgeranylated His_6-Rab3A loaded with GTP-γ-S or (4) 200 μM GTP plus 300 nM geranylgeranylated His_6-Rab27A loaded with GTP-γ-S, for 15 min at 37 °C (Fig. 2e).
6. Continue as described in Subheading 3.3.2 (**steps 5**–7).

3.3.4 Sperm Treatment 3 for Analysis of Rab3-GTP Levels After A23187 Treatment in Non-permeabilized Cells [24]

1. Add 50 μl (350,000–500,000 cells) of capacitated sperm in HTF to each tube.
2. Incubate with 100 μM 2-APB (*see* **Note 24**) for 10 min at 37 °C.
3. Initiate the AR with 10 μM A23187 (*see* **Note 28**) incubating for 15 min at 37 °C.
4. Continue as described in Subheading 3.3.2 (**steps 5**–7).

3.4 Pull-Down Assays for Active Rab27 and Rab3

Immobilization of the activity probes

1. Wash glutathione-Sepharose beads twice with one volume each of pull-down buffer and recover by centrifugation at 1,100 × *g* for 2 min at 4 °C.
2. Incubate 60–90 μl washed beads with 1 ml of bacterial lysates containing GST-Slac2-b-SHD or GST-RIM-RBD for 1 h at 4 °C under constant rocking.
3. Wash the beads twice with one volume each of ice-cold PBS and once with one volume of ice-cold pull-down buffer by centrifugation at 1,100 × *g* for 2 min at 4 °C.
4. Recover the GST-Slac2-b-SHD or GST-RIM-RBD bound-beads by centrifugation at 1,100 × *g* for 2 min 4 °C and continue immediately (as described under Subheading 3.4.2, *see below*).

3.4.1 Preparation of Human Sperm Lysates

1. Wash 5 ml of capacitated sperm (50×10^6 cells) twice with ice-cold PBS by centrifugation at 6,200 × *g* for 2 min at room temperature.
2. Resuspend the pellet in 1 ml HTF medium and incubate with 100 μM 2-APB (*see* **Note 24**) for 10 min at 37 °C.

3. Challenge cells with 10 μM A23187 (*see* **Note 28**) for 15 min at 37 °C.
4. Spin down cells by centrifugation at 12,600 × *g* for 5 min at 4 °C.
5. Resuspend the pellet in 1 ml ice-cold pull-down buffer.
6. Lyse the cells by sonication at 40 Hz twice for 15 s each, with a 10 s interval, keeping them on ice at all times.
7. Extract proteins by incubation for 15 min at 4 °C.
8. Clarify the whole cell detergent extracts by centrifugation at 14,000 × *g* for 5 min at 4 °C and use immediately (continue as described under Subheading 3.4.2).

3.4.2 Pull-Down Assay

1. Add 20–30 μl of glutathione-Sepharose containing 5–10 μg GST-Slac2-b-SHD or GST-RIM-RBD to 1 ml ($20–50 \times 10^6$ cell) whole cell extracts prepared from sperm treated or not with A23187.
2. Incubate the mixtures for 30 min at 4 °C under constant rocking.
3. Wash the beads three times with one volume each of ice-cold pull-down buffer at 1,100 × *g* for 2 min at 4 °C.
4. Dissolve resin-bound proteins by boiling in 60 μl of sample buffer for 3 min at 95 °C.
5. Analyze Rab27-GTP and Rab3A-GTP levels by Western blot using anti-Rab27 and anti-Rab3 antibodies as probes.

3.5 Recruitment of a Human Sperm Rab3A GEF Activity by Immobilized Rab27A

Rab27-GTP (indirectly) activates sperm Rab3 (Fig. 2e and [23]). The following section summarizes the method we designed to unveil the mechanism behind the (indirect) activation of Rab3 by Rab27. The assay consists of two consecutive pull down assays: one to retain a GEF from human sperm extracts on a Rab27A-column and the other to test its activity by measuring the conversion of Rab3A-GDP into Rab3A-GTP (Fig. 3).

3.5.1 Preparation of Human Sperm Extracts

1. Wash 5 ml of capacitated sperm suspensions (50×10^6 cells) twice with a volume of ice-cold PBS by centrifugation at 6,200 × *g* for 2 min at room temperature.
2. Resuspend sperm pellet in 0.5 ml of lysis buffer.
3. Extract proteins by sonication at 40 Hz on ice three times for 15 s each, with a 10 s interval.
4. Incubate cell lysates for 45 min at 4 °C under constant rocking.
5. Clarify whole cell detergent extracts by centrifugation at 14,000 × *g* for 20 min at 4 °C.
6. Recover whole cell extracts (supernatant) and use immediately or store at −20 °C.

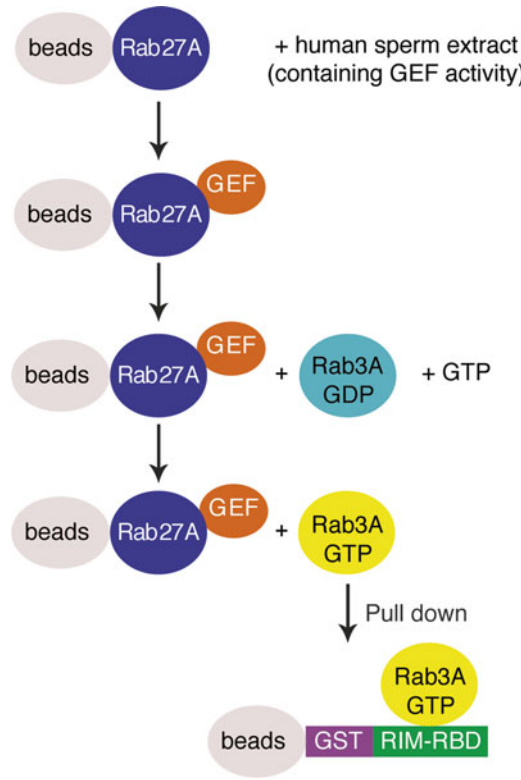

Fig. 3 Schematic representation of the protocol to recruit a Rab3A GEF activity. Geranylgeranylated GST-Rab27A-GTP-γ-S (*violet*) immobilized on glutathione-Sepharose beads (*gray*), are incubated with a human sperm extract with the goal of recruiting a Rab3A GEF protein (*orange*) if present in the lysate. Beads containing sperm proteins that interact with active Rab27A are used as source of Rab3A GEF activity using recombinant His_6-Rab3A-GDP (*blue*) and GTP as substrates. After centrifugation, the reaction product His_6-Rab3A-GTP (*yellow*) is pulled down with the GST-RIM-RBD cassette (*purple-green*), immobilized on fresh glutathione-Sepharose (*gray*)

3.5.2 Immobilization of Rab27A and Pull-Down of a Rab3A GEF Activity

1. Wash glutathione-Sepharose beads twice with one volume each of 20 mM HEPES/K, pH 7.4 and recover by centrifugation at 1,100 × *g* for 2 min at 4 °C.
2. Block non-specific binding sites on glutathione-Sepharose by incubating three times with two volumes each of 0.1 % BSA in 20 mM HEPES/K pH 7.4.
3. Incubate 2.5 μg of purified GST (negative control) or GST-Rab27A (geranylgeranylated and loaded with GTP-γ-S) with 20 μl of glutathione-Sepharose beads in binding buffer (200 μl final volume) for 1 h at 4 °C under constant rocking.

4. Wash beads containing immobilized recombinant proteins three times with 200 µl binding buffer by centrifugation at 1,100 × *g* for 2 min at room temperature.
5. Incubate immobilized recombinant proteins with 0.5 ml of human sperm extract (as described under Subheading 3.5.1) for 30 min at 4 °C under constant rocking.
6. Recover the beads by centrifugation at 2,600 × *g* for 2 min at 4 °C. Precipitate unbound sperm fractions with CCl_3H–CH_3OH–H_2O (*see below*) and analyze by Western blot with anti-α-tubulin as loading control.
7. Wash the beads three times with 0.5 ml ice-cold binding buffer by centrifugation at 1,100 × *g* for 2 min at room temperature.
8. Incubate the beads (containing a Rab3A GEF activity from human sperm retained on immobilized, active Rab27A) with 8 nM geranylgeranylated His_6-Rab3A-GDP (Fig. 3, *see* **Note 29**) in 0.5 ml binding buffer for 10 min at 37 °C under constant rocking.
9. Recover the beads by centrifugation at 3,000 × *g* for 2 min at 4 °C. Dissolve resin-bound proteins by boiling in 60 µl of sample buffer for 3 min at 95 °C and analyze by Western blot with anti-GST antibodies as loading control.
10. Analyze the supernatants from **step 8** by pull-down with the GST-RIM-RBD cassette (*see below* and Fig. 3).

3.5.3 Protein Precipitation

1. Precipitate protein and remove detergent with CCl_3H–CH_3OH–H_2O [29].
2. Dissolve precipitated proteins in 60 µl of sample buffer by heating once for 10 min at 60 °C and once for 3 min at 95 °C.

3.5.4 Analysis of His_6-Rab3A-GTP Levels

1. Add 20 µl of glutathione-Sepharose containing 5–10 µg of GST-RIM-RBD to 0.5 ml supernatants from **step 10** under Subheading 3.5.2.
2. Incubate the mixture for 30 min at 4 °C under constant rocking.
3. Wash the beads three times with one volume each of ice-cold binding buffer by centrifugation at 1,100 × *g* for 2 min at 4 °C.
4. Dissolve resin-bound proteins by boiling in 60 µl of sample buffer for 3 min at 95 °C.
5. Quantify the His_6-Rab3A-GTP levels by Western blot using the anti-Rab3A antibody as probe.

4 Notes

1. To permeabilize sperm we use recombinant SLO, an exotoxin that belongs to the homologous group of thiol-activated toxins that are synthesized by various Gram-positive bacteria. Monomeric toxin binds to cholesterol present in the plasma membranes and oligomerizes into ring-shaped structures, estimated to contain 50–80 subunits, which surround pores of approximately 30 nm diameter [30–33]. The only SLO we could work with reliably was the recombinant protein expressed and sold by the University of Mainz, Mainz, Germany.
2. The units of SLO are calculated for each batch.
3. Use a glass beaker and add the reagents one at the time to water at room temperature while stirring with a magnetic bar. Make sure that each reagent is completely dissolved before adding the next one and bring to final volume. HTF must be filter-sterilized using aseptic processing techniques. Once prepared, you can store aliquots (30 ml) at 2–8 °C until used. Do not freeze or expose to temperatures above 39 °C.
4. The HTF medium we use is buffered with CO_2/$NaHCO_3$. Tubes containing HTF medium should be placed loosely capped into a 5 % CO_2/95 % air incubator overnight to allow for the exchange of gas and pH equilibration. Check pH range (should be from 7.4 to 7.6) before using.
5. The sample should be obtained by masturbation and ejaculated into a sterile, wide-mouthed container made out of glass or plastic, from a batch that has been confirmed to be non-toxic for spermatozoa. Specimen containers should be labeled with an identification number, the date and time of collection.
6. Detailed protocols to assess semen quality are described in WHO laboratory manual for the examination and processing of human semen [34].
7. The direct swim-up technique is the choice for normal semen samples. Sperm are selected by their ability to swim out of semen placed at the bottom of the tube and up into the culture medium phase. Therefore highly motile sperm are obtained free from contaminants such as leukocytes, germ cells, dead or immotile spermatozoa, and seminal plasma.
8. After leaving the testis, mammalian spermatozoa are morphologically differentiated but are immotile and unable to fertilize. Progressive motility is acquired during epididymal transit. However, freshly ejaculated mammalian sperm are not immediately capable of undergoing the AR and fertilizing an egg. They require a period of several hours in the female reproductive tract or in an appropriate medium in vitro to acquire this

ability; this maturation process is termed capacitation [13, 16]. For all the assays present in this chapter, spermatozoa were capacitated in vitro by incubating in HTF/BSA medium for at least 3 h in a 5 % CO_2/95 % air incubator.

9. Alternatively, you can layer 300 μl of semen at the bottom of the tubes and then pipette carefully HTF/BSA medium over the semen.
10. Makler counting chamber is only 10 μm deep (1/10 of the depth of ordinary hemocytometers), making it the shallowest of known chambers. Constructed from two pieces of optically flat glass, the upper one serves as cover glass, with a 1 mm^2 fine grid in the center subdivided into 100 squares of 0.1×0.1 mm each. Spacing is firmly secured by four quartz pins [35].
11. The incubation performed in this step allows SLO binding to cholesterol molecules present in the plasma membrane of sperm.
12. DTT reduces and reversibly activates SLO.
13. Reagents should be mixed in the following order: HEPES/K, pH 7.5, DTT, BSA, $MgCl_2$, GDP, and protease inhibitors (AEBSF, aprotinin, bestatin, E-64, leupeptin, and EDTA). Then add Rab immobilized on beads and finally mouse brain cytosol and GGPP.
14. This step favors the recapture of isoprenylated Rabs dissociated from the resin.
15. The nucleotide must be ten times more concentrated than the protein.
16. Small molecules such as nucleotides will be retained in the column whereas large molecules, such as proteins will come out of the column after centrifugation.
17. Magnesium ions help to stabilize the nucleotide bound to the protein.
18. Recombinant protein concentrations are determined by the Bradford method (Bio-Rad) using BSA as a standard in 96-well microplates and quantified on a BioRad 3550 Microplate Reader or from the intensities of the bands in Coomassie Blue-stained SDS-PAGE gels using ImageJ program.
19. From this step on, every time you are going to add something or wash, first remove all excess liquid on the coverslips with lint-free tissue paper.
20. You can continue the protocol on the same day.
21. PSA lectin has specificity toward α-D-mannosyl-containing oligosaccharides present in the acrosome granule. PSA is coupled to FITC, if the acrosome is present then FITC-PSA binds the mannose residues and the structure shows fluorescent green staining [36]. On the contrary, if the acrosome is lost there is no staining.

22. We use Hoechst 33342, a cell-permeant nuclear dye that emits blue fluorescence when bound to dsDNA, to stain the nuclei and detect all cells.
23. We use an Eclipse TE2000 Nikon microscope equipped with a Plan Apo 40×/1.40 oil objective and a Hamamatsu digital C4742-95 camera operated with MetaMorph 6.1 software (Universal Imaging Corp., USA). We score the presence of red and/or green staining in the acrosomal region by manually counting between 100 and 200 cells either directly at the fluorescence microscope or in digital images from at least ten fields.
24. An IP_3-sensitive calcium channel inhibitor, which blocks calcium mobilization from the acrosome and hence avoids losing membranes by AR.
25. EDTA increases the off rate of nucleotides bound to endogenous Rabs present in sperm because it chelates magnesium ions [37].
26. We have stimulated SLO-permeabilized cells without loading endogenous Rabs with guanine nucleotides and the method also works [25].
27. This dilution renders 10 μM free calcium estimated by MAXCHELATOR, a series of programs to determine the free metal concentration in the presence of chelators available on the World Wide Web at http://maxchelator.stanford.edu.
28. A calcium-ionophore that induces the AR in non-permeabilized cells.
29. To measure Rab3A GEF activity retained in the Rab27A-column, we assayed the exchange of GDP for GTP on His_6-Rab3A by pull-down (3.4.2).

Acknowledgments

This work was supported by grants from Agencia Nacional de Promoción Científica y Tecnológica (grant numbers PICT 2006-1036 and PICT 2010-0342) and Secretaría de Ciencia, Técnica y Posgrado, Universidad Nacional de Cuyo to C.N.T.

References

1. Sudhof TC, Rothman JE (2009) Membrane fusion: grappling with SNARE and SM proteins. Science 323:474–477
2. Rizo J, Sudhof TC (2012) The membrane fusion enigma: SNAREs, Sec1/Munc18 proteins, and their accomplices-guilty as charged? Annu Rev Cell Dev Biol 28:279–308
3. Cherfils J, Zeghouf M (2013) Regulation of small GTPases by GEFs, GAPs, and GDIs. Physiol Rev 93:269–309
4. Wickner W (2010) Membrane fusion: five lipids, four SNAREs, three chaperones, two nucleotides, and a Rab, all dancing in a ring on yeast vacuoles. Annu Rev Cell Dev Biol 26:115–136

5. Barr FA (2013) Review series: Rab GTPases and membrane identity: causal or inconsequential? J Cell Biol 202:191–199
6. Stenmark H (2009) Rab GTPases as coordinators of vesicle traffic. Nat Rev Mol Cell Biol 10:513–525
7. Nottingham RM, Pfeffer SR (2009) Defining the boundaries: Rab GEFs and GAPs. Proc Natl Acad Sci U S A 106:14185–14186
8. Fukuda M (2008) Regulation of secretory vesicle traffic by Rab small GTPases. Cell Mol Life Sci 65:2801–2813
9. Yanagimachi R (2011) Mammalian sperm acrosome reaction: where does it begin before fertilization? Biol Reprod 85:4–5
10. Tomes CN (2007) Molecular mechanisms of exocytosis. In: Regazzi R (ed) Molecular mechanisms of membrane fusion during acrosomal exocytosis, 65th edn. Landes Biosciences and Springer Science + Bussiness Media LLC, New York, pp 275–291
11. Mayorga LS, Tomes CN, Belmonte SA (2007) Acrosomal exocytosis, a special type of regulated secretion. IUBMB Life 59:286–292
12. Florman HM, Jungnickel MK, Sutton KA (2008) Regulating the acrosome reaction. Int J Dev Biol 52:503–510
13. Visconti PE, Krapf D, de la Vega-Beltran JL et al (2011) Ion channels, phosphorylation and mammalian sperm capacitation. Asian J Androl 13:395–405
14. Costello S, Michelangeli F, Nash K et al (2009) Ca^{2+}-stores in sperm: their identities and functions. Reproduction 138:425–437
15. Gadella BM, Luna C (2014) Cell biology and functional dynamics of the mammalian sperm surface. Theriogenology 81:74–84
16. Buffone MG, Ijiri TW, Cao W et al (2012) Heads or tails? Structural events and molecular mechanisms that promote mammalian sperm acrosomal exocytosis and motility. Mol Reprod Dev 79:4–18
17. Aitken RJ, Nixon B (2013) Sperm capacitation: a distant landscape glimpsed but unexplored. Mol Hum Reprod 19:785–793
18. Buffone MG, Hirohashi N, Gerton GL (2014) Unresolved questions concerning mammalian sperm acrosomal exocytosis. Biol Reprod 90:112
19. Pocognoni CA, De Blas GA, Heuck AP et al (2013) Perfringolysin O as a useful tool to study human sperm physiology. Fertil Steril 99:99–106
20. Lopez CI, Belmonte SA, De Blas GA et al (2007) Membrane-permeant Rab3A triggers acrosomal exocytosis in living human sperm. FASEB J 21:4121–4130
21. Yunes R, Tomes C, Michaut M et al (2002) Rab3A and calmodulin regulate acrosomal exocytosis by mechanisms that do not require a direct interaction. FEBS Lett 525:126–130
22. Branham MT, Bustos MA, De Blas GA et al (2009) Epac activates the small G proteins Rap1 and Rab3A to achieve exocytosis. J Biol Chem 284:24825–24839
23. Bustos MA, Lucchesi O, Ruete MC et al (2012) Rab27 and Rab3 sequentially regulate human sperm dense-core granule exocytosis. Proc Natl Acad Sci U S A 109:E2057–E2066
24. Bustos MA, Roggero CM, De la Iglesia PX et al (2014) GTP-bound Rab3A exhibits consecutive positive and negative roles during human sperm dense-core granule exocytosis. J Mol Cell Biol 6:286–298
25. Ruete MC, Lucchesi O, Bustos MA et al (2014) Epac, Rap and Rab3 act in concert to mobilize calcium from sperm's acrosome during exocytosis. Cell Commun Signal 12:43
26. Lopez CI, Pelletan LE, Suhaiman L et al (2012) Diacylglycerol stimulates acrosomal exocytosis by feeding into a PKC- and PLD1-dependent positive loop that continuously supplies phosphatidylinositol 4,5-bisphosphate. Biochim Biophys Acta 1821: 1186–1199
27. Coppola T, Perret-Menoud V, Luthi S et al (1999) Disruption of Rab3-calmodulin interaction, but not other effector interactions, prevents Rab3 inhibition of exocytosis. EMBO J 18:5885–5891
28. Kondo H, Shirakawa R, Higashi T et al (2006) Constitutive GDP/GTP exchange and secretion-dependent GTP hydrolysis activity for Rab27 in platelets. J Biol Chem 281:28657–28665
29. Wessel D, Flugge UI (1984) A method for the quantitative recovery of protein in dilute solution in the presence of detergents and lipids. Anal Biochem 138:141–143
30. Sekiya K, Satoh R, Danbara H et al (1993) A ring-shaped structure with a crown formed by streptolysin O on the erythrocyte membrane. J Bacteriol 175:5953–5961
31. Sekiya K, Danbara H, Futaesaku Y (1993) Mechanism of pore formation on erythrocyte membrane by streptolysin-O. Kansenshogaku Zasshi 67:736–740
32. Sekiya K (1995) Electron-microscopic observation of pore formation in the erythrocyte membrane by streptolysin O. Nihon Saikingaku Zasshi 50:509–517
33. Bhakdi S, Tranum-Jensen J, Sziegoleit A (1985) Mechanism of membrane damage by streptolysin-O. Infect Immun 47:52–60

34. World Health Organization Department of Reproductive Health and Research (2010) WHO laboratory manual for the examination and processing of human semen. WHO Press, Geneva
35. Makler A (1980) The improved ten-micrometer chamber for rapid sperm count and motility evaluation. Fertil Steril 33:337–338
36. Mendoza C, Carreras A, Moos J et al (1992) Distinction between true acrosome reaction and degenerative acrosome loss by a one-step staining method using *Pisum sativum* agglutinin. J Reprod Fertil 95:755–763
37. Burstein ES, Macara IG (1992) Interactions of the ras-like protein p25rab3A with Mg^{2+} and guanine nucleotides. Biochem J 282:387–392