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INVITED RESEARCH HIGHLIGHT

Sperm Biology

Remodeling of the plasma membrane in preparation for sperm–egg recognition: roles of acrosomal proteins

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The interaction of sperm with the egg's extracellular matrix, the zona pellucida (ZP) is the first step of the union between male and female gametes. The molecular mechanisms of this process have been studied for the past six decades with the results obtained being both interesting and confusing. In this article, we describe our recent work, which attempts to address two lines of questions from previous studies. First, because there are numerous ZP binding proteins reported by various researchers, how do these proteins act together in sperm–ZP interaction? Second, why do a number of acrosomal proteins have ZP affinity? Are they involved mainly in the initial sperm–ZP binding or rather in anchoring acrosome reacting/reacted spermatozoa to the ZP? Our studies reveal that a number of ZP binding proteins and chaperones, extracted from the anterior sperm head plasma membrane, coexist as high molecular weight (HMW) complexes, and that these complexes in capacitated spermatozoa have preferential ability to bind to the ZP. Zonadhesin (ZAN), known as an acrosomal protein with ZP affinity, is one of these proteins in the HMW complexes. Immunoprecipitation indicates that ZAN interacts with other acrosomal proteins, proacrosin/acrosin and sp32 (ACRBP), also present in the HMW complexes. Immunodetection of ZAN and proacrosin/acrosin on spermatozoa further indicates that both proteins traffic to the sperm head surface during capacitation where the sperm acrosomal matrix is still intact, and therefore they are likely involved in the initial sperm–ZP binding step.

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SPERM CAPACITATION AND SPERM-ZONA PELLUCIDA INTERACTION– BACKGROUND AND CONFUSION IN THE FIELDS

Sperm capacitation was first described by Chang to be a physiological process occurring in the female reproductive tract whereby spermatozoa gain fertilizing ability.[1,2] Subsequent studies indicate that capacitation can be induced *in vitro* simply by incubating spermatozoa in a medium containing albumin, calcium and bicarbonate.[3–7] The procedures of *in vitro* capacitation and egg culture were then combined to establish the *in vitro* fertilization process, which is now used routinely as part of assisted reproductive technology.[8] On the research side, the ability to induce sperm capacitation *in vitro* has also accelerated studies on the molecular mechanisms of the process. Hyperactivated motility patterns are now known as signature movements of capacitated (Cap) sperm. Increases in sperm tyrosine phosphorylation are other emblems of capacitation-associated signaling events.[9]

Remodeling of the molecular components on the sperm surface is another capacitation-associated event that has unfolded from research from the past few decades. Albumin and high-density lipoproteins present in the female reproductive tract or medium induce the release of cholesterol from the sperm surface during capacitation, thus leading to an enhancement in sperm membrane fluidity.[10,11] This can be detected by a fluorescent dye, merocyanine, which intercalates into the disorganized membrane domains.[7,12,13] This increase in membrane fluidity prepares Cap sperm for the downstream membrane fusion events that are essential for fertilizing ability, that is, the acrosome reaction and sperm–egg plasma membrane fusion.[9]

The ability to culture spermatozoa and eggs *in vitro* has also allowed researchers to identify a number of proteins that are involved in sperm–zona pellucida (ZP) interaction. With success in the purification of the three mouse (m) ZP glycoproteins to homogeneity, Florman and Wassarman confirmed that the mZP3 glycoprotein was a primary receptor binding to acrosome intact sperm, whereas mZP2 was a secondary receptor engaging in adhering acrosome reacted sperm to the ZP.[14] This concept was later questioned by Gerton *et al.* who showed that acrosomal exocytosis occurs in a gradual manner[15–17] and that both

mZP2 and mZP3 engage in the interaction with sperm undergoing acrosomal exocytosis. Recent work of Dean *et al.* further indicated that mZP2 cannot be excluded from the binding of acrosome-intact sperm.[18,19] The assumption that mZP3 binds only to acrosome intact spermatozoa has also been recently challenged by Hirohashi's research group, who showed using high-performance videomicroscopy that acrosomal exocytosis has already initiated by the time that sperm have moved through the cumulus cell layers.[20] In other words, spermatozoa that bind to the ZP do not have their acrosome completely intact. Regardless of this confusion, one finding that still holds true is that ZP glycoproteins are endowed with large carbohydrate moieties and that ZP glycans are important in the initial binding of the ZP to spermatozoa.[9]

On the sperm side, the membrane β-1,4-galactosyltransferase (GalT) is one of the early proteins described by Shur and Hall for its affinity for the ZP and its involvement in sperm–ZP binding was described through a series of *in vitro* experiments.[21–24] Shur *et al.* have hypothesized that GalT is engaged in a "dead-end" reaction. Normally, GalT transfers a galactose from a galactose donor (UDP-Gal) to *N*-acetylglucosamine (GlcNAc) to form a Gal-GlcNAc conjugate. In the female reproductive tract, Shur and Hall have suggested that UDP-Gal was not present and, therefore, the binding of sperm GalT to its substrate GlcNAc on the ZP glycans forms a basis of sperm–ZP interaction without yielding a product.[21,22] The same "dead-end reaction" concept can be applied to a number of sperm surface glyco-enzymes with ZP affinity: namely that they bind to their substrate, which contains a sugar residue present on the ZP glycans. These enzymes include α-D-mannosidase,[25,26] PH-20 (aka SPAM1),[27,28] arylsulfatase A (ARSA, with galactose sulfate as one of its substrates).[29,30] Considering that these glycol-enzymes do not complete their reaction at the time of sperm–ZP binding, they can be considered as "lectins." However, it is possible that the reaction of these enzymes is eventually completed, so that sperm can leave the original binding site and move to the next one, as part of their forward movement through the ZP.

Besides the glycol-enzymes, there exists another set of sperm surface proteins with a direct lectin property. These include proacrosin/acrosin (ACRO),[31,32] sp56 (aka ZP3R),[33] sp38 (aka IAM38, ZP binding protein1 [ZPBP1]),[34–36] zonadhesin (ZAN),[37,38] sp17[39] and spermadhesins (including AQN, AWN).[40–42] In addition, ZP binding proteins on the sperm surface without known information for their ability to interact with the carbohydrate moieties of the ZP have been described, including SED1 (aka MFGM),[43] ZP binding protein2 (ZPBP2),[44] glutathione-S-transferase;[45] ADAM3,[46] carbonyl reductase,[47] basigin,[48] SP10[49] and FA-1.[50,51] Sulfogalactosylglycerolipid (SGG, aka seminolipid)[52,53] is another sperm surface molecule (not a protein) that has affinity for the ZP and is involved in sperm–ZP binding. Of note is the acrosomal location of a number of ZP binding proteins: that is, proacrosin/acrosin, ZAN, ZPBP1, ZPBP2, SP10, and sp56,[35,36,38,44,54–61] and this finding reinforces the concept that these ZP binding proteins are involved in the binding of the reacting acrosome to the ZP. However, before the results described by Jin *et al.*[20] it was thought that the acrosomal exocytosis occurred on the ZP and that the binding of these acrosomal proteins to the ZP occurred after the initial binding of ZP-associated proteins on the sperm head surface. As described below, this concept is now challenged by our recent results.

WHY ARE THERE SO MANY ZONA PELLUCIDA BINDING PROTEINS?

The existence of so many sperm proteins with ZP affinity requires an explanation. One possible explanation is that information derived from the *in vitro* sperm–ZP binding assay does not accurately represent situations *in vivo*. With the rapidly advancing technology of targeted gene deletion (see http://www.genome.gov/12514551), colonies of "knockout" (KO) mice lacking individual genes encoding ZP binding proteins have been produced and fecundity of these male mice was assessed by various measurements that is, their ability to sire offspring, their sperm parameters (sperm number, motility, morphology and *in vitro* fertilizing ability), and their libido and ability to copulate with females.[62] Surprisingly and interestingly, a number of KO mice including *Gal*$^{-/-}$, *Zp3r*$^{-/-}$, *Zan*$^{-/-}$, *SED1*$^{-/-}$, *Acr*$^{-/-}$, *Zpbp2*$^{-/-}$, *Spam1*$^{-/-}$ and *Arsa*$^{-/-}$ mice can sire offspring,[43,44,63–69] although evidence of subfertility is noted in a number of these mouse colonies.[43,44,69,70] In contrast, *Adam3*$^{-/-}$ and *Zpbp1*$^{-/-}$ mice, which still produce spermatozoa, are infertile.[44,46,71,72] Of note, ADAM3 also functions in sperm–egg plasma membrane binding.[72] While spermatozoa from *Adam3*$^{-/-}$ mice lack ADAM3, as expected, they also possess an aberrant amount of proteins that are important for sperm–egg plasma membrane binding on their surface (i.e. no ADAM1b and a lower amount of ADAM2).[72] In addition, spermatozoa from *Adam3*$^{-/-}$ mice are severely defective in their movement through the uterotubal junction.[71] All of these observations indicate the multi-functional roles of ADAM3 in fertilization and it is therefore not surprising that *Adam3*$^{-/-}$ male mice are infertile. In the same vein, spermatozoa from *Zpbp1*$^{-/-}$ mice have grossly abnormal heads (typical of the so-called "globospermia" morphology), suggesting that ZPBP1 is involved in the formation of the sperm acrosome.[44] The infertility status of *Zpbp1*$^{-/-}$ mice is, therefore, to be expected.

Explanations are required for the existence of so many sperm proteins with ZP affinity in the first category, the deletion of whose genes still produces fertile male mice (see above). The results from these KO mouse studies indicate that these proteins are not essential for fecundity, although their relevance in the ZP binding process cannot be denied (especially when a number of these KO male mice are subfertile). Because fertilization is the fundamental process needed to sustain the continuation of a species, a number of ZP binding proteins/molecules may be required to safeguard this. The redundancy of their functions would allow them to back up for one another. Alternatively, they might act together in a synergistic and/or sequential manner, although the disappearance of one specific molecule does not annul the sperm–ZP binding process.[73–75]

EXISTENCE OF ZONA PELLUCIDA BINDING PROTEINS/MOLECULES IN SPERM LIPID RAFTS AND HIGH MOLECULAR WEIGHT COMPLEXES

The interpretation of the presence of many ZP binding proteins/molecules on sperm is consistent with the concept of the existence of lipid rafts, nanoscale liquid-ordered sterol-containing membrane microdomains that are platforms of cell adhesion and signaling molecules.[73,76–78] The method to isolate lipid rafts as detergent resistant membranes (DRMs)[79] has further accelerated the characterizations of their molecular components, results of which have supported the stated concept. Since sperm-egg interaction is fundamentally engaging cell adhesion and signaling processes, a number of investigators in the gamete field, including us, started to characterize sperm lipid rafts. So far, publications on the sperm lipid rafts topic have come out from at least 17 labs,[78] starting with the article from Kitajima's lab[80] describing the ability of sea urchin sperm DRMs to bind to a sperm binding protein on the egg. Likewise, we have shown that pig and mouse sperm DRMs have affinity for homologous ZP glycoproteins and the intact ZP, respectively.[81,82] Our results showing a higher amount and enhanced ZP binding ability of DRMs isolated from Cap sperm as compared with those from noncapacitated (Noncap)

spermatozoa further support the concept that sperm lipid rafts are the ZP interaction domains on the sperm surface.[81] Our lipidomic characterization further indicated that SGG is an integral component of sperm DRM: it plays an important role in the formation of sperm lipid rafts as well as endowing their ZP binding ability. Proteomic analyses further revealed the presence of a number of ZP binding proteins (SED1, GalT, α-D-mannosidase, SPAM1, ARSA, spermadhesins, basigin, proacrosin, SP10) as well as their associated partners (e.g, sp32 or ACRBP) as molecular components of sperm DRMs.[82–84] Significantly, these findings imply that these ZP binding proteins have to be escorted into the lipid raft domains. Therefore, it is not surprising that several chaperone proteins are also present in isolated sperm DRMs including a series of heat shock proteins (e.g, Hsp60 (chaperonin), HspA5, and Hsp90, Hsp90b1 (endoplasmin)), calnexin, protein disulfide isomerase associated 3 and protein disulfide isomerase associated 6.[82] The co-existence of ZP binding proteins and chaperone proteins in isolated sperm lipid rafts suggests that they must be in close proximity and might be associated with one another forming high molecular weight (HMW) complexes within the lipid raft microdomains. This postulate was indeed verified by Dun *et al*. Blue native gel electrophoresis of proteins extracted from whole sperm with a gentle nonionic detergent reveals the presence of HMW complexes, which have affinity for solubilized ZP glycoproteins. In mouse spermatozoa, ZPBP2, ZPBP1, and ZP3R and a series of chaperone proteins, chaperonin-containing TCP complex (CCT/TRiC) are molecular constituents of a 820 kDa HMW complex.[85] A similar type of HMW protein complex (~900 kDa) is also present in human spermatozoa, although ZPBP2 is the only ZP binding protein constituent.[86] A smaller HMW complex (200 kDa) is also present in human sperm comprising two ZP binding proteins, ARSA and SPAM1, and a chaperone protein, HspA2.[87] Of note, only a few ZP binding proteins are found in the sperm HMW complexes with ZP affinity. It is possible that proteins extracted from other regions of spermatozoa besides the anterior head (site of ZP binding) may have diluted out HMW complexes that are directly involved in ZP interaction: this dilution would make it difficult technically to detect these relevant complexes.

PROTEOMIC CHARACTERIZATION OF SPERM ANTERIOR HEAD PLASMA MEMBRANE VESICLES – EXISTENCE OF HIGH MOLECULAR WEIGHT COMPLEXES WITH ZONA PELLUCIDA AFFINITY

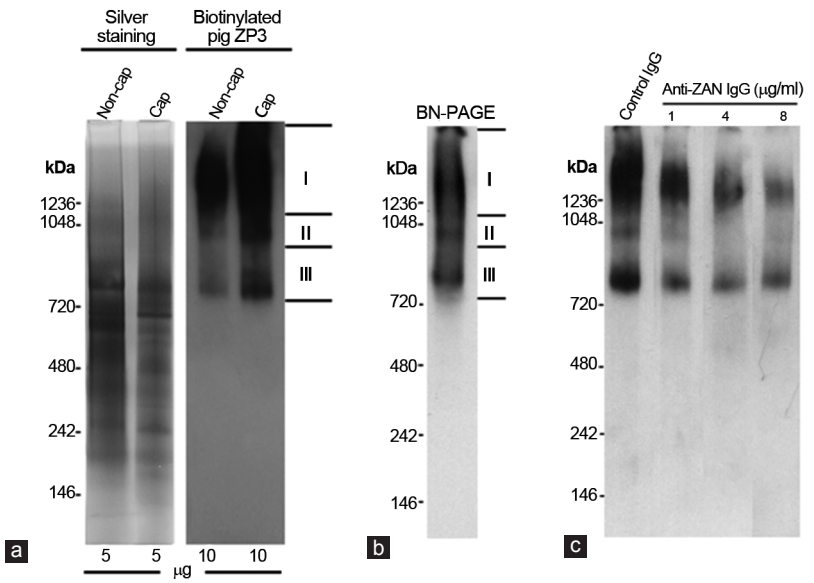
Toward the end of the 2000's, concerns over the use of detergents to isolate lipid rafts were voiced strongly; DRMs can be artifacts from protein aggregation induced by this treatment.[88] Isolation of lipid rafts by physical force such as nitrogen cavitation was suggested.[89] In fact, in the sperm biology field, nitrogen cavitation at 650 psi has been used since the 1980's to specifically prepare vesicles from the pig sperm anterior head plasma membrane (APM), the site of ZP binding.[90,91] Isolated APM vesicles from Cap pig sperm have a direct ability to bind to the homologous ZP glycoproteins with the same K_d value as that measured for Cap sperm DRMs for the parallel ZP interaction.[81] Therefore, we started our proteomic characterization of APM vesicles isolated from Noncap and Cap sperm, with the hope of gaining an insight to the identities of proteins that are relevant in capacitation. Our recent results indicate that the amount of APM vesicles isolated from Cap sperm increased to 160% compared with that from Noncap sperm, and liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS) also indicated an increase in the protein numbers in Cap APM vesicles (127 *vs* 81 in Noncap APM vesicles, with 59 proteins found in common between Noncap and Cap vesicles). Significantly, a number of proteins involved in sperm–egg interaction and two chaperone proteins (heat shock 70 kDa protein 1-like and heat shock protein Hsp90-alpha) rank the highest in their spectral counts among all proteins identified by LC–MS/MS (**Table 1**). The presence of ZP binding proteins in APM vesicles corroborates their known ZP binding properties[81,92] and the increase in the amounts of isolated APM vesicles in Cap sperm further explains the higher capacity of these sperm (compared with Noncap counterparts) for ZP interaction.[93]

Blue native gel electrophoresis further revealed the presence of HMW protein complexes (>200 kDa) in APM vesicles from Noncap and Cap sperm (**Figure 1a, left panel**), but HMW complexes sized 1000–1300 kDa (named Complex I), 850–1000 kDa (Complex II) and 750–850 kDa (Complex III) from Cap sperm had a significantly higher capacity to bind to pig ZP3 glycoproteins (hetero-oligomers of pig ZP3α and pig ZP3β; sperm receptor;

Table 1: LC–MS/MS analyses of Noncap and Cap APM vesicle proteins[a,b]

Protein name/function category[c]	*Swiss-Prot accession*	*Average spectral count*[d]		*Fold difference (Cap/Noncap)*	*Average percentage sequence coverage*[e]	
		Noncap[c]	*Cap*		*Noncap*	*Cap*
Sperm–egg interactions						
Milk fat globule-EGF factor 8 (SED1)	MFGM	66.91	105.78	1.58	48.80	56.10
Carbohydrate-binding protein AQN-3	AQN3	12.03	5.69	0.47	24.47	33.07
Carbohydrate-binding protein AWN	AWN	8.35	6.39	0.77	32.57	51.37
Proacrosin/acrosin	ACRO	7.94	8.68	1.09	11.50	20.80
Angiotensin-converting enzyme	ACE	3.55	4.77	1.34	1.93	4.33
Proacrosin-binding protein precursor (sp32)	ACRBP	2.36	8.49	3.59	3.23	13.10
ZPBP1 (sp38)	ZPBP1	1.17	1.60	1.36	3.50	5.70
ZAN	ZAN	0.00	8.14	∞	0.00	6.20
Chaperones						
Heat shock 70 kDa protein 1-like	HS71L	10.81	4.81	0.44	12.13	12.87
Hsp90-alpha (Hsp86)	HS90A	4.01	4.17	1.04	5.87	10.00

[a]An equal amount (50 μg) of proteins extracted from APM vesicles of Noncap and Cap sperm from each of the three pigs was used for MS analyses; [b]Only highly abundant APM proteins, showing at least five spectral counts in one of the three replicates, are listed; [c]The APM proteins identified are grouped based on their known biological functions and individual proteins in each group were ranked from the highest to the lowest abundance in Noncap samples using normalized spectral counts as relative indexes; [d]Average normalized spectral counts for proteins identified in Noncap and Cap APM samples from three animals. All data from individual pigs are in Kongmanas *et al*.[93]; [e]Average percentage of amino acid sequence coverage for each identified protein. Cap: capacitated; Noncap: noncapacitated; LC–MS/MS: liquid chromatography coupled with tandem mass spectrometry; APM: anterior head plasma membrane; EGF: epidermal growth factor; Hsp: heat shock protein; ZAN: zonadhesin; ZPBP1: zona pellucida binding protein1



d

#	Protein name/Function Category	Swiss-Prot Accession	Total spectral counts of identified peptides in HMW protein complexes* Complex I Non-cap	Complex I Cap	Complex II Non-cap	Complex II Cap	Complex III Non-cap	Complex III Cap
	Sperm-egg interactions							
1	Zonadhesin	ZAN	83	137	7	12	2	18
2	Milk fat globule-EGF factor 8 (SED1)	MFGM	7	15	6	4	15	12
3	Proacrosin/acrosin	ACRO	4	17	3	4	10	3
4	Proacrosin-binding protein precursor (sp32)	ACRBP	5	17	1	2	3	1
5	Sperm acrosomal protein (SP-10)	ASPX	3	6	0	2	0	4
6	Carbohydrate-binding protein AWN	AWN	5	3	3	1	4	0
7	Zona pellucida-binding protein 1 (Sp38)	ZPBP1	0	4	0	0	0	0

*The number of spectral counts in each column is the sum of peptides of each protein in each HMW protein complexes
Data shown were representative of two replicate experiments.

Figure 1: (**a**) Left panel: Presence of HMW complexes in pig APM vesicles as shown by blue native gel electrophoresis/silver staining; Right panel: Far western blotting showing the binding of Complex I (1000-1300 kDa), Complex II (850-1000 kDa) and Complex III (750-850 kDa) to biotinylated pig ZP3 (sperm receptor). (**b**) Immunoblotting of Cap sperm APM proteins separated by blue native gel electrophoresis, showing zonadhesin bands in the three Complexes with patterns similar to the far western bands of ZP3 binding. (**c**) Preincubation of APM Complexes with anti-zonadhesin (anti-ZAN) IgG inhibits the Complex binding to ZP3 in a dose dependent manner. (**d**) Identity and spectral counts of proteins in the three HMW Complexes. For experimental details of results described throughout this article, see Kongmanas *et al*.[93]. All figures shown in this review are also adapted from this article.

Figure 1a, right panel). As expected, LC–MS/MS revealed that proteins known for their affinity for the ZP scored the highest for the spectral counts in all three complexes and the amounts of most of these proteins were higher in Cap HMW complexes (**Figure 1d**). Interestingly, ZAN had the highest spectral counts in the three complexes. This finding was in contrast to the LC–MS/MS results of the whole APM vesicle extracts where SED1 scored the highest in spectral counts and ZAN the lowest in the protein category of sperm–egg interaction (**Table 1**). The presence of ZAN in Complexes I, II and III was confirmed by immunoblotting (**Figure 1b**). The anti-ZAN reactive bands in the three complexes corresponded to the pig ZP3 binding patterns (**Figure 1a**). Significantly, ZAN contributed to the ZP affinity of the three complexes. Preincubation of the complexes with various concentrations of anti-ZAN IgG inhibited their binding to pig ZP3 in a dose-dependent manner (**Figure 1c**).

Chaperones are also present in Complexes I–III, including various subunits of T-complex protein 1 (aka CCT/TRiC).[93] As suggested earlier by Dun *et al*.[85–87] these chaperones might escort the ZP binding proteins to come together to form HMW complexes.

ZONADHESIN AND SELECTIVE ACROSOMAL PROTEINS PLAY ROLES IN SPERM CAPACITATION

Besides ZAN, proacrosin, ACRBP, SP10 and ZPBP1 are ZP binding proteins present in the three HMW complexes (**Figure 1d**). All of these proteins are known to localize in the acrosome. Previous studies indicated that vesicles of hybrid membranes (APM and outer acrosomal membrane) existed in the vesicle preparation from pig sperm subjected to nitrogen cavitation at 650 psi (called APM vesicles in this review).[94] ZAN was localized to the outer acrosomal membrane and acrosomal matrix.[38,57] Therefore, its existence as revealed by LC–MS/MS in the HMW complexes might reflect these previous findings. The question relevant to the physiology of sperm–ZP interaction, however, remains: are acrosomal proteins present in the APM HMW complexes exposed on the sperm head surface, so that they can bind to the ZP? Our immunofluorescence and flow cytometry of ZAN on intact pig sperm indeed revealed that ZAN was not present on the head surface in the majority of spermatozoa resuspended in medium that did not support capacitation. However, the percentage of sperm that were positively labeled with anti-ZAN increased when sperm were incubated in capacitating medium (containing albumin, bicarbonate, and $CaCl_2$) for 30 min (**Figure 2a**). In addition, the immunofluorescence intensity of ZAN increased in these spermatozoa. Both the percentage of anti-ZAN labeled sperm and the immunofluorescence intensity peaked at 60 min incubation in capacitating medium (**Figure 2b**). However, most spermatozoa (≥80%) were still acrosome-intact as shown by the binding of FITC-labeled *Pisum sativum agglutinin* to their acrosomal matrix (**Figure 2b**). Corroborating this result is the observation that ZAN remained in the acrosome of nitrogen-cavitated spermatozoa with a much higher level of immunofluorescence intensity than that present on the head surface of the corresponding Cap acrosome-intact sperm (**Figure 2c**). All of these results indicate that a fraction of ZAN is transported from the acrosome to the sperm head surface during capacitation. Immunofluorescence and flow cytometry of proacrosin/acrosin show the same trend as ZAN in terms of their transport to the sperm head surface.[93] However, the transportation of ACRBP (sp32) to the sperm head surface appeared to be much earlier than that of ZAN and proacrosin/acrosin. ACRBP was present on the head surface of almost all Noncap spermatozoa and this distribution remained the same when sperm were incubated in capacitating medium, although the intensity of the immunofluorescence increased slightly. ACRBP possesses a specific affinity to proacrosin (53 kDa) but not to the intermediate and mature forms of

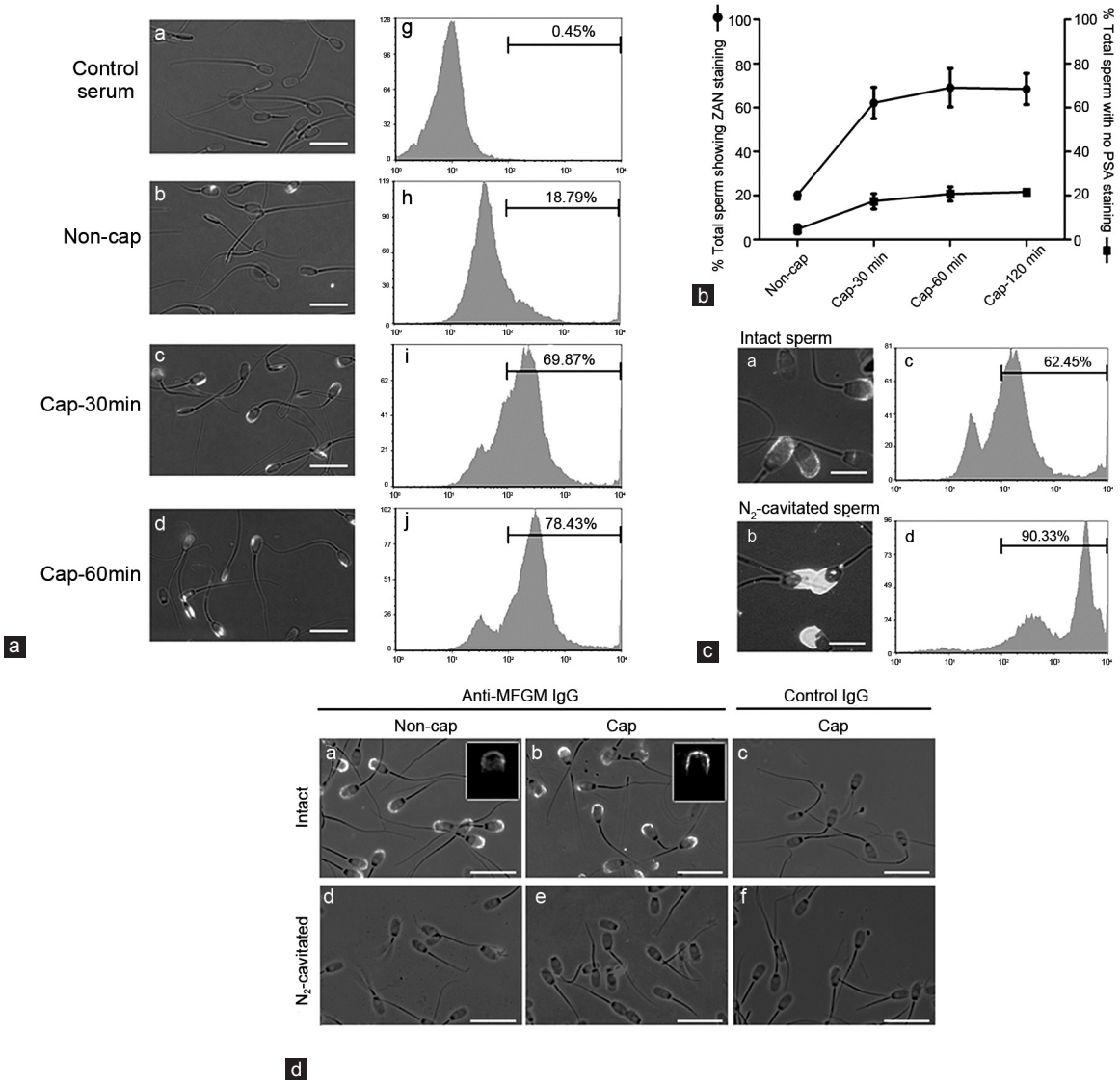


Figure 2: (**a and b**) Zan targets to the pig sperm head surface during incubation in capacitating medium. (**a**) Merged immunofluorescence (panels a–d) and phase contrast (panels g–j) images are shown in the left column, whereas the corresponding flow cytometry histograms are shown on the right. Spermatozoa were incubated in capacitating medium for 30 and 60 min. (**b**) Kinetics of the numbers of sperm with ZAN on the head surface is shown as the function of capacitation time, together with the population of acrosome reacted sperm (negatively stained with PSA). (**c**) Immunofluorescence and flow cytometry indicating that only a fraction of ZAN was targeted to the sperm surface and that most of the protein remained in the acrosome of nitrogen cavitated sperm. (**d**) Immunofluorescence of SED1 (MFGM) on spermatozoa before and after nitrogen cavitation. Results indicate the same fluorescence pattern/intensity of SED1 in Noncap and Cap sperm and the absence of the protein in the acrosome of nitrogen-cavitated gametes. Zan: zonadhesin; Noncap: noncapacitated; Cap: capacitated; PSA: pisum sativum agglutinin.

acrosin (43 and 35 kDa, respectively). All of these proacrosin/acrosin forms are present in Noncap and Cap spermatozoa. Therefore, the overall results suggest that ACRBP targets to the head surface of Noncap sperm independently of proacrosin. While the transport of ZAN and proacrosin (both with known ZP affinity) to the APM region of Cap sperm is likely beneficial for interaction with the ZP, the benefit of having ACRBP is still a matter of investigation. To date, direct affinity of ACRBP for the ZP has not been demonstrated.

As with ACRBP, the presence of SED1 before and after capacitation was of similar pattern and intensity (**Figure 2d**). This result is not surprising, because SED1 is secreted by epididymal epithelial cells into the lumen and is deposited onto the sperm head surface during passage through the epididymis.[43] The absence of SED1 in the acrosome of nitrogen cavitated sperm supports the idea that SED1 is acquired externally during epididymal maturation. The ZP affinity of SED1 would contribute to the baseline ZP interactions by HMW complexes of Noncap sperm (**Figure 1a**).

Zonadhesin is a mosaic protein comprising a number of cell adhesion-related domains (MAM, mucin, and von Willebrand factor D [VWF D]) (**Figure 3a**). It is synthesized in spermatids as a precursor protein and then processed to mature forms p45 and p105, present in mature spermatozoa.[37] Both p45 and p105 still contain VWF D domains: VWF D1 + D2 in p45 and VWF D2 + D3 + D4 in p105. These VWF D domains are likely the basis

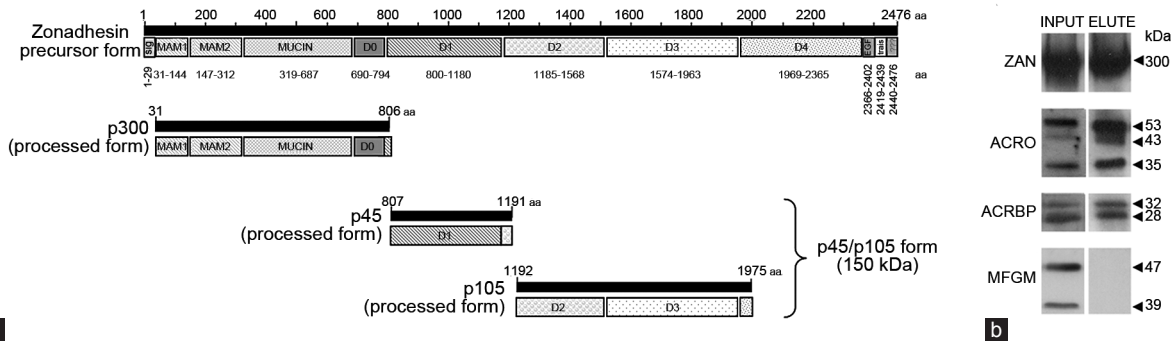


Figure 3: (**a**) Structural domains of ZAN (adapted from Bi *et al*.[38] and Herlyn and Zischler.[98] (**b**) Immunoprecipitation of APM proteins with anti-ZAN IgG captured on Protein G paramagnetic beads[93]. Input = whole APM protein extracts; Elute = APM proteins bound to anti-ZAN beads. Results indicate interaction among ZAN, proacrosin/acrosin (ACRO) and ACRBP but not SED1 (MFGM). ZAN: zonadhesin; APM: anterior head plasma membrane.

for the ZP affinity of p45 + p105, as well as their multimerization and interactions with other proteins.[37,38] Even on SDS–PAGE, HMW forms of *Zan* (300–500 kDa and higher) are present along with p45 and p105 mature forms,[37] a result that corroborates the presence of ZAN using blue native gel electrophoresis (**Figure 1**). With this molecular adhesion property, ZAN might interact with other acrosomal proteins, scaffolding them as HMW complexes for transport to the sperm APM. In fact, our immunoprecipitation results using anti-ZAN IgG captured on paramagnetic beads[93] indicated that ZAN interacts with proacrosin/acrosin and ACRBP in the APM extracts (**Figure 3**). However, it is still unclear whether ZAN interacts directly with ACRBP or through the association of ACRBP with proacrosin. On the other hand, ZAN does not interact with SED1 (**Figure 3**). This result is not surprising considering that ZAN and SED1 on the sperm surface originate from different sources: ZAN from the acrosome, and SED1 from the epididymal lumen. Regardless, the interaction among the three acrosomal proteins (ZAN, proacrosin/acrosin and ACRBP) would form a basis for their co-existence in the APM complexes, and transportation of a fraction of them to the sperm APM region during capacitation would partially account for the increased amount in isolated APM vesicles in Cap sperm and the higher ZP binding affinity of the Cap gametes. A better understanding of the capacitation process should be gained by unraveling the mechanisms of how ZAN moves to the sperm APM site. ZAN should be the focus in such a protein transport study because it is the main component of the APM HMW complexes with ZP affinity, and transport of ZAN to the sperm head surface is observed in mouse spermatozoa during capacitation.[65] Notably, VWF is known to form multimeric complexes, which are stored intracellularly. However, a soluble fraction of these complexes can be secreted upon the rise of $[Ca^{2+}]_i$, intracellular cAMP levels and pH_i.[95] All of these stimulations take place as part of physiological changes during sperm capacitation,[96,97] which might possibly trigger the traffic of ZAN to the sperm APM. In addition, the roles of TCP-1 subunits in chaperoning ZAN and other acrosomal proteins to the sperm head surface cannot be ruled out.

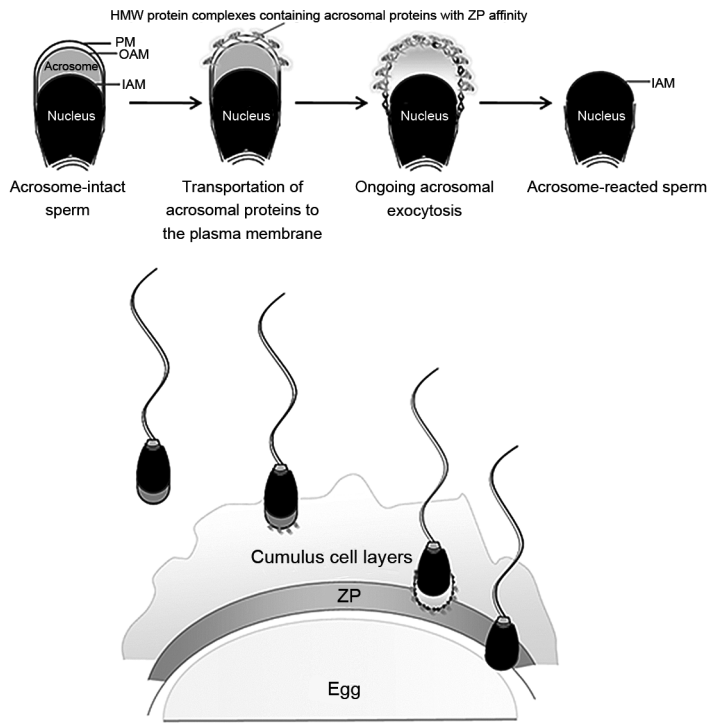


Figure 4: Proposed model of the involvement of acrosomal proteins in sperm–ZP interaction. During capacitation, a fraction of acrosomal proteins with ZP affinity traffics to the anterior sperm head surface as part of the initiation of acrosomal exocytosis. This endows the ability of Cap sperm to start binding to the ZP. Acrosomal exocytosis continues on the ZP with dispersion of the acrosomal matrix. The same acrosomal proteins in the matrix then contribute to the anchoring of acrosome reacting sperm to the ZP. Key: PM: plasma membrane; OAM: outer acrosomal membrane; IAM: inner acrosomal membrane; ZP: zona pellucida.

SUMMARY AND PERSPECTIVES FOR OUR FINDINGS

While pig APM vesicles comprise a number of ZP binding proteins and chaperones, only some of these proteins interact with each other to form HMW complexes. ZAN, SED1, proacrosin/acrosin, ACRBP, SP10 and ZPBP1 are the set of proteins in HMW complexes, which are known to be relevant in

sperm–ZP binding, whereas TCP-1 subunits are chaperones found in these complexes. HMW complexes from Cap sperm have significantly higher capacity to bind to pig ZP3 glycoproteins (sperm receptors), and this is partly because of the higher amounts of both of these protein constituents, compared with HMW complexes of Noncap sperm. As ZAN, proacrosin/acrosin, ACRBP, ZPBP1 and SP10 are known to be acrosomal proteins, we performed studies with the first three of these to determine whether they are targeted to the sperm head surface during capacitation. Our results revealing that a fraction of ZAN and proacrosin/acrosin indeed traffics to the sperm head surface during capacitation is in accordance with the recent finding in the mouse system that acrosomal exocytosis initiates during sperm migration through the cumulus cell layers: that is, prior to the spermatozoon encountering the ZP. It remains to be seen whether the timing of the inception of acrosomal exocytosis is the same in the pig. Nonetheless, the results suggest that ZAN, proacrosin/acrosin and perhaps also other acrosomal proteins (yet to be identified) that have been trafficked to the sperm head surface are involved in the initial interaction between Cap sperm and the ZP (while the sperm acrosomal matrix still remains relatively intact; see our model in **Figure 4**). The remainder of these acrosomal proteins in the acrosome would then participate in the subsequent interaction of acrosome reacting spermatozoa with the ZP. Moreover, if these proteins move to the inner acrosomal membrane following the completion of acrosomal exocytosis, they might also participate in adhering acrosome-reacted sperm to the ZP. Given that the transport of ZAN to the sperm head surface has also been documented in mouse spermatozoa,[65] the event may be used as a bioindex of sperm capacitation and we have research in progress to determine whether this movement occurs in human spermatozoa. Regardless, this phenomenon should be confirmed in oviductal spermatozoa Cap *in vivo*, to validate its biological relevance.

AUTHOR CONTRIBUTIONS

NT, RJA, MB, DH, TB, KFF, JW and JBA designed the experiments in our research work described in this review. KK, HK, AS, CS, JF and PS performed the lab work. NT, KK and AS prepared the review.

COMPETING INTERESTS

The authors declare no competing interests.

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