Cell Tissue Res (2015) 359:895–908
DOI 10.1007/s00441-014-2072-9

REGULAR ARTICLE

Panel of monoclonal antibodies to sperm surface proteins as a tool for monitoring localization and identification of sperm–*zona pellucida* receptors

Michal Zigo · Andriy Dorosh · Alžběta Pohlová · Věra Jonáková · Miroslav Šulc · Pavla Maňásková-Postlerová

Received: 3 May 2014 / Accepted: 17 November 2014 / Published online: 23 December 2014

Abstract Primary binding of the sperm to the *zona pellucida* (ZP) is one of the many steps necessary for successful fertilization. Sperm bind ZP by means of membrane receptors which recognize carbohydrate moieties on ZP glycoproteins according to a well-defined sequential process. Primary binding receptors, many of which have been disclosed in various mammals, are localized throughout the acrosomal region of the sperm surface. A panel of monoclonal antibodies against proteins from the sperm surface was prepared. Antibodies were screened by immunofluorescence for protein localization and Western blotting. Proteins localized on the sperm head and simultaneously detected by Western blotting were further studied in terms of immunolocalization in reproductive tissues and fluids, binding to ZP, immunoprecipitation and sequencing. Of 17 prepared antibodies, 8 recognized proteins localized on the sperm head and also detected proteins of interest by Western blotting. Only three other antibodies recognized proteins that also coincided in binding to ZP. These three antibodies were used for immunoprecipitation, and further protein sequencing of immunoprecipitates revealed that these antibodies distinguished acrosin precursor, RAB-2A protein, and lactadherin P47. This is not the first time we have detected acrosin on the surface of ejaculated and capacitated sperm. However, to our knowledge, this is the first time RAB-2A has been detected on the sperm surface. Lactadherin P47 has already been characterized and its physiological function in reproduction has been proposed.

Keywords Sperm surface proteins · Monoclonal antibodies against sperm surface proteins · *Zona pellucida*-binding receptors · RAB-2A, Lactadherin P47

Introduction

The pig is one of the most studied animal models in the pursuit of elucidating the processes taking place during mammalian fertilization. The fundamental mechanism of gamete recognition seems to be conserved throughout evolution from marine vertebrates to eutherian mammals in the way that the sperm surface molecules interact with the oligosaccharide ligands of the envelope glycoproteins (Töpfer-Petersen et al. 2008). The porcine egg coat, the *zona pellucida* (ZP), is composed of three glycoprotein families, ZP1 (ZPA; 92 kDa), ZP3α (ZPB; 55 kDa) and ZP3β (ZPC; 55 kDa) named by a nomenclature based on apparent molecular weight (Hedrick and Wardrip 1986, 1987). ZP1 is split into two smaller molecules, ZP2 (69 kDa) and ZP4 (23 kDa), under reducing conditions (Hasegawa et al. 1994). Sperm bind ZP by means of membrane receptors which recognize carbohydrate moieties on ZP glycoproteins according to a well-defined sequential process, one of which is the primary binding (Serres et al. 2008). The sperm-binding activity in pigs has been mapped to the neutral tri- and tetra-antennary complex N-glycans of ZPB expressing nonreducing terminal β-galactosyl residues (Kudo et al. 1998; Yonezawa et al. 2005).

Electronic supplementary material The online version of this article (doi:10.1007/s00441-014-2072-9) contains supplementary material, which is available to authorized users.

M. Zigo (✉) · A. Dorosh · A. Pohlová · V. Jonáková · P. Maňásková-Postlerová
Laboratory of Reproductive Biology, Institute of Biotechnology, Academy of Sciences of the Czech Republic, v.v.i., Vídeňská 1083, 142 20 Prague 4, Czech Republic
e-mail: michal_zigo_2000@yahoo.com

M. Zigo · A. Pohlová · M. Šulc
Department of Biochemistry, Faculty of Science, Charles University, Hlavova 8, 128 43 Prague 2, Czech Republic

M. Šulc
Institute of Microbiology, Academy of Sciences of the Czech Republic, v.v.i., Vídeňská 1083, 142 20 Prague 4, Czech Republic

Upon ejaculation, the sperm cell surface is coated with extracellular glycoproteins that form a protective layer and stabilize the sperm cell. Sperm surface-coating factors are believed to mask underlying proteins involved in (1) sperm-ZP binding and (2), especially in pigs, docking of the acrosome required for the initiation of the acrosome reaction (Gadella 2013). This step has a vital purpose in pigs and some other mammals, as reviewed in Suarez (2008). To be able to selectively recognize ZP, the sperm must undergo the capacitation process, during which sperm ZP-binding proteins are ordered in functional protein complexes that only emerge at the apical tip of the sperm head plasma membrane, the exclusive area involved in primary ZP binding (Boerke et al. 2008; Gadella 2008; Gadella et al. 2008). Capacitated porcine spermatozoa exhibit stable docking of the acrosome to the plasma membrane, preparing the sperm for the acrosome reaction (Tsai et al. 2010). The physiological execution of the acrosome reaction is a later event, and, just recently, it has become less clear where this event is initiated.

Characterization of the molecules that mediate primary recognition and adhesion to ZP still remains a difficult task. Several putative ZP receptors have been identified in pig, including spermadhesins AWN, AQN-1 and AQN-3 as well as P47 (lactadherin) and the short Fn-2 type protein pB1 (also DQH) and carbonyl reductase (Ensslin et al. 1995; Jonáková et al. 1998; Maňásková et al. 2000, 2007; van Gestel et al. 2007). These data support the current concept of a multiple receptor involved in primary binding, in which the contributing proteins may act sequentially or synergistically.

Over the last few decades, one of the central dogmas of the fertilization process in mammals has been that once capacitated, acrosome-intact sperm bind to the ZP and then undergo acrosomal exocytosis (Saling et al. 1979). Recent experiments suggest that sperm binding to ZP is not sufficient to induce acrosomal exocytosis, and instead of ZP-triggered acrosomal exocytosis, Baibakov et al. (2007) proposed a mechanosensory mechanism that involved (1) the binding of acrosome-intact sperm to the ZP surface, followed by (2) the loss of the acrosome as the sperm penetrate the ZP. Furthermore, Jin et al. (2011) made a groundbreaking observation that, in the mouse at least, instead of the ZP, the cumulus appears to be the physiological inducer of the acrosome reaction. This was also observed earlier in pigs (Mattioli et al. 1998). Other inducers of acrosome reaction are progesterone (Melendrez et al. 1994) as well as estrogens (Děd et al. 2010).,

Acrosomal exocytosis ensures the exposure and release of soluble and acrosomal matrix proteins. The actual model of the penetration process includes alternating cycles of (1) binding of the acrosome-reacted sperm to the ZP (secondary binding), (2) limited proteolysis of the matrix and (3) release of the sperm and penetration caused by the sperm forward motility (O'Rand et al. 1986). Acrosin was believed to be the main participant of this model; however, skepticism came from the observation that mice sperm null for proacrosin were able to penetrate ZP and to fertilize the egg (reviewed by Honda et al. 2002).

Membrane molecules, which are the main ZP-binding candidates, often represent minor components in total cellular extracts, and therefore sophisticated isolation approaches must be used. Approaches for selective isolation of the surface subproteome have therefore been developed, including nitrogen cavitation (Canvin and Buhr 1989; Flesch et al. 1998; Bongalhardo et al. 2002), isolation of proteins associated with detergent-resistant membranes (DRM) (Cross 2004; van Gestel et al. 2005 and Girouard et al. 2008), and affinity isolation of tagged proteins. A promising technique involving tagging the surface molecules with sulfo-NHS-SS-Biotin [Sulfosuccinimidyl-2-(biotinamido)ethyl-1,3-dithiopropionate] was introduced by Zhao et al. (2004). This approach was successfully implemented for the identification of new potential ZP-binding candidates (Belleannee et al. 2011; Zigo et al. 2013). Numerous studies have characterized sperm membrane receptors responsible for the binding to ZP in various animals, reviewed in Tanphaichitr et al. (2007).

The purpose of this study was to develop an alternative tool for monitoring and identification of ZP-"binding" receptors. This tool comprises a panel of monoclonal antibodies raised against proteins from the sperm surface. The following goals were defined: (1) preparation of the panel of monoclonal antibodies and their testing on epididymal, ejaculated and capacitated sperm for the protein localization; (2) localization of the proteins recognized by the panel in selected reproductive tissues and fluids; (3) screening for co-incidence in binding of the proteins recognized by the panel with ZP glycoproteins; (4) use of the panel for immunoprecipitation of selected proteins that coincide in ZP binding; and (5) sequencing of the precipitated proteins.

Materials and methods

Collection of biological fluids, spermatozoa and tissues from boar reproductive organs

Boar ejaculates from 12 adult animals were obtained from the breeding station PROAGRO Nymburk (Nymburk, Czech Republic) and pooled to obtain a representative "sample". Ejaculates were centrifuged (400*g*, 20 min) to separate seminal plasma from spermatozoa. Spermatozoa were washed three times with phosphate-buffered saline (PBS) and centrifuged for 10 min at 400*g*. Washed sperm samples were used for protein extraction and immunofluorescence.

For the experiments, reproductive fluids and reproductive and non-reproductive organ tissues from five adult fertile boars were collected immediately post-mortem from the Breeding Institute of Animal Physiology and Genetics Liběchov, Academy of Sciences of the Czech Republic, v.v.i., Czech Republic. Boar epididymal fluid together with

epididymal spermatozoa were obtained from the epididymal duct by injection and extrusion of the fluid. Epididymal fluid with spermatozoa was centrifuged for 20 min at 600*g*. Spermatozoa were washed four times with PBS and then centrifuged for 15 min at 400*g*.

Boar seminal vesicle fluid was obtained by the following procedure. The seminal vesicles, separated from connective tissue, were cut away from the urethra and the secretions were collected by applying pressure. After centrifugation (3500*g*, 15 min, 4 °C), the supernatant was frozen and stored at −25 °C.

Tissues of boar urogenital tract (from the testes and prostate) and tissue obtained from non-reproductive organ (kidneys) were homogenized in Tris-buffered solution [pH 7.8, 30 mM Tris, 50 mM KCl, 1 % (v/v) Triton X-100] by homogenizer Precellys 24 (Bertin Technologies, Montigny-le-Bretonneux, France) according to the manufacturer's protocol. Homogenates were centrifuged (20,000*g*, 4 °C) and supernatants were stored at −25 °C.

Sperm capacitation

Sperm capacitation was done as described in Zigo et al. (2011). Briefly, fresh boar ejaculates diluted in KORINAT I (14.3 mM sodium bicarbonate, 12.25 mM sodium citrate, 364 mM glucose, and 12.3 mM EDTA; pH 7.5) were centrifuged at 400*g*. The sperm pellet was washed with Tris-buffered saline (TBS; 50 mM Tris, 150 mM NaCl, pH 7.4) to remove dilutor components, layered on a 40–80 % (v/v) discontinuous Percoll gradient (Sigma-Aldrich, St. Louis, MO, USA) and centrifuged at 200*g* for 45 min. After centrifugation, the 80 % (v/v) layer was diluted in ten times diluted Tyrode's buffer medium (TBM) (pH 7.7; 20 mM Tris, 113.1 mM NaCl, 3 mM KCl, 10 mM $CaCl_2$, 11 mM glucose) (Sigma), 5 mM pyruvic acid (Sigma), 1 ampoule of gentamycin (80 mg/2 ml) (Lek Pharmaceuticals, Ljubljana, Slovenia), and the cells were washed again. The washed spermatozoa were resuspended in TBM, supplemented with 1 mg/ml of bovine serum albumin SL 5 grade (Serva, Heidelberg, Germany), and capacitated (4 h, 37 °C, 5 % (*v/v*) CO_2) (Berger and Horton 1988). Sperm samples after capacitation were used for protein extraction and immunofluorescence.

Sperm stages, before and after capacitation, were studied by indirect immunofluorescence with the use of monoclonal antibody against intra-acrosomal protein proacrosin/acrosin (Acr-2), as described previously by Děd et al. (2010). Non-capacitated, capacitated, and acrosome-reacted sperm were counted from the total of 200 random sperm in both ejaculated and in vitro capacitated sperm samples. In the ejaculated boar samples (before capacitation), the average count of non-capacitated sperm was 78 %, while in the samples after capacitation, the average count of capacitated sperm was 70 % (Fig. S1), which corresponded to the previous results in Děd et al. (2010).

Preparation of solubilized *zona pellucida*

Solubilized ZP was prepared as described in Zigo et al. (2013). Briefly, porcine ovaries were obtained from slaughtered adult sows from the slaughterhouse in Český Brod (Czech Republic). Oocytes were released from frozen porcine ovaries in a meat grinder with ice-cold saline (0.15 M NaCl) and the homogenate was sieved through nylon screens as described by Hedrick and Wardrip (1986). The oocytes were purified by centrifugation in a discontinuous Percoll gradient (Sigma) (Hokke et al. 1994), collected from the 0–10 % (v/v) interface, washed in distilled water, and gently homogenized using a small glass homogenizer. *Zona pellucida* particles were collected on a 40 μm screen and repeatedly washed with saline. Isolated *zonae pellucidae* were heat solubilized in 0.2 M $NaHCO_3$, pH 9 at 73 °C for 30 min, and centrifuged at 350*g* for 10 min. The supernatant was used for biotinylation.

Biotinylation of *zona pellucida* glycoproteins

ZP glycoproteins were biotinylated as described in Zigo et al. (2013). Briefly, solubilized *zona pellucida* was incubated with 0.4 % (w/v) N-hydroxysuccinimidobiotin (Sigma) in dimethylformamide (Sigma) for 30 min at room temperature (Jonáková et al. 1998). *Zona pellucida* glycoproteins were dialyzed in a Spectra/Por MWCO 6/8000 membrane (Spectrum Medical, Laguna Hills, CA, USA) against phosphate-buffered saline (PBS; 20 mM phosphate, 150 mM NaCl, pH 7.2) overnight and stored at -25 °C.

Isolation of proteins from the sperm, preparation of protein extracts from tissues and fluids

The isolation of proteins from the sperm surface was done as described in Zigo et al. (2013). Briefly, a Thermo Scientific Pierce Cell Surface Protein Isolation kit (Rockford, IL, USA) was used according to the manufacturer's protocol. In this method, mammalian cells were first labeled with EZ-Link Sulfo-NHS-SS-Biotin, a thiol-cleavable amine-reactive biotinylation reagent. Cells were subsequently lysed with a mild detergent, and labeled proteins were then isolated with Immobilized NeutrAvidin Gel (agarose beads). The bound proteins were released by incubation with an SDS-PAGE sample buffer (50 mM Tris buffer titrated by HCl to pH 6.8, 1 % (v/v) glycerol, 2 % (*w/v*) SDS (Sigma), 0.002 % (w/v) bromophenol blue) containing 50 mM DTT (Sigma). The release of the bound proteins was completed on a multiple NeutrAvidin Gel column to achieve satisfactory protein yields (this is an extension of the manufacturer's protocol). Samples were stored at −25 °C prior to use.

The whole sperm extracts from the ejaculated and capacitated sperm (50 μl of sperm suspensions) were mixed with an SDS-PAGE sample buffer containing 50 mM DTT (Sigma),

vortexed and left to boil for 5 min. Sperm suspensions were centrifuged at 20,000*g* for 2 min at 4 °C. Supernatants were stored at −25 °C.

Prior to use, protein extracts from the sperm surface, whole sperm extracts, extracts from reproductive and non-reproductive tissues and collected fluids (epididymal, seminal vesicle and seminal plasma) were refined with 2-D Clean-Up Kit (GE Healthcare, Piscataway, NJ, USA) and all refined proteins were resuspended in SDS-PAGE sample buffer containing 50 mM DTT (Sigma).

Preparation of the panel of monoclonal antibodies against proteins from the sperm surface

Prior to immunization, boar sperm surface protein extract was clarified by using Zeba desalting spin columns (Thermo Scientific) to remove excess detergent and DTT, and used for immunization of BALB/c mice (AnLab, Prague, Czech Republic). Three females were immunized subcutaneously with 50 μg of sperm surface protein extract each in complete Freund's adjuvant (Sigma) followed by three additional immunizations with antigen in incomplete Freund's adjuvant at 2-week intervals. After 3 weeks, the final boost injection was performed intraperitoneally with no adjuvant followed by myeloma Sp2/0 and spleen cell fusion 3 days later according to the basic procedure (Harlow and Lane 1988). Positive clones were selected by indirect immunofluorescence on boar sperm cells. Hybridoma cells producing antibodies recognizing the apical part of the sperm head were subcloned and frozen for further use.

Sodium dodecyl polyacrylamide electrophoresis (SDS-PAGE), Western blotting

Protein extracts from the sperm, reproductive and non-reproductive tissues and collected fluids (epididymal, seminal vesicle and seminal plasma) obtained in the SDS sample buffer were used for one-dimensional electrophoresis (1-DE), which is a method for separation and analysis of macromolecules, based on their size and charge. SDS-PAGE was carried out as previously described by Laemmli (1970). A 7–21 % (w/v) gradient gel slab was used and run in a MiniProtean IV apparatus (Bio-Rad, Hercules, CA, USA). The concentration of bisacrylamide was 0.19–0.56 % (w/v), while the concentration of acrylamide was 6.81–20.44 % (w/v) from a total of 7–21 % (w/v). 15 μg of total protein was loaded per well. The molecular masses of the separated proteins were estimated by using prestained Precision Plus Protein Standards All Blue from Bio-Rad run in parallel.

Tris-glycine buffer (pH 9.6) with 20 % (v/v) methanol was used for the transfer of proteins separated by SDS-PAGE onto a PVDF Immobilon Transfer Membrane (Millipore, Bedford, MA, USA) for immunodetection. Electroblotting was carried out for 1.5 h at 500 mA, according to the method described by Towbin et al. (1979).

Protein immunodetection

The PVDF membrane (Millipore) with the transferred proteins was blocked with 1.5 % (w/v) teleostean fish gelatin (Sigma) in PBS for 3 h. After washing with 0.05 % (v/v) Tween 20 (Serva) in PBS, the membrane was incubated with primary antibodies from the panel of monoclonal antibodies against sperm surface proteins (diluted 1:50–1:200 in PBS) at 4 °C overnight. Following a washing step, incubation was performed for 1 h at 37 °C with goat anti-mouse immunoglobulins coupled to horseradish peroxidase (Sigma) diluted 1:12,000 in PBS. After washing, a chemiluminescent substrate, SuperSignal (Thermo Scientific), was applied and the blot was screened with ImageQuant LAS4000 (GE Healthcare) to visualize the corresponding interaction bands. Where not mentioned, procedures were carried out at room temperature. Blots were afterwards stained with Comassie Brilliant Blue (CBB; Serva).

Far-western blot with biotinylated *zona pellucida* glycoproteins

Far-western blot is derived from the standard Western blot method to detect protein–protein interactions in vitro. Far-western blot with biotinylated ZP glycoproteins was done as described in Zigo et al. (2013). Briefly, the PVDF (Millipore) membrane with the transferred proteins was deactivated with 1 % (w/v) teleostean fish gelatin (Sigma) in PBS for 4 h at room temperature. After washing with 0.02 % (v/v) Tween 20, 1 mM $CaCl_2$ in PBS (pH 7.2), the membrane was incubated with biotin-labeled glycoproteins of porcine *zona pellucida* (gpZP) (100 μg/ml in PBS) at 4 °C overnight. Following washing, incubation was performed for 0.5 h at 37 °C with 0.1 μg/ml of avidin-peroxidase solution (Sigma) in PBS. After washing, a chemiluminescent substrate, SuperSignal (Thermo Scientific), was applied and the blot was screened with ImageQuant LAS4000 (GE Healthcare) to visualize the corresponding interaction bands. Afterwards blots were stained with CBB (Serva).

The origin of the sperm surface subproteome was checked with the Western blot detection of acrosin and, within the limit of detection of this method, acrosin was present only in the avidine non-bound fraction after incubation of biotinylated sperm extract with avidine agarose beads. These results have been previously published in Zigo et al. (2013).

Immunoprecipitation

Protein extract (500 μg), dissolved in modified RIPA buffer (pH 7.2, 10 mM sodium phosphate, 150 mM NaCl,

1 % (v/v) Triton X-100, 1 % (w/v) sodium deoxycholate, 0.1 % (w/v) SDS, 1 mM DTT) (Sigma) with proteinase inhibitor cocktail Complete Mini (Roche, Mannheim, Germany), from capacitated boar sperm was incubated with monoclonal antibodies against sperm surface proteins (from the panel) in RPMI-1640 medium (Sigma) in a 7:4 volume ratio (700:400 μl) overnight at 4 °C. Then, 50 μl of Protein G-Sepharose beads (GE Healthcare) was added and incubated for 4 h at 4 °C. After centrifugation at 1000*g* for 1 min, protein G beads were washed six times with 200 μl PBS with 0.05 % (v/v) Tween 20 (Serva) and centrifuged at 1000*g* for 1 min. SDS-PAGE sample buffer containing 50 mM DTT (Sigma) was then added and the beads were boiled for 5 min and then centrifuged at 5000*g* for 10 min.

Supernatants were subjected to SDS-electrophoresis and the corresponding protein bands were detected with specific antibody on PVDF membranes. Immunoprecipitated proteins were subjected to mass spectrometric analysis.

Proteolytic digestion, sample preparation, and mass spectrometric analysis

The protein spots destaining, cysteine residue modification, proteolytic digestion, peptide extraction and sample preparation for mass spectrometry was performed as described previously (Sulc et al. 2009). The protein digestion was carried out in a cleavage buffer containing 0.05 M 4-ethylmorpholine acetate, 10 % (v/v) acetonitrile and sequencing grade trypsine endoprotease (Promega, 50 ng/μl) overnight at 37 °C. The resulting peptides were extracted with 40 % (v/v) acetonitrile/0.1 % (v/v) trifluoroacetic acid (TFA). After extraction, the peptides were directly diluted in 0.1 % (v/v) TFA and subjected to an R3 microcolumn (Applied Bioscience, Foster City, CA, USA) pre-equilibrated with 0.1 % (v/v) TFA. After desalting with the 0.1 % (v/v) TFA solution, the peptides were directly eluted with a 5 mg/ml solution of α-cyano-4-hydroxycinnamic acid in 50 % (v/v) acetonitrile/0.1 % (v/v) TFA from the R3 microcolumn on the MALDI target; and the droplets were allowed to dry at ambient temperature.

Mass spectra were measured in an ultraFLEX III matrix-assisted laser desorption/ionization reflectron time-of-flight (MALDI-TOF/TOF) mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with a nitrogen laser (337 nm). Spectra were calibrated externally using the monoisotopic $[M+H]^+$ ion of peptide standards PepMix I (Bruker). The positive MALDI-TOF spectra and MS/MS LIFT spectra of the selected *m/z* signals were collected in reflectron mode to identify the proteins. MALDI-TOF MS and MS/MS spectra were interpreted using the MASCOT software engine (http://www.matrixscience.com/).

Indirect immunofluorescence technique: localization of the proteins on the surface of epididymal, ejaculated and capacitated sperm

Indirect immunofluorescence for localization of proteins of interest recognized with the panel of monoclonal antibodies on the surface (non-permeabilized membrane) of epididymal, ejaculated and capacitated sperm was used to assess the presence/absence and potential redistribution of these proteins during their individual states. Sperm suspensions were smeared onto glass slides and left to desiccate at ambient temperature. Primary antibodies from the panel were applied. Slides were left to incubate for 1 h at 37 °C in a moist chamber. After washing, incubation was carried out with a secondary antibody against the mouse IgG Fc fragment, conjugated with fluorescein isothiocyanate (FITC; Sigma) diluted 1:160 in PBS, for 1 h at 37 °C. Finally, after washing with PBS and distilled water, slides were incubated for 12 min with 1.5 μg/ml of VectaShield-DAPI (Vector Laboratories, Burlingame, CA, USA). Samples were viewed and evaluated with a Nikon Eclipse E400 fluorescent microscope with a ×100 Nikon Plan Fluor lens and a VDS CCD-1300 camera (VDS Vosskuhler, Osnabruck, Germany) with the aid of LUCIA imaging software (Laboratory Imaging, Prague, Czech Republic). In the controls, a non-sense primary antibody with a matched immunoglobulin subclass at the same concentration as the test antibody was used, and the procedure was followed as previously described; no reactions were observed (data not shown).

Additionally, a control to validate surface labeling by the antibodies to a known internal antigen, β tubulin, and phosphotyrosine, was performed to exclude the possibility that air-dried sperm smears could have cracked or perforated membranes so that antibodies could gain access to internal proteins. This control study is included in the Electronic supplementary material.

Results and discussion

Panel of monoclonal antibodies against proteins from the sperm surface

A panel of monoclonal antibodies against sperm surface proteins was prepared. The reason we decided to operate with the surface subproteome and not the whole sperm proteome was to increase our chances in obtaining antibodies against surface proteins, as these proteins often represent minor components in total cellular extracts. The panel comprised the 17 antibodies listed in Table 1, which also summarizes the experiments performed. Using the indirect immunofluorescence technique, of the total number of 17 antibodies 2 did not recognize and 4 recognized parts of the sperm tail, and the rest of the

Table 1 Characterization of panel of monoclonal antibodies

Ab	Stained region in ejaculated sperm	WB of sperm proteome	Immunofluorescence intensities of the sperm[a]			Ab recognized protein localization
			Epididymal	Ejaculated	Capacitated	
3B10	Midpiece	–	–	+	+	Not tested
1C6	Non-recognizing	–	–	–	–	Not tested
4C7	Acrosomal region	45 kDa	–	++++	+++	Surface of ejaculated and capacitated sperm
4C11	Acrosomal region	45 kDa	–	++++	+++	Surface of ejaculated and capacitated sperm
5C5	Acrosomal region	24, 27 kDa	+	++	++++	Surface of capacitated sperm, epididymal fluid
1D1	Acrosomal region	51 kDa	–	+	++++	Surface of capacitated sperm
2D10	Acrosomal region	200 kDa	–	++	+++	Surface of capacitated sperm
3D5	Principal piece	–	+	++	++	Not tested
3D7	Acrosomal region	–	–	+	+	No reaction
1E3	Acrosomal region	32, 35, 38 kDa	–	++	+++	Surface of ejaculated and capacitated sperm, epididymal fluid, prostate
2E1	Acrosomal region	200 kDa	+	++	+++	Surface of ejaculated and capacitated sperm
4E8	Midpiece	–	++	++	+++	Not tested
5 F2	Acrosomal region	–	+++	+++	++++	No reaction
1G7	Midpiece	–	+	++	++	Not tested
2G9	Non-recognizing	–	–	–	–	Not tested
1H9	Apical region	35, 45 kDa	–	++++	+++	Surface of capacitated sperm, kidney
2H10	Postacrosomal region	70 kDa	+	++	++	Not tested

[a] Immunofluorescence intensities: –no, + weak, ++ medium, +++ good, ++++ strong

antibodies stained the apical region of the sperm head (Table 1), where the primary receptors for ZP binding are located (Boerke et al. 2008). Therefore, the six above-mentioned antibodies were excluded from the panel. The next question was whether the remaining 11 antibodies were robust enough to also detect the protein of interest by Western blotting. Table 1 clearly shows that out of 11 antibodies recognizing the acrosomal part of the sperm head by indirect immunofluorescence, 8 also detected the protein of interest by Western blot (corresponding molecular masses of proteins recognized by the remaining 8 antibodies are listed in Table 1). These 8 monoclonal antibodies recognizing the acrosomal part of the sperm head and detecting the proteins of interest by Western blot were further studied and are discussed in the following text and the Electronic supplementary material.

Recently, the model of primary binding caused by the induction of acrosome reaction has become disputed, as it was found in mice that the majority of the sperm reaching ZP are already acrosomally reacted, and the acrosomal exocytosis is probably induced by cumulus (Jin et al. 2011). However, without knowing the exact locations where acrosomal exocytosis occurs during the course of normal fertilization, a role of the ZP in stimulating or inducing this sperm secretory event cannot be excluded. It may be that, during the biogenesis of the ZP within the ovarian follicle, ZP proteins diffuse into the extracellular of the cumulus cells surrounding the oocyte, either by not being incorporated into the particulate zona during assembly or by the slight degradation of ZP proteins after insertion into the zona. Further studies are required to investigate this possibility.

Antibody recognizing the protein of 45 kDa: 4C7

The monoclonal antibody from the panel termed 4C7, recognizing the protein of molecular mass ~45 kDa, was further studied. Using indirect immunofluorescence, the protein recognized by the 4C7 antibody was localized on the surface of non-permeabilized sperm. This protein was present both on ejaculated (Fig. 1a') and capacitated sperm (Fig. 1a"), but not on the surface of epididymal sperm (Fig. 1a). The signal was relatively strong on both ejaculated and capacitated sperm. Additionally, we searched for the origin of this protein in reproductive tissues and fluids. The result is depicted in Fig. 1b, clearly showing that the protein recognized by the 4C7 antibody was present solely on the surface of both ejaculated and capacitated sperm (Fig. 1b,– lanes 1), but in no other location. The whole sperm proteome extracts (Fig. 1b, lane 2) and extract from the kidney (Fig. 1b, lane 8) served as positive and negative controls, respectively. The molecular mass shifts of the proteins isolated from the sperm surface towards higher masses with respect to proteins isolated from the whole sperms are due to biotinylation. Next, we studied whether the protein recognized by the 4C7 antibody could bind ZP glycoproteins. We employed far-western blot assay of

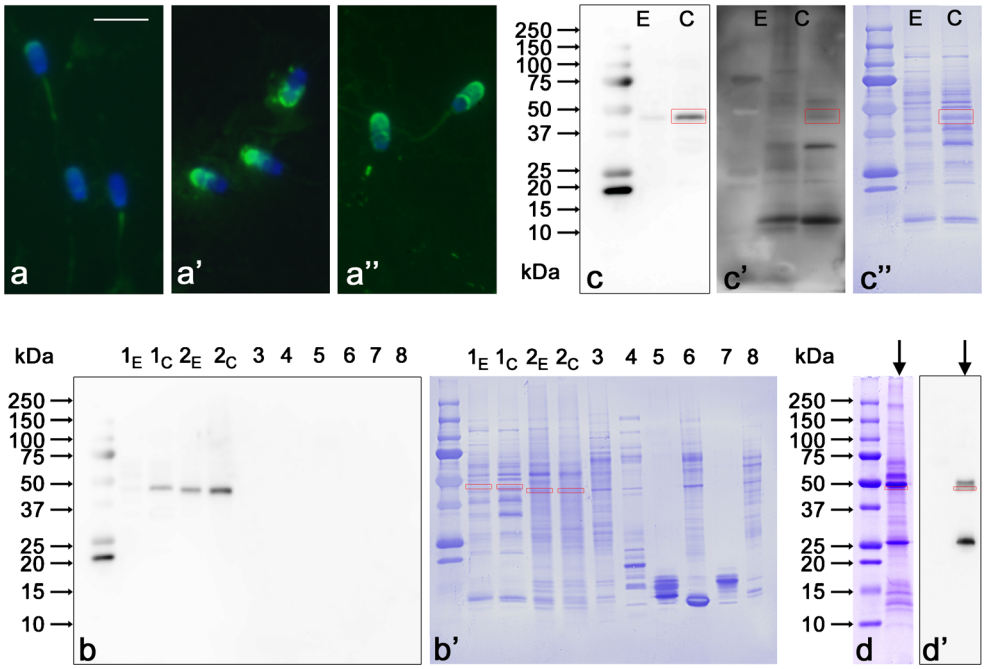

Fig. 1 Characterization of antibody termed 4C7 by immunofluorescence of epididymal (**a**), ejaculated (**a'**) and capacitated (**a"**) sperm; immunolocalization by Western blotting (**b**) in the sperm surface subproteome (*lanes 1*) from ejaculated (*with subscript E*) and capacitated (*with subscript C*) sperm, SDS extract (*lanes 2*) from ejaculated (*with subscript E*) and capacitated (*with subscript C*) sperm, extract from the testis (*lane 3*), epididymal fluid (*lane 4*), seminal vesicle fluid (*lane 5*), extract from the prostate (*lane 6*), seminal plasma (*lane 7*), and extract from the kidney (*lane 8*) followed by CBB staining (**b'**); comparison of Western blot detection (**c**) with the far-western blot using biotinylated *zona pellucida* glycoproteins (**c'**) of the sperm surface subproteome from ejaculated (*lane E*) and capacitated (*lane C*) sperm, respectively, followed by CBB staining (**c"**); and immunoprecipitation from capacitated sperm extract (**d**) confirmed by the Western blot (**d'**). All corresponding bands are indicated by *red rectangles*. The first lane in (**b**, **b'**, **c**, **c'**, **c"** and **d**, **d'**) represents molecular mass standards. *Bar* in immunofluorescence 10 μm

proteins isolated from the sperm surface with ZP glycoproteins (Fig. 1c') and the interactions were compared with Western blot detections (Fig. 1c). The results depicted clearly show that the protein recognized by 4C7 antibody coincides in binding to the *zona pellucida* at the molecular mass of 49 kDa. The interaction was more apparent with capacitated sperm (Fig. 1c', lane C) than with ejaculated sperm (Fig. 1c', lane E), which can be attributed to greater abundance of the protein in the capacitated fraction. Prior to mass spectrometry analysis, the protein was immunoprecipitated for higher purity and concentration (Fig. 1d) and the immunoprecipitate was tested by Western blot (Fig. 1d'). After SDS-PAGE, the protein band was located just under the heavy chain (50 kDa) of the 4C7 antibody, which was further confirmed by Western blotting (Fig. 1d, d'), and the third signal belonged to the light chain (25 kDa) of the 4C7 antibody. The protein of interest was identified after in-gel proteolysis and following MS and MS/MS analysis of formed peptides using the Mascot Peptide Mass Fingerprint tool to be an acrosin precursor (EC 3.4.21.10) (gi|164703 from *Sus scrofa*, with MW 46 kDa, pI 9.66), significant probability Mowse score 164 [protein scores greater than 72 are significant ($p<0.05$), sequence coverage 34 %, matched 10 from 13 searched *m/z* values]. The acrosin precursor identification was confirmed using MS/MS Ion Search of acquired MS/MS spectra at *m/z* 2578.389 [probability Mowse score 96, scores greater than 34 are significant ($p<0.05$), mass error 7.0 ppm, identified sequence R.LIFGANEVVWGSNKPVKPPLQER.F] and *m/z* 2139.089 [probability Mowse score 98; scores greater than 40 are significant ($p<0.05$), mass error 3.5 ppm, identified sequence K.RPGVYTSTWPYLNWIASK.I], respectively.

The identified protein is a major component of the acrosomal content, localized both in the inner acrosomal membrane and acrosomal matrix, which also plays a role in reproduction as a secondary binding receptor to the ZP (Tesařík et al. 1988; Jones and Williams 1990; Töpfer-Petersen and Calvete 1995, 1996). It has been shown in mice that knockout of the acrosin gene does not affect fertility (Baba et al. 1994), although it may provide a competitive advantage to wild-type relative to acrosin-null mouse sperm by promoting dispersion of the acrosomal matrix (Adham et al. 1997; Yamagata et al. 1998). In domestic animals such as the pig, which have oocytes surrounded by a thick ZP (16–20 μm), acrosin seems to essentially contribute to the secondary binding interaction and sperm penetration through the ZP (Töpfer-Petersen et al. 2008). This is not the first time we have detected acrosin on the surface of ejaculated and capacitated sperm (Zigo et al. 2013). The explanation may be that, in both ejaculated and

capacitated sperm samples, acrosomally reacted sperm were also present (Supplemental Fig. 1) and the released acrosin remained adherent to the sperm surface regardless of washing (Straus et al. 1981; Straus and Polakoski 1982). At this point, we believe that the presence of proacrosin/acrosin on the surface of the sperm, preferentially capacitated (Fig. 1b), is not coincidental, and that the spontaneous (false) acrosome reaction also has its meaning in the process of fertilization. We suggest that a portion of sperm which undergo spontaneous acrosome reaction also have a physiological function that may allow the released proacrosin/acrosin adhere to other acrosomally non-reacted sperm. The ability of acrosin to bind ZP is not a new attribute. Acrosin has been shown to participate in the secondary binding of the sperm to oocyte in multiple animal models (with acrosin localization on the inner acrosomal membrane). This is why the presence of acrosin on the surface of sperm is also of interest. Acrosin will certainly also bind ZP on the sperm surface, but the purpose of this will be different from the secondary binding of the sperm to oocyte. In this case, we presume that acrosin would most probably participate as a mediating molecule or even as a primary binding molecule. However, further experiments are required to confirm this hypothesis.

Antibody recognizing the protein of 24 and 27 kDa: 5C5

The monoclonal antibody termed 5C5 from the panel recognizing the protein of molecular masses ~24 and 27 kDa was further studied. Using immunofluorescence, the protein recognized by the 5C5 antibody was localized on the surface of non-permeabilized sperm. The protein was present on the surface of all epididymal, ejaculated, and capacitated sperm. The detected signal was strong in case of capacitated sperm (Fig. 2a"), while in epididymal (Fig. 2a) and ejaculated sperm (Fig. 2a') it was of medium strength. Additionally, we searched for the origin of this protein in reproductive tissues and fluids. The result is depicted in Fig. 2b, showing clearly that the protein is present only on capacitated sperm, with only the 24-kDa form present, with no signal on ejaculated sperm (Fig. 2b, lanes 1). The protein originates from epididymal fluid, where both 24- and 27-kDa forms are present (Fig. 2b, lane 4). The whole sperm proteome extracts (Fig. 2b, lanes 2) and extract from the kidney (Fig. 2b, lane 8) served as positive and negative controls, respectively. Extraction of proteins from ejaculated and capacitated sperm with SDS yielded both 24- and 27-kDa forms of the protein (Fig. 2b, lanes 2). The extraction conditions during isolation from the sperm surface allowed us to obtain only the 24-kDa form from the surface of capacitated sperm (Fig. 2b, lanes 1). Overall, this suggests that during the passage via epididymis the protein recognized by the 5C5 antibody is integrated from the epididymal fluid into the surface of the sperm. Further, we were unable to detect the protein by Western blot during ejaculation, when the proteins from seminal plasma are bound to the sperm surface and make the protein of interest inaccessible to biotinylation. During capacitation, when the bound proteins are released from the sperm surface, the protein was biotinylated and observed. However, the fact that only the 24-kDa form was obtained by the method for isolating proteins from the sperm surface indicates that the 27-kDa form is firmly anchored in the sperm plasma membrane.

We next investigated whether the protein recognized by the 5C5 antibody could bind ZP glycoproteins. As previously, we also used the far-western blot with ZP glycoproteins (Fig. 2c'), but with the whole sperm proteome because the protein was more abundant in these extracts, as evidenced by Fig. 2b. The interactions were compared with Western blot detections (Fig. 2c). The results show that the protein recognized by 5C5 antibody coincides in binding to *zona pellucida* of capacitated sperm (lane C) at the molecular mass of 24 kDa. The interaction is more apparent with capacitated sperm (Fig. 2c', lane C) than with ejaculated sperm (Fig. 2c', lane E), which again can be attributed to greater abundance of the protein in the capacitated fraction (Fig. 2b). We assume that the 27-kDa form also coincides in binding to *zona pellucida*, but the direct statement based on Fig 2c' is rather speculative. We were not able to clarify this even after multiple far-western blot assays, and we therefore concentrated only on the 24-kDa form. Prior to mass spectrometry analysis, the protein was immunoprecipitated for higher purity and concentration (Fig. 2d) and the immunoprecipitate was tested by Western blot (Fig. 2d'). After SDS-PAGE, the protein band was located under the light chain of the 5C5 antibody, which was further confirmed by Western blotting (Fig. 2d, d'), and the third signal of 50 kDa belonged to the heavy chain of the 5C5 antibody. The protein of interest was identified after in-gel proteolysis and following MS and MS/MS analysis of formed mixture of peptides using the Mascot Peptide Mass Fingerprint tool to be a Ras-related protein RAB-2A (gi|311253799 from *Sus scrofa*, with MW 24 kDa, pI 6.08), significant probability Mowse score 91 [protein scores greater than 76 are significant ($p<0.05$), sequence coverage 31 %, matched 5 from 7 searched *m/z* values]. The protein identification was confirmed using MS/MS Ion Search of acquired MS/MS spectra at *m/z* 1550.739 [probability Mowse score 103, scores greater than 45 are significant ($p<0.05$), mass error 12.8 ppm, identified sequence K.LQIWDTAGQESFR.S] and *m/z* 1785.894 [probability Mowse score 118, scores greater than 45 are significant ($p<0.05$), mass error 3.52 ppm, identified sequence R.FQPVHDLTIGVEFGAR.M], respectively.

RAB proteins belong to a subgroup of the Ras superfamily, whose four members were first identified in the brain (Touchot et al. 1987). Presently, the RAB family includes over 60 members in the human genome (Bock et al. 2001), thus being the largest branch of the Ras-related family of low-molecular-weight GTP-binding proteins. RAB proteins have been shown

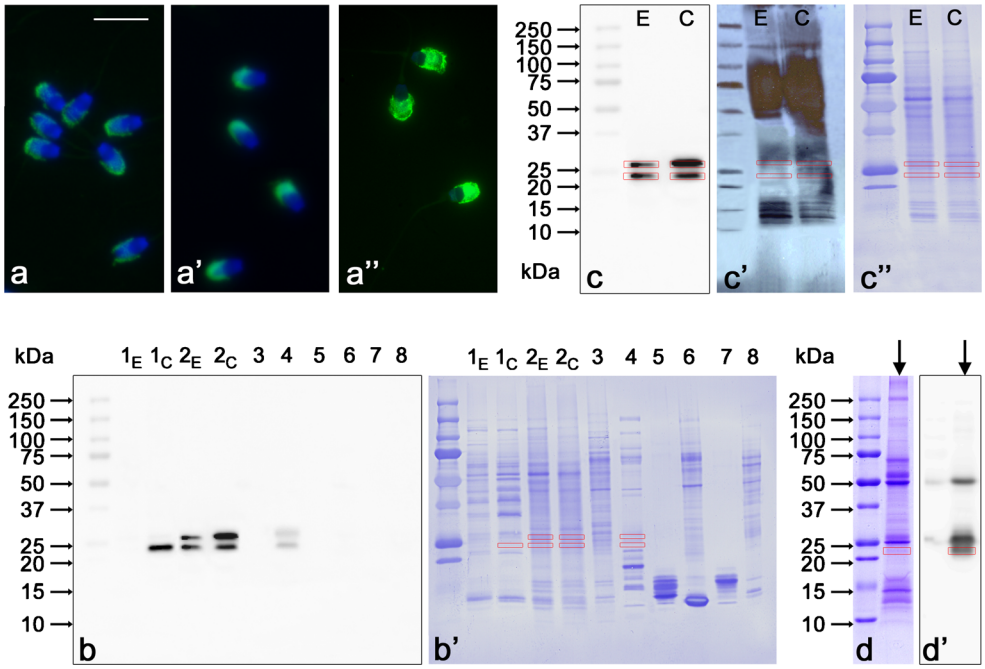

Fig. 2 Characterization of antibody termed 5C5 by immunofluorescence of epididymal (**a**), ejaculated (**a'**) and capacitated (**a"**) sperm; immunolocalization by Western blotting (**b**) in the sperm surface subproteome (*lanes 1*) from ejaculated (*with subscript E*) and capacitated (*with subscript C*) sperm, SDS extract (*lanes 2*) from ejaculated (*with subscript E*) and capacitated (*with subscript C*) sperm, extract from the testis (*lane 3*), epididymal fluid (*lane 4*), seminal vesicle fluid (*lane 5*), extract from the prostate (*lane 6*), seminal plasma (*lane 7*), and extract from the kidney (*lane 8*) followed by CBB staining (**b'**); comparison of Western blot detection (**c**) with the far-western blot using biotinylated *zona pellucida* glycoproteins (**c'**) of the sperm surface subproteome from ejaculated (*lane E*) and capacitated (*lane C*) sperm, respectively, followed by CBB staining (**c"**); and immunoprecipitation from capacitated sperm extract (**d**) confirmed by the Western blot (**d'**). All corresponding bands are indicated by *red rectangles*. The first lane in (**b**, **b'**, **c**, **c'**, **c"** and **d**, **d'**) represents molecular mass standards. *Bar* in immunofluorescence 10 μm

to play an essential role as regulators of vesicular transport pathways (Pereira-Leal and Seabra 2000, 2001). They are involved in many stages of vesicular transport including vesicle formation, actin- and tubulin-dependent vesicle movement, and targeting to and fusion with membranes (Stenmark and Olkkonen 2001), enabling them to accomplish a diverse set of functions by interacting with a multitude of effectors. When RABs are first produced, they are prenylated by the addition of one or two 20-carbon geranylgeranyl moieties to the protein's carboxyl terminus (Stenmark and Olkkonen 2001), which are used to anchor the RABs into membranes. Multiple targeting determining regions and factors contribute to the specificity and regulation of RAB recruitment and localization (Ali and Seabra 2005). RAB2 proteins are typically found between the cis-Golgi saccule and the endoplasmic reticulum (Stenmark and Olkkonen 2001). In this cytosolic location, they are normally involved in orchestrating both anterograde and retrograde transport between these two membrane compartments (Short et al. 2001; Cheung et al. 2002). However, recently, it was found that RAB-2A may also participate in events localized within the germ cell. Mountjoy et al. (2008) demonstrated that RAB-2A protein is involved in acrosomal biogenesis, where it regulates the transport and fusion of small secretory vesicles to the growing proacrosomic and acrosomic vesicles and ensures their fusion. After completion of acrosomal biogenesis, RAB-2A serves as a part of the perinuclear theca protein complex that binds the acrosome firmly to the nucleus, thus stabilizing the acrosome. Additionally, Mountjoy et al. (2008) also showed the difference in orientation or cellular polarity of the Golgi apparatus in the spermatid versus the somatic cell.

We found the RAB-2A protein on the surface of boar sperm. To our knowledge, we were the first to detect its presence in the pig germ cell. In contrast to Mountjoy et al. (2008), we detected the RAB-2A protein on the surface of the sperm plasma membrane. Immunofluorescent microscopy study revealed that RAB-2A was localized on the surface of all epididymal, ejaculated and capacitated sperm. Extraction of the whole sperm proteome showed that two forms of RAB-2A are present in the pig sperm, of 24 and 27 kDa. The presence of more than one RAB-2A form may be explained by hypervariability of the C-terminal domain, as shown in Chavrier et al. (1991). However, only the 24-kDa form was obtained from the surface subproteome, and from capacitated sperm only. We believe that the 27-kDa form, in contrast to the 24-kDa form, possesses additional geranylgeranyl moieties, as previously shown in RAB proteins (Stenmark and Olkkonen 2001), and therefore is more resistant to isolation

under mild conditions. As previously described, RAB-2A originates from the epididymal fluid. Considering that RAB proteins are commonly prenylated to be anchored in the membranes, the most probable way how RAB-2A is secreted into the epididymal lumen is through the membranous secretory vesicles—exosomes called epididymosomes, reviewed in Simpson et al. (2008). Epididymosomes are small membranous vesicles secreted in an apocrine manner in the intraluminal compartment of the epididymis, which also play a major role in the acquisition of new proteins by the maturing spermatozoa (Sullivan et al. 2007). Despite the association of RAB-2A protein with epididymosomes not yet having been proved, Girouard et al. (2011) found other RAB family proteins to be associated with these membranous vesicles in bull. Furthermore, Utleg et al. (2003) have shown that RAB-2 proteins in humans are associated with exosomes originating from the prostate, so-called prostasomes. The function of RAB-2A on the sperm surface still remains unsolved. According to Integrative Multi-species Prediction (Wong et al. 2012), there is 35 % probability that RAB-2A has a role in reproduction, as it was predicted that RAB-2A participates in gamete generation (Mountjoy et al. 2008) with the same probability. We have shown that RAB-2A, at least its 24-kDa form, coincides in binding to ZP. However, if RAB-2A can bind the ZP, it should be further clarified whether it binds as a primary ZP receptor or a primary binding-mediating molecule.

Antibody recognizing the protein of 35 and 45 kDa: 1H9

The monoclonal antibody termed 1H9 from the panel recognizing the protein of molecular mass ~35 and 45 kDa was further studied. Using indirect immunofluorescence, the protein recognized by the 1H9 antibody was localized on the surface of non-permeabilized sperm. The protein was present both on ejaculated (Fig. 3a') and capacitated sperm (Fig. 3a"), but the signal on the surface of epididymal sperm was absent (Fig. 3a). The signal was relatively strong both on ejaculated and capacitated sperm, and was shifted from the apical region of the ejaculated sperm to the postacrosomal region. We also searched for the origin of the protein recognized by the 1H9 antibody in the reproductive tissues and fluids. The result is depicted in Fig. 3b, which clearly shows that the protein is present on the surface of capacitated sperm (Fig. 3b, lanes 1), and its faint signal was detected on the surface of ejaculated sperm and also in the kidney. The whole sperm proteome extracts (Fig. 3b, lanes 2) and extract from the kidney (Fig. 3b, lane 8) served as positive and negative controls, respectively.

We next investigated whether the protein could bind ZP glycoproteins. Similarly, as in the case of 4C7 and 5C5 antibodies, we employed far-western blot assay of proteins isolated from the sperm surface with ZP glycoproteins (Fig. 3c'), and the interactions were compared with Western blot detections (Fig. 3c). The results clearly show that the protein recognized by the 1H9 antibody coincides in binding to *zona pellucida* at the molecular masses of both 35 and 45 kDa. The interaction is more apparent with capacitated sperm (Fig. 3c', lane C) rather than with ejaculated sperm (Fig. 3c', lane E), probably due to greater abundance of the protein isolated in the capacitated fraction. Prior to mass spectrometry analysis, the protein was immunoprecipitated for higher purity and concentration (Fig. 3d) and the immunoprecipitate was tested by Western blot (Fig. 3d'). After SDS-PAGE, the protein band was located between the heavy and light chains of the 1H9 antibody, which was further confirmed by Western blotting (Fig. 3d, d'). Both bands of the interesting protein were identified after in-gel trypsinization and following MS and MS/MS analysis of formed peptides using the Mascot Peptide Mass Fingerprint tool to be a sperm surface protein SP47 (gi|2851513 from *Sus scrofa*, with MW 46 kDa, pI 6.15). The protein band at the molecular weight 45 kDa revealed significant probability Mowse score 235 [protein scores greater than 76 are significant ($p<0.05$), sequence coverage 41 %, matched 14 from 16 searched *m/z* values]. The protein identification was confirmed in this protein band using MS/MS Ion Search of acquired MS/MS spectra at *m/z* 1704.922 [probability Mowse score 119, scores greater than 45 are significant ($p<0.05$), mass error 7.0 ppm, identified sequence K.VNLFEVPLEVQYVR.L] and *m/z* 1851.868 [probability Mowse score 101, scores greater than 43 are significant ($p<0.05$), mass error 8.3 ppm, identified sequence R.TWGLSAFSWYPFYAR.L], respectively. Similarly, the significant probability Mowse score 224 [protein scores greater than 76 are significant ($p<0.05$), sequence coverage 34 %, matched 12 from 12 searched *m/z* values] was obtained for the protein band at the molecular weight 35 kDa and for the verification of protein identification the MS/MS Ion Search of acquired MS/MS spectra was performed. The MS/MS signal at *m/z* 1537.728 revealed probability Mowse score 83 [scores greater than 44 are significant ($p<0.05$), mass error 6.0 ppm, identified sequence R.AGIVNAWTASNYDR.N] and *m/z* 1704.92 [corresponding probability Mowse score 100, scores greater than 45 are significant ($p<0.05$), mass error 7.0 ppm, identified sequence K.VNLFEVPLEVQYVR.L], respectively.

The identified protein was previously described by Ensslin et al. (1998) as a novel peripherally associated 47-kDa protein of pig spermatozoa, P47, isolated by affinity chromatography from solubilized sperm plasma membrane proteins bound to immobilized *zona pellucida* glycoproteins. The pig sperm protein is homologous to lactadherins, major components of the milk fat globule membrane of the mammary gland (formerly known as bovine PAS 6/7 or MGP 53/57 and mouse MFG-E8; Larocca et al. 1991; Aoki et al. 1995; for review and recommended nomenclature, see Mather 2000). We were able to detect the P47/SP47 protein by immunofluorescence microscopy on the apical ridge of ejaculated sperm, which is in agreement with the results of Ensslin et al. (1998) and Petrunkina et al. (2003). Further, Petrunkina et al. (2003) showed that, after

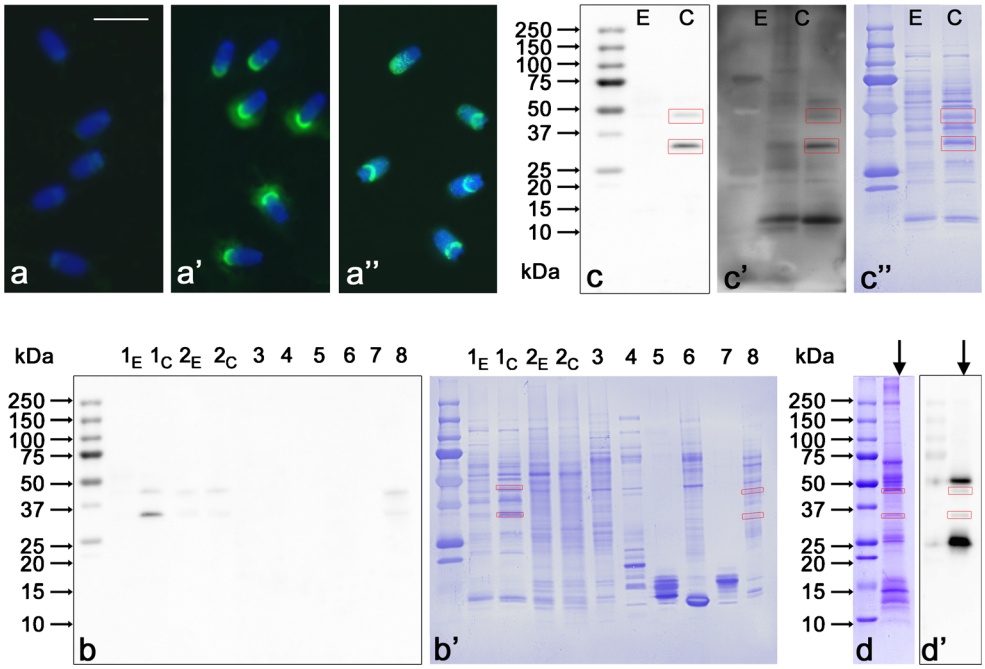

Fig. 3 Characterization of antibody termed 1H9 by immunofluorescence of epididymal (*a*), ejaculated (**a'**) and capacitated (**a"**) sperm; immunolocalization by Western blotting (**b**) in the sperm surface subproteome (*lanes 1*) from ejaculated (*with subscript E*) and capacitated (*with subscript C*) sperm, SDS extract (*lanes 2*) from ejaculated (*with subscript E*) and capacitated (*with subscript C*) sperm, extract from the testis (*lane 3*), epididymal fluid (*lane 4*), seminal vesicle fluid (*lane 5*), extract from the prostate (*lane 6*), seminal plasma (*lane 7*), and extract from the kidney (*lane 8*) followed by CBB staining (*b'*); comparison of Western blot detection (*c*) with the far-western blot using biotinylated *zona pellucida* glycoproteins (*c'*) of the sperm surface subproteome from ejaculated (*lane E*) and capacitated (*lane C*) sperm, respectively, followed by CBB staining (**c"**); and immunoprecipitation from capacitated sperm extract (**d**) confirmed by the Western blot (**d'**). All corresponding bands are indicated by *red rectangles*. The first lane in (**b**, **b'**, **c**, **c'**, **c"** and **d**, **d'**) represents molecular mass standards. *Bar* in immunofluorescence 10 μm

capacitation, the P47 signal shifted to the entire acrosomal distribution. Although in capacitated sperm we detected a small subpopulation of sperm having P47/SP47 unmasked, with resulting acrosomal appearance of the protein, the majority of the sperm displayed the fluorescence signal shifted to the postacrosomal region. In contrast to Ensslin et al. (1998), we were unable to obtain adequate fluorescent signals from epididymal sperm, most probably due to the inaccessibility of the epitope, as sperm were neither fixed nor permeabilized. We attempted to locate P47/SP47 in the reproductive organs and fluids, but due to relatively weak binding of the prepared antibody to the epitope, we were able to obtain a clear signal only with the capacitated sperm surface subproteome, as the isolation protocol for sperm surface proteins was modified to obtain enriched yields. In the ejaculated surface subproteome, only a faint signal was observed, which agrees with the results from immunofluorescence showing that only the protein located in the apical ridge was accessible for biotinylation. We also localized the presence of P47/SP47 in the kidney, in accordance with Ensslin et al. (1998), who observed that the protein is also expressed in non-reproductive organs such as muscle, heart, kidney, etc. Moreover, Ensslin et al. (1998) also showed that P47/SP47 is expressed in the following reproductive organs: uterus, cauda, corpus, caput epididymis, and testes. Furthermore, we detected two forms of P47/SP47: higher molecular mass form of 46 kDa (according to MASCOT software engine) and lower molar mass form of 35 kDa, not reported previously. Both forms were detected in ejaculated and capacitated sperm extracts. We believe that the 35-kDa form is either a truncated version of P47/SP47 or a processing product.

The function of lactadherin in association with spermatozoa still remains unclear. The possible function of boar membrane P47 as an integrin RGD-dependent ligand was suggested by Ensslin et al. (1998), previously indirectly supported by the studies of Andersen et al. (1997). Moreover, these investigators have recently shown that lactadherin can act as a link between two surfaces by binding to integrin receptors through its N-terminal RGD-binding sites in the second EGF-like domain and to phospholipids through its C-terminal C1/C2-like domains (Andersen et al. 2000). Taylor et al. (2000) demonstrated that human lactadherin (formerly BA46) expressed in human milk and breast carcinomas promotes RGD-dependent cell adhesion via integrins. Petrunkina et al. (2003) suggested that lactadherin is involved in other aspects of sperm physiology such as capacitation and acrosome reaction. However, further study is required to determine whether lactadherin epitopes are triggered or integrated in a further signal cascade priming the acrosome reaction and preparation of the sperm–egg fusion.

Fertilization in mammals is far from being completely understood, and recently new results have shown that one of the central dogmas of the fertilization process in mammals has become questioned. That is why it is necessary to continue with unrelenting efforts in order to move forward on the path of knowledge. This study dealt with an employment of monoclonal antibodies raised against the sperm surface proteins, localized on the apical tip of the sperm head plasma membrane, where the molecules for the ZP interaction are localized. We identified three proteins coincident with ZP binding: acrosin, RAB-2A and P47/SP47 lactadherin. Physiological function of P47 was proposed earlier. Due to the fact that acrosin stays adherent on the sperm plasma membrane, we propose an additional function, which is different from the secondary binding of sperm to oocyte. This is the first time to our knowledge that RAB-2A has been reported on the sperm surface, and the function of it remains undisclosed. The additional function of acrosin and the function of RAB-2A on the sperm surface are subjects for further studies.

Acknowledgments This work was supported by grants Nos. P503/12/1834 and P502/14/05547S of the Grant Agency of the Czech Republic, by Charles University in Prague (UNCE204025/2012), by the Institutional Research Project RVO61388971, and by the project BIOCEV CZ.1.05/1.1.00/02.0109 from the ERDF.

Conflict of interest The authors declare no conflicts of interest.

References

Adham IM, Nayernia K, Engel W (1997) Spermatozoa lacking acrosin protein show delayed fertilization. Mol Reprod Dev 46:370–376

Ali BR, Seabra MC (2005) Targeting of Rab GTPases to cellular membranes. Biochem Soc Trans 33:652–656

Andersen MH, Berglund JT, Rasmussen JT, Petersen TE (1997) Bovine PAS 6/7 binds $\alpha_v\beta_5$ integrin and ionic phospholipids through two domains. Biochemistry 36:5441–5446

Andersen MH, Graversen H, Fedosov SN, Petersen TE, Rasmussen JT (2000) Functional analyses of two cellular binding domains of bovine lactadherin. Biochemistry 39:6200–6206

Aoki N, Kishi M, Taniguchi Y, Adachi T, Nakamura R, Matsuda T (1995) Molecular cloning of glycoprotein antigens MGP 57/53 recognized by monoclonal antibodies raised against milk fat globule membrane. Biochim Biophys Acta 1245:385–391

Baba T, Azuma S, Kashiwabara S, Toyoda Y (1994) Sperm from mice carrying a targeted mutation of the acrosin gene can penetrate the oocyte zona pellucida and effect fertilization. J Biol Chem 269: 31845–31849

Baibakov B, Gauthier L, Talbot P, Rankin TL, Dean J (2007) Sperm binding to the zona pellucida is not sufficient to induce acrosome exocytosis. Development 134:933–943

Belleannee C, Belghazi M, Labas V, Teixeira-Gomes AP, Gatti JL, Dacheux JL, Dacheux F (2011) Purification and identification of sperm surface proteins and changes during epididymal maturation. Proteomics 11:1952–1964

Berger T, Horton MB (1988) Evaluation of assay conditions for the zona-free hamster ova bioassay of boar sperm fertility. Gamete Res 19: 101–111

Bock JB, Matern HT, Peden AA, Scheller RH (2001) A genomic perspective on membrane compartment organization. Nature 409:839–841

Boerke A, Tsai PS, Garcia-Gil N, Brewis IA, Gadella BM (2008) Capacitation-dependent reorganization of microdomains in the apical sperm head plasma membrane: functional relationship with zona binding and the zona-induced acrosome reaction. Theriogenology 70:1188–1196

Bongalhardo DC, Somnapan-Kakuda N, Buhr MM (2002) Isolation and unique composition of purified head plasma membrane from rooster sperm. Poult Sci 81:1877–1883

Canvin AT, Buhr MM (1989) Effect of temperature on the fluidity of boar sperm membranes. J Reprod Fertil 85:533–540

Chavrier P, Gorvel JP, Stelzer E, Simons K, Gruenberg J, Zerial M (1991) Hypervariable C-terminal domain of rab proteins acts as a targeting signal. Nature 353:769–772

Cheung AY, Chen CY, Glaven RH, de Graaf BH, Vidali L, Hepler PK, Wu HM (2002) Rab2 GTPase regulates vesicle trafficking between the endoplasmic reticulum and the Golgi bodies and is important to pollen tube growth. Plant Cell 14:945–962

Cross NL (2004) Reorganization of lipid rafts during capacitation of human sperm. Biol Reprod 71:1367–1373

Děd L, Dostálová P, Dorosh A, Dvořáková-Hortová K, Pěknicová J (2010) Effect of estrogens on boar sperm capacitation in vitro. Reprod Biol Endocrinol 8:87

Ensslin M, Calvete JJ, Thole HH, Sierralta WD, Adermann K, Sanz L, Töpfer-Petersen E (1995) Identification by affinity chromatography of boar sperm membrane-associated proteins bound to immobilized porcine zona pellucida. Mapping of the phosphorylethanolamine-binding region of spermadhesin AWN. Biol Chem Hoppe Seyler 376:733–738

Ensslin M, Vogel T, Calvete JJ, Thole HH, Schmidtke J, Matsuda T, Töpfer-Petersen E (1998) Molecular cloning and characterization of P47, a novel boar sperm-associated zona pellucida-binding protein homologous to a family of mammalian secretory proteins. Biol Reprod 58:1057–1064

Flesch FM, Voorhout WF, Colenbrander B, van Golde LM, Gadella BM (1998) Use of lectins to characterize plasma membrane preparations from boar spermatozoa: a novel technique for monitoring membrane purity and quantity. Biol Reprod 59:1530–1539

Gadella BM (2008) The assembly of a zona pellucida binding protein complex in sperm. Reprod Domest Anim 43:12–19

Gadella BM (2013) Dynamic regulation of sperm interactions with the zona pellucida prior to and after fertilisation. Reprod Fertil Dev 25:26–37

Gadella BM, Tsai PS, Boerke A, Brewis IA (2008) Sperm head membrane reorganisation during capacitation. Int J Dev Biol 52:473–480, Review

Girouard J, Frenette G, Sullivan R (2008) Seminal plasma proteins regulate the association of lipids and proteins within detergent-resistant membrane domains of bovine spermatozoa. Biol Reprod 78:921–931

Girouard J, Frenette G, Sullivan R (2011) Comparative proteome and lipid profiles of bovine epididymosomes collected in the intraluminal compartment of the caput and cauda epididymidis. Int J Androl 34(5 Pt 2):e475–e486

Harlow E, Lane D (1988) Antibodies: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor

Hasegawa A, Koyama K, Okazaki Y, Sugimoto M, Isojima S (1994) Amino acid sequence of a porcine zona pellucida glycoprotein ZP4 determined by peptide mapping and cDNA cloning. J Reprod Fertil 100:245–255

Hedrick JL, Wardrip NJ (1986) Isolation of the zona pellucida and purification of its glycoprotein families from pig oocytes. Anal Biochem 157:63–70

Hedrick JL, Wardrip NJ (1987) On the macromolecular composition of the zona pellucida from porcine oocytes. Dev Biol 121:478–488

Hokke CH, Damm JB, Penninkhof B, Aitken RJ, Kamerling JP, Vliegenthart JF (1994) Structure of the O-linked carbohydrate chains of porcine zona pellucida glycoproteins. Eur J Biochem 221:491–512

Honda A, Siruntawinwti J, Baba T (2002) Role of acrosomal matrix proteinases in sperm-zona pellucida interactions. Hum Reprod Update 5:405–412

Jin M, Fujiwara E, Kakiuchi Y, Okabe M, Satouh Y, Baba SA, Chiba K, Hirohashi N (2011) Most fertilizing mouse spermatozoa begin their acrosome reaction before contact with the zona pellucida during in vitro fertilization. Proc Natl Acad Sci U S A 108:4892–4896

Jonáková V, Kraus M, Veselský L, Čechová D, Bezouška K, Tichá M (1998) Spermadhesins of the AQN and AWN families, DQH sperm surface protein and HNK protein in the heparin-binding fraction of boar seminal plasma. J Reprod Fertil 114:25–34

Jones R, Williams RM (1990) Identification of zona- and fucoidan-binding proteins in guinea pig spermatozoa and mechanism of recognition. Development 109:41–50

Kudo K, Yonezawy N, Katsumata T, Aoki H, Nakano M (1998) Localization of carbohydrate chains of pig sperm ligand in the glycoprotein ZPB of egg zona pellucida. Eur J Biol 252:492–499

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685

Larocca D, Peterson JA, Urrea R, Kuniyoshi R, Bistrain AM, Ceriani RL (1991) A Mr 46,000 human milk fat globule protein that is highly expressed in human breast tumors contains factor VIII-like domains. Cancer Res 51:4994–4998

Maňásková P, Liberda J, Tichá M, Jonáková V (2000) Aggregated and monomeric forms of proteins in boar seminal plasma: characterization and binding properties. Folia Biol (Praha) 46:143–151

Maňásková P, Pěknicová J, Elzeinová F, Tichá M, Jonáková V (2007) Origin, localization and binding abilities of boar DQH sperm surface protein tested by specific monoclonal antibodies. J Reprod Immunol 74:103–113

Mather IH (2000) A review and proposed nomenclature for major proteins of the milk-fat globule membrane. J Dairy Sci 83:203–247

Mattioli M, Lucidi P, Barboni B (1998) Expanded cumuli induce acrosome reaction in boar sperm. Mol Reprod Dev 51:445–453

Melendrez CS, Meizel S, Berger T (1994) Comparison of the ability of progesterone and heat solubilized porcine zona pellucida to initiate the porcine sperm acrosome reaction in vitro. Mol Reprod Dev 39: 433–438

Mountjoy JR, Xu W, McLeod D, Hyndman D, Oko R (2008) RAB-2A: a major subacrosomal protein of bovine spermatozoa implicated in acrosomal biogenesis. Biol Reprod 79:223–232

O'Rand M, Welch JE, Fisher SJ (1986) Sperm membrane and zona pellucida interaction during fertilization. In: Dhindsa DS, Bahl O (eds) Molecular and cellular aspects of reproduction. Plenum, New York, pp 131–144

Pereira-Leal JB, Seabra MC (2000) The mammalian Rab family of small GTPases: definition of family and subfamily sequence motifs suggests a mechanism for functional specificity in the Ras superfamily. J Mol Biol 301:1077–1087

Pereira-Leal JB, Seabra MC (2001) Evolution of the Rab family of small GTP binding proteins. J Mol Biol 313:889–901

Petrunkina AM, Läkamp A, Gentzel M, Ekhlasi-Hundrieser M, Töpfer-Petersen E (2003) Fate of lactadherin P47 during post-testicular maturation and capacitation of boar spermatozoa. Reproduction 3: 377–387

Saling PM, Sowinski J, Storey BT (1979) An ultrastructural study of epididymal mouse spermatozoa binding to zonae pellucidae in vitro: sequential relationship to the acrosome reaction. J Exp Zool 209: 229–238

Serres C, Auer J, Petit F, Patrat C, Jouannet P (2008) Molecules involved in sperm-zona pellucida interaction in mammals. Role in human fertility. J Soc Biol 202:119–128

Short B, Preisinger C, Korner R, Kopajtich R, Byron O, Barr FA (2001) A GRASP55-rab2 effector complex linking Golgi structure to membrane traffic. J Cell Biol 155:877–883

Simpson RJ, Jensen SS, Lim JW (2008) Proteomic profiling of exosomes: current perspectives. Proteomics 8:4083–4099

Stenmark H, Olkkonen VM (2001) The Rab GTPase family. Genome Biol 2:reviews3007.1–reviews3007.7

Straus JW, Polakoski KL (1982) Association of proacrosin with phospholipid membranes. Biochem J 201:657–660

Straus JW, Parrish RF, Polakoski KL (1981) Boar acrosin. Association of an endogenous membrane proteinase with phospholipid membranes. J Biol Chem 256:5662–5668

Suarez SS (2008) Regulation of sperm storage and movement in the mammalian oviduct. Int J Dev Biol 52:455–462

Sulc M, Peslova K, Zabka M, Hajduch M, Havlicek V (2009) Biomarkers of aspergillus spores: strain typing and protein identification. Int J Mass Spectrom 280:162–168

Sullivan R, Frenette G, Girouard J (2007) Epididymosomes are involved in the acquisition of new sperm proteins during epididymal transit. Asian J Androl 9:483–491

Tanphaichitr N, Carmona E, Bou Khalil M, Xu H, Berger T, Gerton GL (2007) New insights into sperm-zona pellucida interaction: involvement of sperm lipid rafts. Front Biosci 12:1748–1766, Review

Taylor MR, Couto JR, Scallan CD, Ceriani RL, Peterson JA (2000) Lactadherin (formerly BA46), a membrane-associated glycoprotein expressed in human milk and breast carcinomas, promotes Arg-Gly-Asp (RGD)-dependent cell adhesion. DNA Cell Biol 16:861–869

Tesařík J, Drahorád J, Pěknicová J (1988) Subcellular immunochemical localization of acrosin in human spermatozoa during the acrosome reaction and zona pellucida penetration. Fertil Steril 50:133–141

Töpfer-Petersen E, Calvete JJ (1995) Molecular mechanisms of the interaction between sperm and the zona pellucida in mammals: studies on the pig. Int J Androl 18:20–26

Töpfer-Petersen E, Calvete JJ (1996) Sperm-associated protein candidates for primary zona pellucida-binding molecules: structure-function correlations of boar spermadhesins. J Reprod Fertil Suppl 50:55–61

Töpfer-Petersen E, Ekhlasi-Hundrieser M, Tsolova M (2008) Glycobiology of fertilization in the pig. Int J Dev Biol 52:717–736

Touchot N, Chardin P, Tavitian A (1987) Four additional members of the ras gene superfamily isolated by an oligonucleotide strategy: molecular cloning of YPT-related cDNAs from a rat brain library. Proc Natl Acad Sci U S A 84:8210–8214

Towbin H, Staehelin T, Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci U S A 76:4350–4354

Tsai PS, Garcia-Gil N, van Haeften T, Gadella BM (2010) How pig sperm prepares to fertilize: stable acrosome docking to the plasma membrane. PLoS ONE 5:e11204

Utleg AG, Yi EC, Xie T, Shannon P, White JT, Goodlett DR, Hood L, Lin B (2003) Proteomic analysis of human prostasomes. Prostate 56: 150–161

van Gestel RA, Brewis IA, Ashton PR, Helms JB, Brouwers JF, Gadella BM (2005) Capacitation-dependent concentration of lipid rafts in the apical ridge head area of porcine sperm cells. Mol Hum Reprod 11:583–590

van Gestel RA, Brewis IA, Ashton PR, Brouwers JF, Gadella BM (2007) Multiple proteins present in purified porcine sperm apical plasma membranes interact with the zona pellucida of the oocyte. Mol Hum Reprod 13:445–454

Wong AK, Park CY, Greene CS, Bongo LA, Guan Y, Troyanskaya OG (2012) IMP: a multi-species functional genomics portal for integration, visualization and prediction of protein functions and networks. Nucleic Acids Res 40:W484–W490

Yamagata K, Murayama K, Okabe M, Toshimori K, Nakanishi T, Kashiwabara S, Baba T (1998) Acrosin accelerates the dispersal of sperm acrosomal proteins during acrosome reaction. J Biol Chem 273:10470–10474

Yonezawa N, Amari S, Takahashi K, Ikeda K, Imai FL, Kanai S, Kikichi K, Nakano M (2005) Participation of the non-reducing terminal β-galactosyl residues of the neutral N-linked carbohydrate chains of porcine zona pellucida glycoproteins in sperm-egg binding. Mol Reprod Dev 70:222–227

Zhao Y, Zhang W, Kho Y, Zhao Y (2004) Proteomic analysis of integral plasma membrane proteins. Anal Chem 76:1817–1823

Zigo M, Jonáková V, Maňásková-Postlerová P (2011) Electrophoretic and zymographic characterization of proteins isolated by various extraction methods from ejaculated and capacitated boar sperms. Electrophoresis 32:1309–1318

Zigo M, Jonáková V, Šulc M, Maňásková-Postlerová P (2013) Characterization of sperm surface protein patterns of ejaculated and capacitated boar sperm, with the detection of ZP binding candidates. Int J Biol Macromol 61:322–328