USP8/UBPy-regulated sorting and the development of sperm acrosome: the recruitment of MET

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Abstract

The acrosome is a peculiar vacuole that at fertilization undergoes the acrosome reaction (AR), an event unique in the sperm life. Contents released promote sperm penetration through oocyte's investments; membranous components are involved in sperm–egg interaction/fusion. Therefore, both constituents play a role in fertilization. The biogenesis of this vacuole, however, has not been clarified yet; recently, it has been proposed as a novel lysosome-related organelle (LRO). Our research focuses on the involvement of the endosomal pathway in acrosomogenesis starting from the early phases. The trafficking sorted by USP8/UBPy, an endosomal regulator recently described as a compelling candidate for male fertility gene, was investigated in comparison to that of SP56, a marker of the biosynthetic pathway. Mouse spermatids were double/triple immunolabeled and examined by confocal microscopy. The contribution of the vesicular traffic assisted by the cortical microtubule array was also evaluated in nocodazole-treated spermatids. USP8/UBPy-sorted cargo contributes early to acrosomogenesis and its trafficking is microtubule mediated. It was identified, through co-immunoprecipitation/co-immunolocalization assays, that the membrane receptor MET, described herein for the first time in spermatids, as an USP8/UBPy-target substrate is delivered to the acrosome. MET and USP8/UBPy still colocalize in epididymal spermatozoa. Following the AR, MET and USP8/UBPy show a distinct fate. MET, in particular, translocates at the PAS, the post acrosomal segment known to harbor sperm-borne factors involved in oocyte activation. Overall, our results support the concept of the acrosome as a LRO and provide evidence for the identification of MET as a tyrosine kinase receptor that may play a role in fertilization.


Introduction

The acrosome is a unique membranous organelle located over the anterior part of the sperm nucleus that is essential for normal fertilization because spermatozoa lacking these structures are infertile (Holstein et al. 1973). To penetrate into the oocyte's investments and to fertilize the oocyte, the spermatozoon must undergo a dramatic morphological change known as the acrosome reaction (AR; Yanagimachi 2011). At the triggering of the AR, the outer acrosomal membrane (OAM) fuses with the overlying plasma membrane and both are lost as hybrid vesicles, thus allowing the release of the soluble acrosomal content to open the sperm's passage. The luminal content also includes a particulate material, the acrosomal matrix, that provides a stable scaffold for interactions between the sperm and oocyte (Lin et al. 2007, Guyonnet et al. 2012). The inner acrosomal membrane (IAM), on its own, does not vesiculate, but remains intact as a cap to protect the underlying nuclear envelope. With the restructuring of sperm membranes in order to promote the exposure of the sperm fusion factor(s) and the internalization of the invaginated acrosomal membrane that encapsulates the paternal genome, fertilization is then fully accomplished (Satouh et al. 2012). Each acrosomal component is consequently crucial for successful fertilization; this functional importance is strengthened by the fact that they are conserved in spermatozoa across mammalian species including human. Notwithstanding this, the precise mechanism through which the acrosome develops and forms is not fully known as yet. Traditionally, acrosomogenesis is subdivided into four sequential phases that cumulatively last long about 2 weeks in the mouse and 1 month in the human (Lin et al. 2007). The basic premise to fulfill the entire process is that defined protein cargoes have to be recognized and delivered to the appropriate acrosomal compartment; this could occur with the aid of selected effectors of vesicular traffic, including both sorting/tethering factors and motor protein-assisted cytoskeletal tracks (Berruti & Paiardi 2011). The biosynthetic pathway through the Golgi apparatus is actively involved (Tang et al. 1982, Fujihara et al. 2012), but contribution of extra-Golgi pathways has also been documented (Berruti & Paiardi 2011). The finding that the endosomal-sorting complex required for transport (ESCRT)-deubiquitinating (DUB) enzyme USP8/UBPy is involved in the acrosome biogenesis (Berruti & Martegani 2005) provides...
et al (2010) has led to the proposal of the acrosome as a novel lysosome-related-organelle (LRO) (Berruti et al. 2010). LROs are cell type-specific membrane-bound organelles that arise by cargo delivery from early endosomal intermediates, the organelles that connect the endocytic and biosynthetic pathways (Huizing et al. 2008, Delevoye et al. 2009). In support of the acrosome as an LRO, there are recent proteomic studies providing the overlap between the profiling of LRO (Hu et al. 2007) and acrosomal matrix (Guyonnet et al. 2012) proteomes. USP8/UBPy is a key regulator of protein sorting at the endocytic route; in particular, it controls the dynamics and fate of membrane growth factor receptors by regulating the level of their ubiquitination on endosomes (Mizuno et al. 2006, Row et al. 2006, Niendorf et al. 2007, Wright et al. 2011, Berruti & Martegani 2013). Among the first receptors reported to be the target of USP8/UBPy, there is the membrane tyrosine kinase, MET, also known as the hepatocyte growth factor (HGF) receptor (Row et al. 2006). MET signaling regulates crucial biological activities such as cell proliferation, morphogenesis, motogenesis and invasion in the embryo as well as in the adult (Birchmeier et al. 2003, Trusolino et al. 2010, Woodard et al. 2013). HGF/MET system is expressed also in the testis (Ricci & Catizone 2014).

In this study, we have defined the contribution of USP8/UBPy to the acrosome biogenesis in a better way, demonstrating the early involvement, microtubule-mediated, of the endosomal pathway. Moreover, we have identified MET, described herein for the first time in post-meiotic spermatids, as an USP8/UBPy-sorted molecular target recruited to the acrosome. In epididymal spermatozoa, MET and USP8/UBPy still colocalize. With the induction of the AR, MET relocates to a sperm region, the post-acrosomal segment (PAS), known to harbor sperm-borne factors involved in oocyte activation.

**Materials and methods**

**Animals**

Testes and epididymides were isolated from CD1 male mice purchased from Charles River Italia (Calco, Lecco, Italy). The animals were housed at the standard conditions (temperature of 21±1 °C with relative humidity of 55±10% and 12 h light:12 h darkness cycle) and killed by CO₂ asphyxiation at the selected time-points. All efforts were made to minimize the number of animals used in this study as organ donors.

All procedures were carried out in accordance with the National and European directives for the care and use of laboratory animals (D.L. 116/1992; 86/609/CEE) and the NIH Guide for the Care and Use of Laboratory Animals. Animal protocols were reviewed and approved by the ethical committee for animal experimentation of the University of Milan and by the Italian Ministry of Health.

**Antibodies**

Primary antibodies used are as follows: rabbit anti-mUBPy/USP8 was prepared as described previously (Gnesutta et al. 2001); rabbit polyclonal anti-MET (SP260, sc-162) with its blocking peptide sc-162 P and mouse monoclonal anti-MET (B-2, sc-8057) with its blocking peptide sc-8057 P were purchased from Santa Cruz Biotechnology; mouse monoclonal anti-SP56 antibody (clone 7C5, MA1-10866) was obtained from Thermo Fisher Scientific, Inc. (Rockford, IL, USA); mouse monoclonal anti-β-tubulin (clone TUB 2.1, T4026) was purchased from Sigma–Aldrich Chemical Company and sheep polyclonal anti-tubulin (ATN02) was obtained from Cytoskeleton (Denver, CO, USA).

Secondary antibodies used for immunofluorescence (goat anti-rabbit IgG Alexa Fluor 488 and Alexa Fluor 568, goat anti-mouse IgG Alexa Fluor 488 and Alexa Fluor 568 and donkey anti-sheep IgG Alexa Fluor 488) were purchased from Invitrogen. HRP-coupled anti-rabbit and anti-mouse secondary antibodies were obtained from GE Healthcare (Buckinghamshire, UK).

**Isolation of spermatogenic cells, sperm collection and cell counts**

Spermatogenic cells were isolated from testis in RPMI-1640 medium (Sigma–Aldrich Chemical Company) by sequential enzymatic treatments according to well-standardized protocols (Berruti & Martegani 2005, Aivatiadou et al. 2009, Berruti et al. 2010). For sperm collection, epididymes were dissected, cut in to small pieces and the sperm were allowed to swim out in calcium-free motility buffer for 30 min as described previously (Paiardi et al. 2011). The numbers of cells were determined by hemocytometer counts either undiluted or diluted tenfold. All counts were made in duplicate and averaged (Berruti & Martegani 2005, Aivatiadou et al. 2009). Cell viability was assessed by the Trypan blue exclusion test (Aivatiadou et al. 2009).

**Nocodazole treatment**

Freshly isolated spermatogenic cells were cultured in Falcon petri dishes in RPMI-1640 medium supplemented with 25 mM HEPES, 0.5% BSA (Sigma–Aldrich Chemical Company), 100 µg/ml streptomycin and 100 µg/ml penicillin (Euroclone Life Sciences, Milan, Italy) at a density of 1–3×10⁶ cells/ml. Nocodazole (Sigma–Aldrich Chemical Company) was added to the cell culture until a final concentration of 5 µM was achieved. Cells were incubated with either nocodazole or vehicle only (DMSO) for 1 h in a 5% CO₂ incubator at 33 °C. The cells were collected by centrifugation and washed twice in RPMI to remove nocodazole before being processed for paraformaldehyde fixation.

**Protein extracts and western blotting**

One-percent Triton X-100 protein lysates were obtained as described (Berruti & Martegani 2005, Aivatiadou et al. 2009). Alternatively, freshly isolated spermatogenic and sperm cells were lysed in RIPA buffer (50 mM HEPES, pH 7.5, 150 mM...
NaCl, 0.5% NP-40, 0.1% SDS, 0.5% deoxycholate, 1 mM EDTA, 30 mM NaF, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM phenylmethylsulfonyl fluoride (PMSF) and Complete Mini Protease Inhibitor Cocktail for 15 min at 4 °C under gentle shaking. Occasionally, some samples were subjected to sonication (Berruti & Martegani 2005). Lysates were clarified by centrifugation at 15 700 g for 10 min at 4 °C and supernatants recovered. For tests and liver homogenates, the tissues were mechanically homogenized in RIPA buffer and then processed as described earlier in this study.

Protein concentration was determined using a standard protein assay (Bio-Rad DC, Bio-Rad). Protein extracts were resolved on SDS-PAGE and transferred onto a nitrocellulose membrane (Hybond–ECL, GE Healthcare). Western blotting or processed for immunoprecipitation. Equivalent aliquots of sperm suspension were removed at 0, 5, 15 and 30 min from A23187 addition, washed in tris buffered saline (TBS), smeared on glass slides and allowed to adhere at room temperature to be processed afterwards for immunocytochemistry. Parallel aliquots of sperm suspension at the same time-points were processed for Coomassie Brilliant blue (Sigma–Aldrich) staining technique (Feng et al. 1998) to assess the progression of the AR. At least 200 glass slide-smeread sperm/time-points were scored using an Axiosvert 200 M inverted microscope equipped with bright-field light optics. The percentage of acrosome-reacted (no blue acrosome staining) sperm vs acrosome-intact (blue acrosome cap) sperm at each of the above time-points was calculated and the results are expressed as mean ± S.E.M.

### Immunocytochemistry

Freshly isolated and nocodazole-treated spermatogenic cells were processed for indirect immunofluorescence as described (Berruti et al. 2010). Briefly, 4% (v/v) paraformaldehyde-fixed, ice-cold acetone-postfixed, preblocked spermatogenic cells were immunostained with the indicated primary antibodies, that is, anti-UBPy/USP8 (1:300), anti-SP56 (1:250), anti-β/α-tubulin (1:300) and anti-MET (1:50) followed by the appropriate Alexa Fluor 488- or Alexa Fluor 568-conjugated secondary antibodies. Nuclei counterstaining was carried out with 4',6-diamidino-2-phenylindole (DAPI; 2 μg/ml, Sigma–Aldrich Chemical Company) or with Draq5 (5 μM, Biostatus Limited, Shepshed, UK).

In control samples, the primary antibodies were omitted or, in the case of MET, replaced with primary antibody neutralized with blocking peptide according to the manufacturing procedures.

Epididymal sperm cells were fixed in ice-cold methanol for 10 min, post-fixed in ice-cold acetone for 5 s and processed for immunostaining as described earlier in this study. For spermatogenic cells isolated without enzymatic treatment, fragmented material was prepared essentially according to Guttman et al. (2004). Decapsulated testes/seminiferous tubules were minced into small pieces in RPMI-1640 medium. The pieces were fixed in 4% (v/v) paraformaldehyde for 2 h at 4 °C and then transferred into a tube containing ~5 ml of TBS. The material was gently passed first through an 18-gauge and then through a 21-gauge needle for five times per each. The larger fragmented material was left to sediment by gravity at room temperature for 5 min; the upper layer was thus transferred to another tube for centrifugation. The pellet was resuspended into a small volume of TBS and the suspension was smeared onto slides and allowed to adhere. The slides were post-fixed with ice-cold acetone for 5 min, rinsed in TBS and, after the blocking step, processed for immunofluorescence as described earlier in this study.

### Immunohistochemistry

For traditional immunohistochemistry, testes were fixed in Bouin’s fixative at 4 °C overnight, embedded in paraffin wax and sectioned at a thickness of 6 μm (Paiardi et al. 2011).

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**Acrosome reaction**

Epididymal sperm were obtained by ‘swim-out’ from snipped caudae kept in HEPES-buffered Whitten medium without BSA as described (Sosnik et al. 2010). Following a rinsing, sperm were resuspended in the modified Whitten medium at a concentration of 2–3×10<sup>6</sup> cells/ml and maintained at 37 °C for 1 h. The AR was then induced with 10 μM calcium ionophore A23187 (Sigma–Aldrich) and 2.4 mM CaCl<sub>2</sub>. Equivalent aliquots of sperm suspension were removed at 0, 5, 15 and 30 min from A23187 addition, washed in tris buffered saline (TBS), smeared on glass slides and allowed to adhere at room temperature to be processed afterwards for immunocytochemistry. Parallel aliquots of sperm suspension at the same time-points were processed for Coomassie Brilliant blue (Sigma–Aldrich) staining technique (Feng et al. 1998) to assess the progression of the AR.
Deparaffinized sections, after blocking with 3% BSA for 45 min and with 3% BSA + 0.1% Triton X-100 for 15 min, were incubated with anti-MET antibodies (1:50), followed by an Alexa-conjugated secondary antibody. Nuclei counterstaining was carried out with 2 μg/ml DAPI. Seminiferous tubules fixed in 4% (v/v) paraformaldehyde to be used for surface antigen detection were incubated with an anti-MET antibody essentially as described by Catizone et al. (2012). Immunostained tubules were then mounted onto slides, covered with coverslips and squashed to allow cell to flow out. Controls were with the MET primary antibody neutralized with its blocking peptide.

**Microscopy**

For simultaneous double immunolabeling, cells were examined under a Leica TCS SPAOBS scanning laser confocal fluorescent microscope (Leica Lasertechnik GmbH, Heidelberg, Germany) equipped with laser Ar/Kr (488 nm), laser He/Ne (568 nm) and laser u.v. (361–365 nm) for green, red and blue fluorescence respectively. Images were captured under 63× magnification using identical laser power, light pathways and band passes by a computerized system (Leica Power Scan Software, Heidelberg, Germany). Captured images were elaborated with Adobe Photoshop. For single immunofluorescence analysis, the samples were analyzed under an Axiovert 200 M inverted microscope (Zeiss Microscopy GmbH, Jena, Germany), equipped with standard filter sets for green (Alexa Fluor 488), red (Alexa Fluor 568) and blue (DAPI) fluorescence and a Zeiss AxioCam, CCD camera. Acquired images were elaborated with Adobe Photoshop.

**Results**

**Endocytic vs biosynthetic route in acrosome biogenesis**

To understand the possible origin of components of the acrosome, we carried out an extensive immunofluorescence analysis to determine first, by reciprocal comparison, the cellular localization of USP8/UBPy and SP56, a protein of the acrosomal matrix used as a marker of the biosynthetic pathway (Kim et al. 2001). Double-fluorescent protein immunostaining was matched with DAPI to visualize nuclear spermatid morphology during the distinct phases of acrosomogenesis. Representative images are provided in Fig. 1. At the Golgi phase, USP8/UBPy and SP56 do not colocalize at the early acrosomogenic phases. Confocal microscopy was performed on spermatids at the Golgi phase (A) and cap phase (B) after immunostaining against USP8/UBPy and SP56 and counterstaining with DAPI. Images at each strips in both A and B represent a single slice captured starting from cell periphery toward a deeper cell localization. Triple-color overlays are shown at the right. A’ and B’ show: top left, the sum of the optical sections of A and B respectively; bottom left, the light transmission image where it is possible to appreciate the proacrosomal granule and acrosomal cap respectively (arrow); right, the orthogonal projections on Z axis (scanned along Z axis). Results are representative of at least eight cells at the specified differentiation step in three different, separate experiments. Bar = 8 μm.

**Figure 1** USP8/UBPy and SP56 do not colocalize at the early acrosomogenic phases. Confocal microscopy was performed on spermatids at the Golgi phase (A) and cap phase (B) after immunostaining against USP8/UBPy and SP56 and counterstaining with DAPI. Images at each strips in both A and B represent a single slice captured starting from cell periphery toward a deeper cell localization. Triple-color overlays are shown at the right. A’ and B’ show: top left, the sum of the optical sections of A and B respectively; bottom left, the light transmission image where it is possible to appreciate the proacrosomal granule and acrosomal cap respectively (arrow); right, the orthogonal projections on Z axis (scanned along Z axis). Results are representative of at least eight cells at the specified differentiation step in three different, separate experiments. Bar = 8 μm.
characterized by the fusion of Golgi-derived proacrosomal granules with a single acrosomal granule that then attaches to the cell nucleus, SP56 was detected only within the developing acrosomal granule next to contact the nuclear envelope (Fig. 1A). On the contrary, USP8/UBPy exhibited a diffuse localization marked, especially at the cell periphery, by scattered spots ascribable to vesicular structures (Fig. 1A). No USP8/UBPy–SP56 overlapping was ever detected at any focal plane in Golgi-phase spermatids as indicated also by the Z-axis stacks (Fig. 1A and A'). During the cap phase, fusion of further vesicles around the granule leads to the formation of the acrosomal vesicle. Triple-fluorescent labeling showed that, in cap-phase spermatids (Fig. 1B), both USP8/UBPy and SP56 are resident at the acrosomal vesicle. Superimposition of the respective images examined at more focal planes, however, revealed that while SP56 marks the acrosomal matrix as expected, USP8/UBPy signs predominantly the profile of the vesicle lining the OAM and IAM, oriented toward the cell surface and acroplaxoma-nucleus respectively. Accordingly, no significant USP8/UBPy–SP56 overlapping was yielded also by the Z-axis stacks (Fig. 1B and B'). It follows that at least two pathways of vesicle trafficking contribute molecules to the developing acrosome in round spermatids.

The successive phases are characterized by morphological/structural changes. The acrosome phase is signed by nucleus/acrosome elongation while at the maturation phase the acrosome acquires its final shape; at spermiatation, mature spermatids are released as spermatozoa within the tubule lumen. Figure 2 shows USP8/UBPy and SP56 relationship in germ cells at these developmental stages. Notwithstanding the structural remodeling of the spermatid head in progress, USP8/UBPy and SP56 still maintain distinct distribution. At the acrosomal phase (Fig. 2A), USP8/UBPy marks gradually the shaping of the acrosomal vesicle now embracing the apical one-third of the nucleus; on the other hand, SP56 remains luminally confined to the central portion of the acrosomal matrix. This occurs also successively when the acrosome has acquired its typical hook-like shape and spermatozoa are then released (Fig. 2B and C). Superimposition of the immunofluorescence images (Fig. 2) did not result in any significant protein overlapping also considering the consistent thinning of the sperm acrosome.

**The microtubule array and acrosomogenesis**

Acrosome biogenesis is a microtubule-mediated event (Moreno et al. 2006). Spermatids at the early (Golgi and cap) phases of acrosomogenesis exhibit a rather unusual microtubule array, i.e., a cortical network without any apparent nucleating foci and with undistinguishable plus- and minus-ends; however, some microtubules penetrate deep into the cytoplasm. Another spermatid peculiarity is the spatial orientation and fate of the Golgi complex. As a consequence of the Golgi rotation during early spermiogenesis, the cis-Golgi and trans-Golgi-network (TGN) locate toward the cell surface and the developing acrosome/nucleus respectively (Griffiths et al. 1994, Moreno et al. 2006). We double-stained, USP8/UBPy–tubulin and SP56/tubulin, respectively, mouse spermatids that were then examined by confocal microscopy. During the Golgi phase, there were detected USP8/UBPy-labeled vesicular structures in close contact with the cortical microtubule network (Fig. 3A, left). Indeed, USP8/UBPy possesses an microtubule interacting and transport (MIT) domain through which it could bind to microtubules (Row et al. 2007, Berruti et al. 2010). SP56, on its own, stained the proacrosomal granule that is more deeply located; no apparent SP56/microtubule array overlapping was revealed (Fig. 3B, left). The cortical microtubule array starts to disassemble at the cap phase when USP8/UBPy

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**Figure 2** USP8/UBPy and SP56 do not colocalize at late acrosomogenic phases. Spermatids at advanced stages of acrosome biogenesis (A, acrosomal phase; B, maturation phase and C, testis spermatozoa) were immunolabeled against USP8/UBPy and SP56 and examined by confocal microscopy. Yellow signals in the merge of C can be likely due to the remarkable thinning of the sperm anterior acrosome as highlighted by the light transmission image. Results are representative of at least six cells at each stage in three separate experiments. Bar = 5 μm.
already labels the cap; neither acrosomal USP8/UBPy (Fig. 3A, left) nor SP56 (Fig. 3B, left) colocalizes with tubulin-marked structures. Taken together, these findings suggest that the cortical network could supply the microtubule tracks along which USP8/UBPy-signed endocytic vesicles are transported to reach the forming acrosome.

To evaluate this hypothesis, we subjected spermatids to nocodazole treatment. At the Golgi phase, nocodazole, a microtubule de-polymerizing drug, induced a fragmentation of the cortical microtubule network; this apparently did not interfere with the association of USP8/UBPy-sorted cargo with the fragmented microtubules (Fig. 3A, right). Nocodazole treatment did not affect the positioning of SP56-marked proacrosomal granule toward the nuclear envelope (Fig. 3B, right). The effects of the drug were, however, evident in cap-phase spermatids. The nocodazole-induced disassembling of the cortical array in Golgi-phase spermatids prevented the contribution of USP8/UBPy-sorted protein cargo(es) to the proacrosomal granule; consequently, this last did not evolve into a normal cap (Fig. 3A, compare untreated, left vs nocodazole-treated, right, spermatids). On the other hand, the SP56-signed biosynthetic route (Fig. 3B, right) seemed to be unaffected by the treatment.

**MET, USP8/UBPy and their relationship during acrosome biogenesis**

To accomplish with success spermatogenesis, spermatogenic cells have to divide, differentiate and concomitantly migrate within the seminiferous epithelium. MET signaling can regulate cellular proliferation, cellular motogenesis and morphogenesis (Hammond et al. 2001). We thus focused attention on MET, already described as a candidate substrate for USP8/UBPy (Row et al. 2006). Previous immunohistochemical studies reported MET immunopositivity in the embryonic...
(Ricci et al. 1999), postnatal (Catizone et al. 2001) and pre-pubertal (Catizone et al. 1999) testis, however, without any information about MET in post-meiotic spermatids. We analyzed testis from post-pubertal mice at different ages probing two different commercial anti-mouse MET antibodies directed against the mouse 140 kDa β-chain and using more immunofluorescence approaches.

Fresh suspensions of spermatogenic cells isolated from testis through enzymatic steps were examined. Independently from the antibody used, MET immunolocalization did not differ consistently; it is summarized in Fig. 4. In spermatocytes (primary and secondary, Fig. 4B and C), MET exhibited mostly a spotted distribution, in particular at the sub-plasmalemmal region. The spot-like distribution apparently increased in round spermatids (Fig. 4D). With the progression of acrosomogenesis, MET was found to label the acrosome (Fig. 4E and F). The acrosome was the only cellular structure to be immunostained in testis spermatids (Fig. 4G). MET immunolabeling was also carried out on spermatogenic cells isolated avoiding any enzymatic treatment (Guttman et al. 2004); similar results were, however, obtained (Supplementary Figure 1A, see section on supplementary data given at the end of this article). To possibly preserve cell–cell contacts, we directly immunolabeled fragments of seminiferous tubules squashed onto cover-slips. MET immunopositivity was found at/near the plasma membrane in meiotic cells, whereas post-meiotic cells appeared essentially MET immunonegative (Supplementary Figure 1B). Conversely, with traditional immunohistochemistry, the fluorescence of the crescent-shaped acrosomal vesicle in elongating spermatids dimmed the weaker signal from spermatocytes (Supplementary Figure 1C). Overall, immunofluorescence analysis suggests that MET undergoes a re-routing pathway that delivers it from the cell periphery to the acrosome during the acrosome biogenesis. We thus double-immunostained spermatids, previously subjected to nocodazole treatment, against MET and tubulin. As it is shown in Fig. 5D, the nocodazole-induced disassembling of the cortical microtubule array interferes with the labeling of the acrosomal cap by MET.

The presence of MET was also evaluated by western immunoblotting. The most strong signal was elicited by a band of ~105 kDa from testis homogenate, isolated spermatogenic cells and epididymal spermatozoa; this band, however, was also detected in liver homogenate, used as a positive control (Fig. 5A). Further bands of lower molecular weights (68 and 46 kDa, in particular) were occasionally detected, especially when sonication plus Triton X-100 extraction was used (Fig. 5B, left). Parallel blots probed with preadsorbed antibodies were negative, suggesting that the immunodetected molecular bands could be proteolytic products of the canonical 140 kDa β-chain (Fig. 5A, lower panel, and B, right).

Then, we searched for a possible USP8/UBPy–MET association under physiological conditions. We examined this possibility by co-immunoprecipitation experiments. Lysates from freshly isolated spermatogenic cells immunostained for endogenous MET and counterstained with DAPI. Images were captured under a fluorescence microscope at 63 × magnification. Representative images are provided for primary spermatocytes (B), secondary spermatocytes (C) and spermatids at each acrosomogenic phase (D, E, F and G). Bar = 5 μm.

Figure 4 MET redistributes in spermatogenic cells at different stages of cell differentiation. (A) Controls performed with a pre-saturated MET antibody. (B, C, D, E, F and G) Freshly isolated spermatogenic cells immunostained for endogenous MET and counterstained with DAPI. Images were captured under a fluorescence microscope at 63 × magnification. Representative images are provided for primary spermatocytes (B), secondary spermatocytes (C) and spermatids at each acrosomogenic phase (D, E, F and G). Bar = 5 μm.
to be processed as the non-stimulated cells. USP8/UBPy immunoprecipitates, analyzed by SDS–PAGE and blotted, were probed with anti-MET antibodies. It was found that MET co-immunoprecipitates with USP8/UBPy apparently independently of HGF stimulation (Fig. 5C, upper panel). Finally, we subjected freshly isolated spermatids to double USP8/UBPy–MET immunolabeling. USP8/UBPy–MET colocalization was already detected in cap-phase spermatids and consistently revealed at the acrosome of both elongating spermatids and epididymal spermatozoa (Fig. 5E). Altogether, experimental evidence suggests that USP8/UBPy could be involved in the re-routing of MET toward the developing acrosome.

**USP8/UBPy, MET and the acrosome reaction**

At fertilization, spermatozoa undergo the AR, a prerequisite for sperm–egg fusion. During the AR, not all the acrosomal proteins are lost; some proteins such as IZUMO (Satoh et al. 2012) and equatorin (Yoshida et al. 2010), known also as MN9 antigen (Toshimori et al. 1992) and/or AFAF (Li et al. 2006), change their localization. The relocation of equatorin and IZUMO from the acrosome to the cell surface is thought to be important for priming sperm molecules for sperm–egg interaction and fusion respectively. We planned to follow the fate of USP8/UBPy and MET at the AR. Cauda epididymal spermatozoa were thus immunolabeled with anti-USP8/UBPy or anti-MET antibodies after artificial induction of the AR (Sosnik et al. 2010) at 0, 5, 15 and 30 min. In parallel, we followed the progression of the AR at each time-point by Coomassie blue staining (Feng et al. 1998). To classify the different patterns of USP8/UBPy and MET localization, we resorted to a nomenclature of AR stages in agreement with Yoshida et al. (2010). At the initial stage (time 0 min), when ~88% of spermatozoa were acrosome intact, USP8/UBPy (Fig. 6A, left) and MET (Fig. 6A, right) immunostainings were similar to those observed in freshly isolated epididymal spermatozoa (Fig. 5E): the DUB and the receptor marked strongly the dorsal acrosomal cap area of acrosome-intact spermatozoa. We noticed that when the anti-MET SP260, but not the anti-MET B-2, was used, the tail mid-piece of cauda epididymal spermatozoa was also fluorescently labeled. At an early stage (time 5 min), USP8/UBPy showed almost the same staining pattern as in the initial stage (Fig. 6A, left), while MET signal resulted already in an apparent change of protein distribution. It was reduced at the equator, while labeling at the marginal sub-acrosomic ring was evidenced. At 15 min of induction of AR, more than 40% spermatozoa were acrosome reacted (ARd). The ARd spermatozoa showed a modified USP8/UBPy labeling: most of USP8/UBPy was lost with the reacted material, while the remnant was restricted selectively to the anterior ventral beak (Fig. 6A, left). On its own, MET exhibited a further translocation to the post-acrosomal region/post-acrosomal segment (PAR/PAS) in...
spermatozoa we considered ARd (Fig. 6A, right); for the others, MET immunostaining was ranged between that described for 5 min-induction and the labeling of the marginal ring (Fig. 6A, right low magnification). At an advanced final stage (time 30 min; ~75% ARd spermatozoa), residual USP8/UBPy labeled the head ventral side including the ventral spur. MET, in turn, located exclusively at the PAR/PAS of ARd sperm (Fig. 6A, right). Based on the changes in the staining pattern according to the progression of AR, we schematically summarized the results as illustrated in the drawing of Fig. 6B.

Discussion

A former study established that the ESCRT–DUB USP8/UBPy is involved in the acrosome biogenesis and that acrosomes of mature spermatozoa are USP8/UBPy immunopositive (Berruti et al. 2010). This supported the concept of the acrosome as a novel LRO and suggested that the deubiquitinase could be functional also for fertilizing spermatozoa. Herein, we show that USP8/UBPy-regulated endocytic transport concurs with acrosomogenesis already at the early stages and that the disassembling of the microtubule-based transport of USP8/UBPy-signed cargo addressed to the acrosome affects acrosome biogenesis. The identification of the membrane receptor MET as an USP8/UBPy-sorted cargo recruited to the acrosome and the finding of its relocation after the sperm AR could be results of great potential interest for understanding molecular mechanisms at fertilization.

According to the traditional interpretation of acrosomogenesis, the proacrosomal granule enlarges to become a cap only through the contribution of Golgi-derived material. This is believed although it is known that the Golgi pathway is dropping in consequence of its migration toward the opposite pole of the cell (Moreno et al. 2000). Herein, it is shown that the membrane fold-like extensions, typical of the cap phase, of the OAM and IAM are USP8/UBPy positive and SP56 negative. At the previous phase, the Golgi phase, USP8/UBPy stains vesicular structures that are always well distinct from the SP56-positive proacrosomal granules. As no USP8/UBPy–SP56 overlapping was ever revealed at the Golgi as well as cap phase, we can conclude that USP8/UBPy-sorted trafficking and SP56 delivery to the developing acrosome occur via two distinct transport routes. Other proteins resident at the acrosome follow an extra-Golgi pathway. The list includes MN9/equatorin/AFAF (Toshimori et al. 1992, Li et al. 2006, Yoshida et al. 2010), SPE-39 (Zhu et al. 2009) and SMAP2 (Funaki et al. 2013), i.e., proteins that in somatic cells localize to early endosomes and/ or function in early endosome-to-TGN transport. Hence, the acrosome could be effectively thought as an LRO, i.e., a peculiar membranous organelle indispensable for carrying on a cell type-specific physiological function.

As known, the biogenesis of LROs (Huizing et al. 2008, Delevoye et al. 2009) relies on protein vesicular trafficking assisted by microtubule tracks. Herein, we find that, during acrosomogenesis, nocodazole-induced fragmentation of the cortical microtubule array blocks the contribution from the USP8/UBPy-signed endocytic route rather than that derived directly from Golgi. In the presence of nocodazole, in fact, the proacrosomal granule does not evolve into a normal acrosomal cap. Moreno et al. (2006) had already investigated about the effects of nocodazole with the conclusion that the lack of
assembly of the acrosome in nocodazole-treated spermatids is the consequence of the Golgi fragmentation. In detail, these authors discussed that the drug disorganizes specifically the cortical microtubule array and that this disorganization leads to TGN scattering and TGN separation from the cis-mediated Golgi. It follows that their observations are in line with our interpretation. Their findings from azh/azh mice also support our conclusion (Moreno et al. 2006). HOOK1, the protein mutated in the azh/azh mouse used as an animal model for detrimental acrosomogenesis, is in fact known to link endocytic, and not biosynthetic, membrane trafficking to the microtubule cytoskeleton (Maldonado-Báez et al. 2013). Moreover, experimental evidence has revealed that other proteins such as AGFG1(HRB)/GOPC, KIFC1 and RAB27A, all suggested to work in the Golgi-to-acrosome pathway in male germ cells (Yang & Sperry 2003, Kierszenbaum et al. 2011), work indeed at endosomal compartments (Hu et al. 2007, Chaineau et al. 2008).

If the compartment(s) from which specific but undefined cargoes are delivered to the acrosome can be defined, this might aid to identify the cargoes themselves. Thus, we reasoned that if we assume that the acrosome is an LRO and take into consideration that USP8/UBPy governs the trafficking of tyrosine kinase receptors at endosomes, we can succeed in identifying an USP8/UBPy-sorted protein delivered to the acrosome. MET being present in the testis and reported as an USP8/UBPy-sorted protein delivered to the acrosome, the receptor is expressed throughout the grounds of our immunofluorescence study, we can conclude that the receptor is expressed throughout spermatogenesis exhibiting distinct subcellular localization according to the stage of cell differentiation. In particular, in post-meiotic cells, MET undergoes a drastic change of localization, i.e., from a diffuse scattered distribution in early round spermatids to the acrosome in elongating spermatids/spermatozoa. The most immunoreactive band in immunoblot assays is due to a 105 kDa β-chain that we confide to correspond to a proteolytic product of the predicted 140 kDa β-chain. It has to be pointed out, however, that lower molecular weight variants of the MET receptor due to the shedding of extracellular fragments have already been reported (Ancot et al. 2012). Intriguingly, we succeeded to find that MET is able to interact with USP8/UBPy. MET co-immunoprecipitates with USP8/UBPy in freshly isolated as well as cultured, HGF-stimulated, spermatogenic cells. With regard to this, we remember that HGF is produced in testis (van der Wee & Hofmann 1999), but internalization/trafficking of membrane MET could also occur through ligand-independent pathways of downregulation (Ancot et al. 2012). With further demonstration of MET and USP8/UBPy interaction, there is the protein colocalization in elongating spermatids. Moreover, it is noteworthy that nocodazole was found to prevent MET trafficking toward the developing acrosome. Altogether, experimental evidence is consistent with an USP8/UBPy-regulated sorting of MET toward the forming acrosome.

It follows consequently that not only USP8/UBPy is involved in the biogenesis of a fully formed acrosome, but also the ESCRT–DUB could act as a key regulator of endosomal traffic by directing toward the acrosome molecular players that may be crucial for a successful fertilization. A recent genome-wide association study has identified USP8 as a compelling candidate for male fertility gene (Kosova et al. 2012). The strong USP8/UBPy–MET colocalization at the acrosome in cauda epididymal spermatozoa was unexpected. The association of ‘endocytosed receptor/ESCRT DUB’ is usually lost when the sorted receptor has reached its final destination. The acrosome may not be the final destination for the MET receptor. Herein, we show that, at the AR, a mandatory step for fertilization, the DUB and the receptor have a different fate. While most of USP8/UBPy is lost in ARd sperm, MET has translocated massively at the PAS, the posterior head region known to harbor proteins that regulate oocyte activation/early development (Sutovsky et al. 2003). Protein relocation/translocation is not an unusual event in sperm biology as demonstrated for IZUMO that translocates from the acrosomal membrane to the equatorial segment plasma membrane (Satoh et al. 2012) and MN13 that diffuses from the acrosome to the PAS (Ito et al. 2010). MN13 is one of the numerous candidates to be the sperm factor that triggers oocyte activation (for a review, see ex Amdani et al. 2013)). Currently, however, the most quoted is PLCζ, the sperm-specific phospholipase C that generates repetitive Ca2+ transients into the ooplasm of the fertilized oocyte, thus leading to oocyte activation (Saunders et al. 2002). A key, as yet unanswered, question is, however, how the enzyme, inactive within sperm, is activated into the oocyte (Amdani et al. 2013).

It may be hypothesized that MET, once translocated at the PAS surface in ARd sperm, could activate PLCζ located at the PAS. In support of this hypothesis, the MET is known to exert its effects through more signaling cascades, including that through the PLCs (Trusolino et al. 2010). PLCs, once tyrosine phosphorylated, increase remarkably their enzymatic activity (Cattaneo et al. 2013). It may be noteworthy to mention that globozoospermic spermatozoa lacking a real acrosome but expressing PLCζ, as the wobbler mouse spermatozoa (Chianese et al. 2010), fail to fertilize even after intracytoplasmic sperm injection (ICSI) (Heytens et al. 2010).

In conclusion, herein we provide further experimental evidence for the acrosome as an LRO. The identification of sperm MET as an USP8/UBPy-specific target sorted
to the acrosome and the discovery of its translocation at the AR are original findings that could help to decipher our incomplete understanding of sperm differentiation and fertility potential.

Supplementary data
This is linked to the online version of the paper at http://dx.doi.org/10.1530/REP-14-0671.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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References
Berruti G, Ripolone M & Ceriani M 2010 USP8, a regulator of endosomal sorting, is involved in mouse acrosome biogenesis through interaction with the spermatid ESCRT-0 complex and microtubules. Biology of Reproduction 82 930–939. (doi:10.1095/biolreprod.100.081679)

Kim KS, Cha MC & Gerton GL 2001 Mouse sperm protein sp56 is a component of the acrosomal matrix. *Biology of Reproduction* 64 36–43. (doi:10.1095/biolreprod64.1.36)


