Chapter 5
Membrane Fusions During Mammalian Fertilization

Bart M. Gadella and Janice P. Evans

Abstract Successful completion of fertilization in mammals requires three different types of membrane fusion events. Firstly, the sperm cell will need to secrete its acrosome contents (acrosome exocytosis; also known as the acrosome reaction); this allows the sperm to penetrate the extracellular matrix of the oocyte (zona pellucida) and to reach the oocyte plasma membrane, the site of fertilization. Next the sperm cell will bind and fuse with the oocyte plasma membrane (also known as the oolemma), which is a different type of fusion in which two different cells fuse together. Finally, the fertilized oocyte needs to prevent polyspermic fertilization, or fertilization by more than one sperm. To this end, the oocyte secretes the contents of cortical granules by exocytotic fusions of these vesicles with the oocyte plasma membrane over the entire oocyte cell surface (also known as the cortical reaction or cortical granule exocytosis). The secreted cortical contents modify the zona pellucida, converting it to a state that is unreceptive to sperm, constituting a block to polyspermy. In addition, there is a block at the level of the oolemma (also known as the membrane block to polyspermy).

5.1 Introduction

Fertilization of the oocyte involves three membrane fusion events [1] namely, (1) a preparative series of secretion membrane fusions at the apical sperm surface known as acrosome exocytosis [2]. The membrane fusions are induced when the sperm cell binds to specific zona binding proteins at the sperm surface [3–7]. The acrosome exocytosis is a multipoint membrane fusion event between the sperm plasma membrane and the outer acrosomal membrane (see Fig. 5.1 [8, 9]) and the exposed acrosomal content is required for sperm to penetrate the zona pellucida [10–12]. This so-called zona drilling effectively takes place because the sperm at this stage also has acquired hyperactivated motility [13]. (2) After zona penetration the sperm enters the perivitelline space where it can bind and fuse with the oocyte plasma membrane [14, 15]. This is the actual fertilization fusion in which the contents of the sperm are delivered into the oocyte cytoplasm. The plasma membrane of the equatorial segment (see Fig. 5.1) is the site where proteins are located that orchestrate sperm-oocyte binding and fusion [16]. (3) In order to prevent polyspermy the oocyte has to activate defense systems to block redundant sperm-oocyte fusion [17]. To this end the first fertilizing sperm delivers activation factors

B.M. Gadella (✉)
Departments of Biochemistry and Cell Biology and of Farm Animal Health, Research Program: Biology of Reproductive Cells, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands
e-mail: b.m.gadella@uu.nl

T. Dittmar, K.S. Zänker (eds.), *Cell Fusion in Health and Disease*, Advances in Experimental Medicine and Biology 713, DOI 10.1007/978-94-007-0763-4_5,

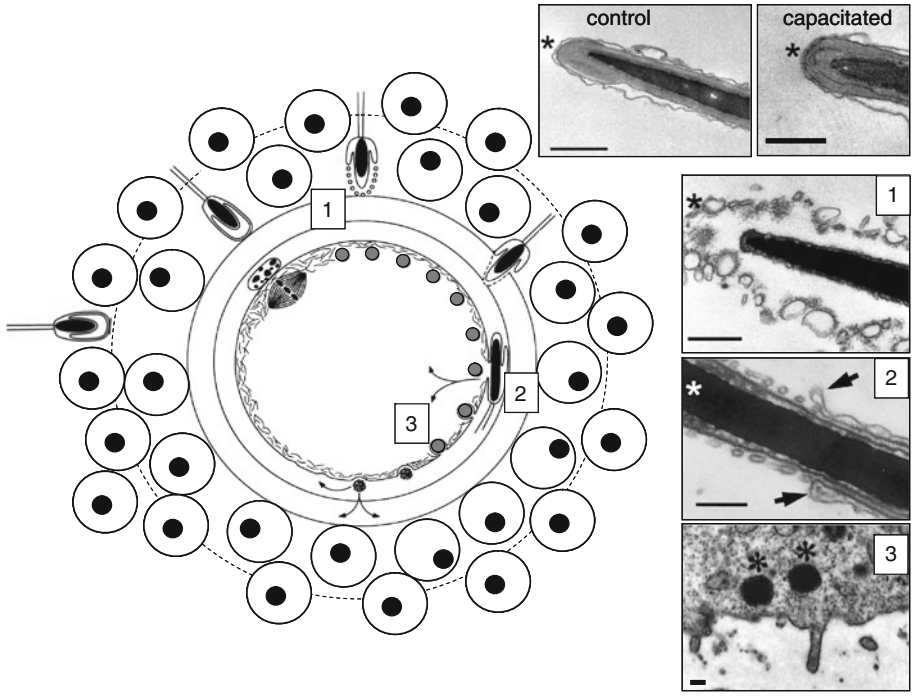

Fig. 5.1 The proposed sequence of events around the three fusions involved in monospermic fertilization. Sperm that have entered the oviduct will shed off decapacitation factors that were adhered peripherally to the sperm surface. During this process the acrosome is docked to the sperm plasma membrane and at the docked area the formation of high affinity zona binding complexes are formed [9, 35]. The control (control) versus capacitated (capacitated) sperm show the very close apposition of the sperm plasma membrane with the outer acrosomal membrane a feature emerging at the apical tip of the sperm head (*astrix*). It is not clear whether the resulting sperm which can associate with the extracellular matrix of the expanded cumulus mass surrounding the unfertilized oocyte induces some early steps of acrosomal fusion (proposed by 27) or that acrosome intact sperm are penetrating through the cumulus by the use of hyperactivated motility in combination with surface proteins [116]. Oviductal secreted proteins are also reported to be important for cumulus and zona pellucida properties [29]. *1*. The recognition of the zona pellucida (primary zona binding to ZP1/ZP3/ZP4) and subsequent inititation of the acrosome reaction (or of the acute secretory phase of it) which is induced by the zona pellucida. The unique multipointfusion of one organelle with the sperm surface generates mixed vesicles at the apical side of the sperm head. *2*. The acrosome reaction causes local modifications of zona proteins and the hyperactivated sperm can penetrate this structure due to secondary zona binding (to ZP2 and ZP3). The surface of the penetrating sperm will be further remodeled and this probably serves to enable the fertilization fusion [30]. Note that the equatorial segment of the sperm head (indicated with *arrows*) has remained resistant to exocytotic fusion. The plasma membrane and the outer acrosomal membrane have become continuous at this point. This needs to be protected from the acrosome reaction as it specifically contains the machinery to fertilize the oocyte. *3*. After the fertilization fusion the cortical reaction (induced by soluble sperm factors now diffusing into the oocyte cortex) causes an overall coating of the oolemma as well as the hardening of the zona pellucida by chemically altering zona proteins. The cleavage of ZP2 and ZP3 appears to be particularly instrumental for the release of sperm from the zona pullicida and to elicit an efficient block to polyspermy [23]. At the time of ovulation the MII phase oocytes have their cortical granules stored just under the oocytes plasma membrane. The fertilization fusion is followed up by a massive series of single point exocytotic fusions of the cortical granules (indicated with *asterix*). The distance bar indicates 50 nm. Panels with transmission electron microscopy micrographs are modified from [9, 113], the line drawing is modified from [7]

into the oocyte cytoplasm. The signaling cascade activated by these factors induce fusions of secretory granules that tightly located under the oocyte plasma membrane (known as cortical granules) [18, 19]. After the secretion of the cortical content into the perivitelline space, this will lead to modifications of the zona pellucida structure [20–23]. In some species, this has been characterized as “zona hardening”

defined as resistence to proteolytic digestion resulting in a zona pellucida that cannot be penetrated by acrosome-reacted sperm, and also cannot be recognized by acrosome-intact sperm [24–26].

Therefore, successful fertilization of an oocyte depends on three independent and quite differently organized membrane fusion events. The current understanding of membrane fusion and fertilization will be overviewed in this chapter.

5.2 Surface Remodeling of Gametes Prior to Zona Binding

5.2.1 The Cumulus-Oocyte Complex in the Oviduct

In mammals fertilization takes place in the oviduct near the ampulla region. The oocyte enters this site after ovulation and is surrounded by a thick (7 μm) extracellular matrix called the zona pellucida [27] and by a multi-cellular layer of cumulus mass (cells and extracellular matrix material, see Fig. 5.1). The oviduct probably modifies these extracellular structures to some extent [25, 28, 29].

5.2.2 Sperm Cell Surface Remodeling

Before the sperm cells enter the oviduct they have already passed a lengthy trajectory of transport and coinciding post-testicular modifications [30]. Sperm cells that are released in the testis from the Sertoli cells into the lumen of seminiferous tubules have shut down transcription and translation processes as well as membrane recycling (no endocytosis or exocytosis [31]). With respect to sperm-zona binding it is of special interest the identified transmembrane proteins with affinity for the zona pellucida originate from sperm forming precursor cells in the testis [32–35]. However, more recent approaches revealed that additional proteins are attached to the sperm surface (especially the epididymis, where sperm cells further mature and acquire motility) that serve to bind the zona pellucida [35–40]. After the ejaculation of sperm a specific coat of proteins containing decapacitation factors serve to stabilize sperm [41–45]. This is required to allow maximum sperm survival during their lengthy transport through the female genital tract (cervix, uterus) and to reach the oviduct intact. At this site sperm release their protective coat and become capacitated (i.e. capable to fertilize because they can recognize the zona pellucida). Therefore, the sperm regain fertilization capacity they originally had in the cauda epididymis (prior to ejaculation) and can induce the acrosome reaction after eventual zona pellucida binding. This capacitation process is guided in the oviduct by sperm-oviduct epithelial interactions (for review see [13]). After a certain period the sperm is released from the oviduct epithelial cell and has hyperactivated motility characteristics and demonstrates efficient zona binding behaviour.

During in vitro fertilization the capacitation of ejaculated sperm is mimicked by washing sperm through discontinuous density gradients (to remove decapacitation factors) and to incubate sperm for a couple of hours in an in vitro capacitation medium which mimics the ionic and metabolic composition of oviductal fluid (also known as synthetic oviductal fluid (SOF)) [7, 20, 46]. Mammalian sperm becomes activated by three principle capacitation factors namely (1) bicarbonate which activates adenylate cyclase/protein kinase A and tyrosine kinase signaling pathways, (2) albumin which specifically extracts sterols from the sperm plasma membrane and (3) extracellular calcium allowing Ca^{2+} mediated signaling cascades [46, 47]. For some species additional glycosaminoglycans are required to remove persistent decapacitation factors from the sperm surface [48]. Taken together sperm capacitation results in the induction of glycolysis in the sperm tail required for the hyperactivated motility (more instant and local production of ATP in the lengthy sperm tail which does not contain mitochondria [49–51]). In the sperm head it causes the redistribution of surface molecules. Most notably this results in the aggregation of lipid rafts and therein the formation of a functional

zona pellucida binding protein complex [30, 46, 52–54]. This zona binding complex not only functionally allows sperm zona binding but also mediates the acrosome exocytosis after this binding (see Section 5.3).

5.3 Zona Binding and Initiation of the Acrosome Reaction

5.3.1 Zona Pellucida Contains Acrosome Exocytosis Inducing Binding Sites

Traditionally it was thought that sperm-zona binding is a simple ligand receptor like interaction in which one zona receptor (namely ZP3) binds to one sperm ligand [55]. This concept appears to be oversimplified. The sperm surface has recently been shown to bind to at least three of the four human zona proteins (namely ZP1, ZP3 and ZP4) and most likely the species-specific zona protein matrix quartenary native state is important for sperm recognition [7]. The sperm cell also binds to this zona protein matrix with multiple proteins, most likely organized into zona binding protein complexes. Some of the identified proteins may be required for the induction of the acrosome reaction. For instance the presence of a potassium channel [35] may indicate that zona binding could induce a K^+ dependent sperm membrane hyperpolarization which in turn allows the opening or a voltage dependent Ca^{2+} channel and by doing so cause elevated cytosolic Ca^{2+} levels required for initiation of acrosome exocytosis [56]. Beyond this, the presence of a phosphatase [35] may indicate that binding may activate specific signaling events that are required for the induction of the acrosome reaction.

5.3.2 Acrosome Exocytosis

Acrosome exocytosis itself is the result of SNARE interactions between the outer acrosomal membrane and the plasma membrane of the so-called pre-equatorial region of the sperm head [57, 58]. Remarkably the two membranes fuse with each other at this entire surface domain which encompasses more than half of the sperm head surface [8, 9]. The multipoint fusion secretion event results in the generation of mixed vesicles that contain acrosomal outer membrane and plasma membrane material. The remaining unfused acrosomal membranes (at the equatorial area of the acrosome and the acrosomal inner membrane covering the apical part of the sperm nucleus) now take over the surface function of the plasma membrane [15, 16]. The vesiculated part of the apical acrosome membrane and plasma membrane are removed from the sperm. The group of Gadella has studied how SNARE proteins are orchestrating this multiple membrane fusion event. In freshly ejaculated sperm SNARE interactions between the apical sperm plasma membrane and the outer acrosomal membrane are not yet established [9]. However, during sperm capacitation these two membranes become stably docked by the formation of a *trans* ternary SNARE complex of proteins from the sperm plasma membrane as well as from the outer acrosmal membrane. The complex consisted of syntaxin1, VAMP1 and SNAP23 in a 1:1 stoichiometry [9]. The docked membranes could even be isolated as bilamellar structures. Related to this stability the capacitated acrosome becomes docked but does not fuse with the plasma membrane. For the execution of the acrosome fusions additional Ca^{2+} entry (in vitro by use of Ca^{2+} ionophores, in vivo after zona binding) is required [9]. Diverse groups have shown that SNARE complex interacting proteins such as complexins [9, 59, 60], dynamins [61], Rab 3A [62], synaptotagmins [63], multi-PDZ domain protein MUPP1, Calmodulin and CaMKIIalpha [64, 65], Rab-2a, syntaxin binding proteins and Munc-18 (Tsai et al., unpublished results) have been discovered in sperm (see Fig. 5.2). When and how they interact with SNARE proteins and whether they are involved in stabilizing the *trans* SNARE complex or are involved in the Ca^{2+} conversion to *cis* complexes (thus eliciting the acrosome plasma membrane fusions) is matter of future research (see also Fig. 5.2).

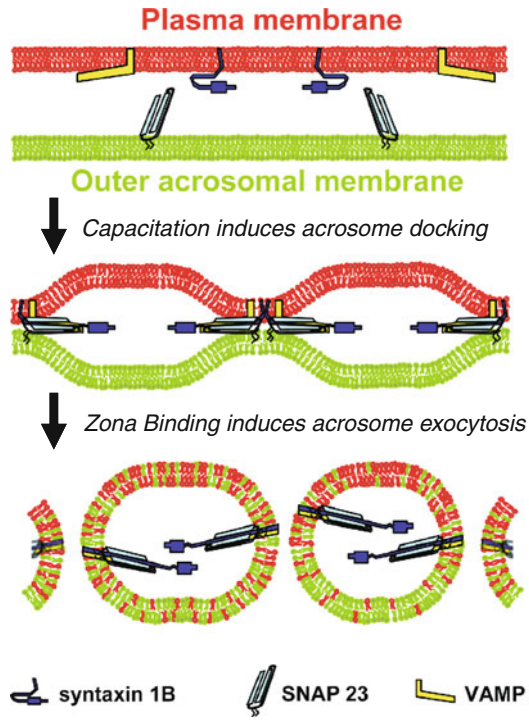

Fig. 5.2 Two step model for SNARE mediated acrosome exocytosis of the sperm. *Sperm capacitation* induces the stable docking of the sperm plasma membrane with the outer acrosomal membrane. The multiple docking of these two membranes does not lead to premature exocytosis. The identified interaction partners are for porcine sperm [9] but may differ between mammalian species. Two mechanisms have been described to stabilize the *trans* ternary SNARE protein complexes. (i) During sperm capacitation an aggregation of lipid rafts at the apical ridge area of the sperm head. This is the site where the sperm binds to the zona pellucida and where the acrosome exocytosis as a response of that binding is inititated [46]. In that area within the aggregating lipid rafts MUPP1/CaMKIIα have been reported to interact with the *trans* ternary SNARE protein complex and this association functions as a fusion clamp [64, 65]. (ii) The important factor in mouse sperm is the phosphorylated form of synaptotagmin, which appears to be important for preventing the acrosome exocytosis [63]. Beyond these factors also complexin and dynamin are interacting with the trans SNARE complex [59–61] but are not able at this stage to induce the *trans* to *cis* conformational shift of the complex. Munc18b is also associated to the *trans* ternary SNARE complex (unpublished observation). It is not clear whether or not Rab3A [62] is already associated to the *trans* SNARE complex at this stage. The current concept is that complexin, Munc18b (which can bind to syntaxin) are stabilizing the *trans* SNARE protein complex and prevent spontaneous acrosome exocytosis. *Zona binding* evokes Ca^{2+} entry (see Section 5.3.1) and this causes both the dissociation of the MUPP1/CaMKII fusion clamp [64] and a calcineurin-mediated dephosphorylation of synaptotagmin VI [63]. The dephosphorylation of synaptotagmins also appear to be essential for the acrosome reaction [59, 62, 117, 63] (unpublished observation). The role of Rab3A [62] and Rab2A (unpublished observation) in the formation of the *cis* ternary SNARE complex conformation is not yet clear. It is possible that these GTPase forming proteins were already recruited during sperm capacitation to the *trans* complex and by the zona-induced changes help to create the *cis* configuration either by dissociation (of Munc 18b) and the coinciding intrinsic Ca^{2+} sensing properties of the dephosphorylated synaptotagmin with the aid of Rab3A [62] or Rab2A. Figure is modified from [9]

5.4 Zona Penetration After the Acrosome Reaction

A result of the above described acrosome exocytosis (Section 5.3) is exposure of the acrosomal content at the front surface of the sperm head where primary zona binding initiated the acrosome reaction. The now exposed intra-acrosomal layer of proteins consists of an array of proteins that interact with the zona pellucida (for review see [7]). This so-called secondary zona binding was thought to be specific to ZP2 [66–68] but recently it has been shown that the intra-acrosomal protein sp56 binds to ZP3 [4, 12].

Beyond the more massive secondary zona binding (compared to the primary zona recognition binding at the sperm surface), the exposed intra-acrosomal proteins also cause a local enzymatic cleavage of this network of 3–4 heavily glycosylated zona proteins [66, 69–75]. For a review on secondary zona binding proteins from the acrosome see [7]. Note that acrosome exocytosis and consequent zona drilling coincides with the generation of hyperactivated motility of sperm [13]. Together they form the pre-requisites for a recycling modus: secondary zona binding followed by local digestion of the ZP network, zona penetration and subsequent rebinding to the ZP. Thus acrosome exocytosis enables the sperm to reach the perivitelline space and exposes the inner acrosomal membrane and the equatorial segment of the sperm head (i.e., that area where the acrosome outer membrane was inert to fuse with the sperm plasma membrane), which is required for the sperm to be capable of fusing with the oocyte membrane (see Section 5.5).

5.5 Gamete Membrane Fusion and the Oocyte-to-Embryo Transition

Sperm-oocyte fusion is one of the best-known extracellular membrane fusion events, and yet it is one of the most poorly understood. Especially in comparison to other types of extracellular fusion events and to SNARE-mediated vesicle fusion events, relatively little is known about the mechanisms underlying sperm-oocyte fusion in any species, particularly mammalian. The identification of fusion-mediating factors in fertilization also has been difficult, but there are multiple possible explanations for this. From the standpoint of genetic/knockout studies, perhaps there are multiple factors with substantial functional overlap, and this redundancy has made it difficult to identify gamete fusion-defective phenotypes. Alternatively, the fusion-mediating factors may play critical roles in other cell types, making it impossible to assess gamete fusion (e.g., embryonic or neonatal lethality) without use of a conditional knockout. From the standpoint of biochemistry or developing function-blocking antibodies as a means to identify these fusion-mediating factors, it is possible that these factors are few in number, unstable, and/or only transiently exposed. These considerations are also valid for proteins involved in sperm-zona interactions described in Section 4.3.1 and 4.4.

Only two proteins, the tetraspanin CD9 on the mouse oocyte and the immunoglobulin superfamily member IZUMO1 (previously known as Izumo) on mouse sperm, have been shown by gene knockout studies as being essential specifically for sperm-egg interaction (Fig. 5.3). Note: It is unclear if CD9 and/or different tetraspanins function in other species' oocytes in gamete membrane fusion [76]. Other mouse knockouts have less severe defects in gamete interactions or fertilization, or have multiple gamete function defects (e.g., [77]). The discovery of CD9's role in murine fertilization occurred rather serendipitously, when the knockout mouse lacking this member of the protein family was found to have greatly reduced female fertility. This is rather remarkable, since CD9 is expressed in numerous cell types in the body, but there is only an obvious phenotype with oocytes showing a significantly reduced ability to fuse with sperm [78–80]. The discovery of the role of IZUMO1 came as a result of persistence and hard work, with 17 years between the report of the function-blocking activity of the monoclonal antibody OBF13 on sperm-oocyte fusion [81] and the report of

Fig. 5.3 (continued) 14 (SAMP14; also known as sperm acrosome associated 4, SPACA4), SAMP32 (also known as SPACA1), and Sperm Lysosomal-Like Protein 1 (SLLP1; also known as SPACA3) [70, 135–137], all of which are novel proteins. Finally, zinc metalloprotease (MP) activity has been implicated by the finding that mouse sperm-egg fusion is reduced in the presence of various metalloprotease inhibitors [138]. Reagents that disrupt the action of enzymes that mediate thiol-disulfide exchanged in proteins (protein disulfide isomerases, PDIs) also reduce the incidence of sperm-egg fusion [139, 140]. The results with N-ethyl-maleimide (NEM) and 5,5′-dithiobis (2-nitro-benzoic acid) (DTNB) [139, 140] suggest that sulfhydryl groups may be a common element involved in fusion systems, as they are in vesicle fusion [141], and certain viral fusion events [142, 143]

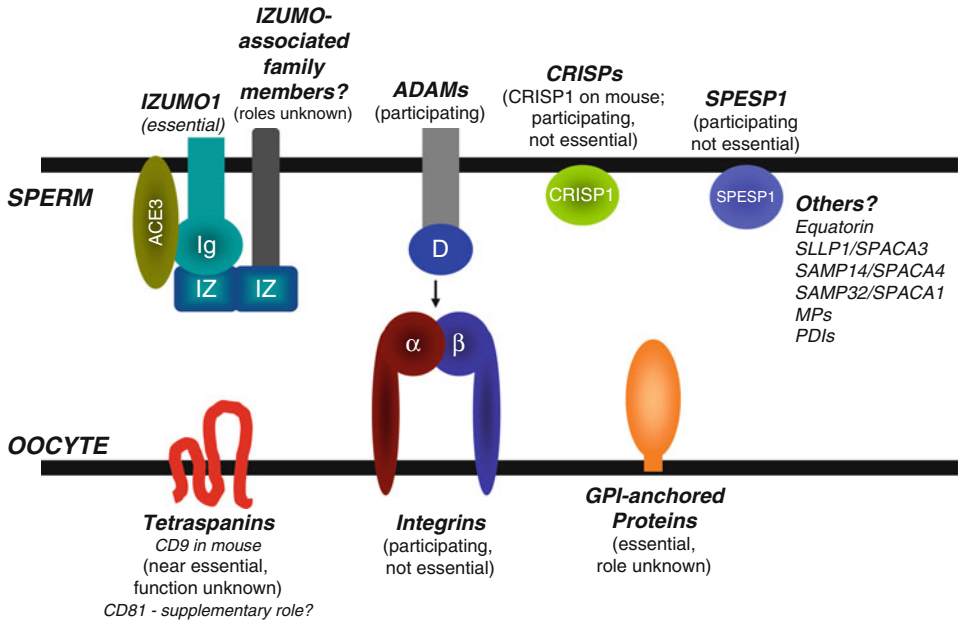

Fig. 5.3 Schematic diagram illustrating molecules implicated in gamete membrane binding and fusion. This diagram illustrates the molecules proposed to participate in sperm-oocyte membrane interactions (i.e., binding and/or fusion). CD9 is the major player identified thus far on the oocyte. CD9$^{-/-}$ females are severely subfertile (only 60% of CD9$^{-/-}$ females become pregnant, litters sizes are 75% smaller, and achieving these pregnancies takes nearly four times longer than it does for control mice [84]). IVF assays show that sperm binding to *CD9*$^{-/-}$ oocytes appears to be unaffected, but sperm rarely fuse [78, 79, 118]. Mouse egg CD9 is likely to function in conjunction with another tetraspanin, CD81; CD9/CD81 double knockout female mice are completely infertile, suggesting that CD9 and CD81 play complementary roles in fertilization [84]. GPI-anchored proteins on the oocyte are also implicated. Mice with oocytes deficient in GPI-anchored proteins are infertile, and these oocytes poorly support sperm-oocyte binding and fusion [86], but it remains unknown which GPI-anchored protein(s) are crucial and what role(s) oocyte GPI-anchored proteins could play. The last main candidates on oocytes are members of the integrin family. Integrins are heterodimeric membrane proteins, made up of an α and a β subunit, with 18 α subunits and eight β subunits combining to make at least 24 different combinations. α6, α3, and β1 in oocytes are not essential for fertility [119, 120], but in vitro studies of certain subunits have revealed defects in sperm-oocyte binding [121–123]. Oocytes with reduced amounts of α9 support sperm binding and fusion less well than do control eggs [123], in agreement with the finding that several ADAMs can interact with α9β1 [124]. Oocytes deficient in β1-deficient show defects in sperm-oocyte binding [121]. On the sperm, IZUMO1 has been shown to be essential for sperm-oocyte fusion [125]. IZUMO1 is member of the immunoglobulin superfamily (IgSF) proteins, and contains an immunoglobulin-like domain (Ig). IZUMO1 also has a ~150 amino acid domain that has been called the Izumo domain (IZ in the diagram), and this domain has been found in three other proteins [87]. Interestingly, IZUMO1 is associated with other Izumo domain proteins, although the functions of these in gamete fusion are not known. ACE3 (Angiotensin Converting Enzyme 3) is another IZUMO1-associated protein [82]; the *Ace3* knockout did not show any defects in male fertility or sperm function in vitro, although there is a slight abnormality in the localization of IZUMO1 [82]. Several sperm ADAMs have been implicated in sperm-oocyte interaction; while no single ADAM is essential, there appears a correlation between the ability of sperm to bind and fuse with the egg membrane and the levels of certain ADAM proteins (see [15] for more information). This suggests that ADAMs could function in redundant roles, consistent with the fact that ADAMs have similar adhesion-mediating motifs to interact with integrins via their disintegrin domain (D in the diagram) [124, 126–128]. Cysteine-rich Secretory Protein 1 was implicated in sperm-oocyte fusion by antibody studies in the 1980s [129, 130], and the *Crisp1* knockout was recently reported. Sperm from *Crisp1*$^{-/-}$ males show a modest decrease in sperm-oocyte fusion in in vitro fertilization assays, although male fertility appears normal [131]. Finally, as noted in the text, acrosome exocytosis exposes the inner acrosomal membrane and the equatorial segment of the sperm head, rendering the sperm capable of interacting with the oolemma. Thus, proteins in the equatorial segment of the sperm head have been of interest. SPESP1 (sperm equatorial segment protein 1; [132]) is a candidate, based on the finding that *Spesp1*$^{-/-}$ males produce slightly smaller litters than wild type controls (22%), and have sperm with reduced (although not completely deficient) ability to undergo sperm-oocyte fusion [77]. Other proteins associated with sperm-oocyte fusion and exposure or rearrangement after acrosome exocytosis include equatorin [133, 134], Sperm Acrosomal Membrane-Associated

the phenotype of the knockout [82]. Numerous other molecules have also been suggested to participate in mammalian gamete membrane interaction (sperm binding and/or sperm-oocyte membrane fusion; Fig. 5.3). Mouse knockouts have been made of several of these, and many have less dramatic phenotypes that the *Cd9*$^{-/-}$ and *Izumo1*$^{-/-}$ mice, namely often only partial loss of function in sperm-oocyte interaction (and sometimes little or no impairment of fertility). These are addressed in the figure legend for Fig. 5.3.

One theme that seems to be emerging in mammalian gamete membrane fusion that is conserved with other examples of membrane fusion is that membrane order, multimeric protein complexes, and the gamete surface proteome may prove to play critical roles. CD9 and other tetraspanins are known to function as organizers of membrane domains, known as tetraspanin-enriched microdomains [83]. Mouse egg CD9 is likely to function in conjunction with another tetraspanin, CD81. CD9/CD81 double knockout female mice are completely infertile, suggesting that CD9 and CD81 play complementary roles in fertilization [84]. Interestingly, glycosylphosphatidyl inositol (GPI)-anchored proteins in oocytes have been implicated by an oocyte-specific knockout of *Piga*, a subunit of an N-acetyl glucosaminyl transferase that participates in first steps of the synthesis of GPI-anchored proteins [85]; female mice with this oocyte-specific *Piga* knockout are infertile [86]. It is possible that the *Piga* deficiency and the resulting lack of GPI-anchored proteins in the oocyte membrane alters membrane composition and/or organization so that sperm interactions are not favored. GPI-anchored proteins are enriched in lipid microdomains, raising the possibility that the microdomain structure of the egg plasma membrane could be perturbed in the absence of GPI-anchored proteins. The importance of membrane order may also extend to sperm. IZUMO1 has recently been described to associate with other membrane proteins [87, 88]. Likewise, members of the ADAM (A Disintegrin and A Metalloprotease domain) family are other sperm proteins implicated in gamete membrane interactions, and the genetic deletion of one *Adam* can affect the expression of multiple ADAM proteins on the sperm surface [15, 89], and protein trafficking during spermatogenesis [90], suggestive of a role of ADAMs in sperm membrane order. Finally, another knockout, *Tssk6*, is defective in sperm-oocyte fusion and has an abnormality in IZUMO1 localization [91], also possibly indicative of aberrant membrane order.

5.6 The Membrane (Oolemma) Block to Polyspermy

5.6.1 Redundant Sperm Around the Fertilized Oocyte

Mammalian oocytes regulate their ability to interact with sperm, namely the membrane block to polyspermy, by altering the receptivity of the oolemma to sperm after fertilization. This was demonstrated by classic studies in which fertilized oocytes recovered from natural matings were found to have extra sperm in the perivitelline space, apparently unable to penetrate the oolemma [92–94]. The numbers of supernumerary perivitelline sperm vary by species, suggestive that there are differences in the reliance on the various polyspermy prevention mechanisms between different species. The oocytes of some species such as rabbit, pika, pocket gopher, and mole have tens to hundreds of sperm in the perivitelline space, suggestive of a highly effective membrane block and a relatively ineffective ZP block. Species in which perivitelline sperm are rare (dog, sheep, field vole) likely have a highly effective ZP block. Numerous species (including mouse, human, rat, guinea pig, cat, pig, cattle) appear to use both blocks to polyspermy; in these oocytes, one or two or up to ~10 sperm are found in the perivitelline space of early zygotes [92–97].

5.6.2 Prevention of Polyspermy at the Oolemma

The basis of the membrane block to polyspermy – i.e., what is different about the zygote membrane that prevents additional sperm fusions – is not known. In mouse oocytes, this membrane transition

occurs gradually; the membrane block is not yet established by 0.75 h post-insemination, but is established by 1.5 h post-insemination [17]. Experiments using fluorescent tags in mouse oocytes to track membrane lipids or protein diffusion suggest that fertilization-induced changes do occur, although such changes have not been well characterized [98, 99]. It has recently been shown that cortical tension is higher in zygotes than in unfertilized oocytes [100], although the exact role that this may play in the membrane block to polyspermy remains to be determined. The mechanism by which the membrane block is triggered also is an active area of investigation. One key finding is that this membrane block appears to be largely independent of cortical granule exocytosis, although it is possible the contents of the cortical granules may augment the membrane block, even if cortical granule exocytosis is not an essential component. Oocytes that are activated in ways that induce increased cytosolic Ca^{2+} concentration and the cortical reaction (calcium ionophore, strontium chloride, injection of a soluble sperm extract, or by fertilization by intracytoplasmic sperm injection) maintain membranes that are receptive to sperm [17, 101–104]. The failure of ICSI-generated embryos to establish a membrane block to polyspermy has been interpreted to indicate that sperm membrane incorporation into the oolemma is linked with membrane block establishment [103], or that membrane block establishment occurs as a result of changes in the oocyte occurring with the process of gamete fusion [104]. In the mouse, the sperm head surface area is only ~0.14% of the oocyte surface area, and thus a membrane block mechanism involving dilution of the oolemma with sperm membrane seems unlikely. Instead, establishment of the membrane block may involve signaling occurring with gamete fusion, although the injection of a soluble sperm extract fails to trigger membrane block establishment, indicating that membrane block establishment is not solely controlled by the sperm-induced increase in cytosolic Ca^{2+} [104].

5.7 Cortical Reaction and the Zona Pellucida Block to Polyspermic Fertilization

5.7.1 Cortical Granules Content Can Modify the Zona Pellucida Structure

The concern for a just fertilized oocyte is to prevent additional sperm to bind to and fuse with the oolemma (polyspemy). The just-fused first sperm introduces soluble cytosolic factors like phospholipase C zeta into the oocyte [105]. These factors induce intracellular Ca^{2+} events and the oocyte plasma membrane depolarization and both are triggers for the cortical granule exocytosis [18]. The secretory granules that reside in the cortex (the area just under the oocyte plasma membrane) fuse with the oocyte plasma membrane and the content of the granules is released into the perivitelline space [106, 107]. Although the contents of the cortical granules are very poorly characterized [106, 108–111], it is known that the release of these materials results in the cleavage of ZP2 and ZP3 into the truncated $ZP2_f$ and $ZP3_f$ forms [20–22]. These alterations are associated with zona hardening (defined as resistance to proteolysis in certain in vitro assays). As a result sperm stop penetrating the hardened zona and do not show affinity for the zona pellucida.

5.7.2 Maturation Dependent Exocytotic Fusion Machinery of the Cortical Reaction

Cortical granule exocytosis resembles to some extent the exocytosis of the acrosome (Section 5.3.2) in that in both cases secretory granule exocytosis takes place at the surface of a gamete. The main difference between the two exocytosis events is that cortical granule exocytosis is a series of single point fusion events of many cortical granules with the oolemma, while the acrosome exocytosis is a multiple point fusion event of one acrosome with the sperm plasma membrane. The majority of

secretory granules migrate towards the oocyte plasma membrane during pre-ovulatory maturation of oocytes somewhere between the germinal vesicle stage (GV, oocytes that are arrested at the prophase of meiosis I) and the arrested stage at metaphase of meiosis II (MII) [107, 112]. Nevertheless, some of the secretory granules already reside in the cortex of GV oocytes but fail to be competent to fuse with the oocyte surface at that stage which appears to depent on too low activity of calcium/calmodulin dependent kinase II activity which becomes activated in MII oocytes [113] and related to this MAPK activity seems to be inviolved in activating the cortical granule exocytosis as well [114]. In addition we have shown recently that cortical granules become docked at the oocytes plasma membrane during these two meiotic maturation stages [107]. The SNARE proteins SNAP23, VAMP-1 and syntaxin 2 are involved and probably form a similar trimeric *trans* complex prior to fertilization (which therefore is analogous to acrosome docking during sperm capacitation). The docked cortical granules are also decorated with complexins (probably stabilizing the SNARE complex) and with clathrin (Fig. 5.4). After fertilization the Ca^{2+} mobilization and related signaling and cytoskeletal rearrangements [18, 115] cause the *trans* to *cis* ternary configuration of the trimeric SNARE complex is turned on and explains the cortical reaction. Remarkably complexin and clathrin dissociate from the cortex and relocate to intracellular structures and this is probably required to re-establish endocytosis and membrane recycling [107]. In case of polyspermic fertilization of pig oocytes, we observed a normal cortical reaction but this is not followed by a release of clathrin which may indicate that at the level of the oolemma fusion the inhibition of endocytosis may have a relationship with the fusion properties of the oolemma [107]. This observation confirms the finding that the polyspermy block is at least not immediately dependent on cortical exocytosis (see Section 5.6.2). As noted above, little to nothing is known about the content of the cortical granules although in general its content should resemble that of other secretory vesicles. Like the acrosomal enzymes also the cortical granule enzymes are capable to alter the zona pellucida structure. But the cortical granule enzymes differ from acrosomal enzymes in that acrosomal enzymes digest the zona pellucida matrix locally (allowing sperm penetration) whereas the cortical granule enzymes make the zona pellucida impermeable for acrosome-reacted sperm (the so called slow polyspermy block).

5.8 Conclusion

This chapter provides an overview about the three fusion events involved in mammalian fertilization. Acrosome exocytosis is first hurdle, allowing the sperm to fertilize the oocyte by resulting in localized digestion of the zona pellucida and thus permitting the sperm to gain access to the oocyte plasma membrane. The first sperm to interact with the oocyte plasma membrane and to execute actual fertilization fusion delivers its male haploid genome to the oocyte. This sperm also activates the oocyte, leading to the oocyte-to-embryo transition, including the establishment of blocks to polyspermy, with the zona pellucida block being mediated by the third membrane fusion event of fertilization (i.e., cortical granule exocytosis). Both the secretion of the acrosome and the cortical granules can be considered as classical exocytotic events in which trimeric SNARE complex formation cause vesicle docking to the gametes plasma membrane and Ca^{2+}-dependent configuration to a *cis* trimeric SNARE complex causes exocytosis. However, the acrosome exocytosis is unique in showing multiple fusions of only one large secretory vesicle with the sperm plasma membrane, whereas the cortical granule exocytosis likely initiaties with single point fusions of an array of cortical vesicles over the entire the oocyte plasma membrane. The regulation machinery for vesicle docking and fusion with the plasma membrane for both gametes needs to be studied into greater detail. In between the two exocytotic membrane fusions lays the actual sperm-oocyte membrane fusion, which remains to be poorly understood. The fusion between two gamete plasma membranes may share similarities to other extracellular fusion events (e.g., myofibril formation, syncytia-forming transformed cancer cells in culture), or viral fusion.

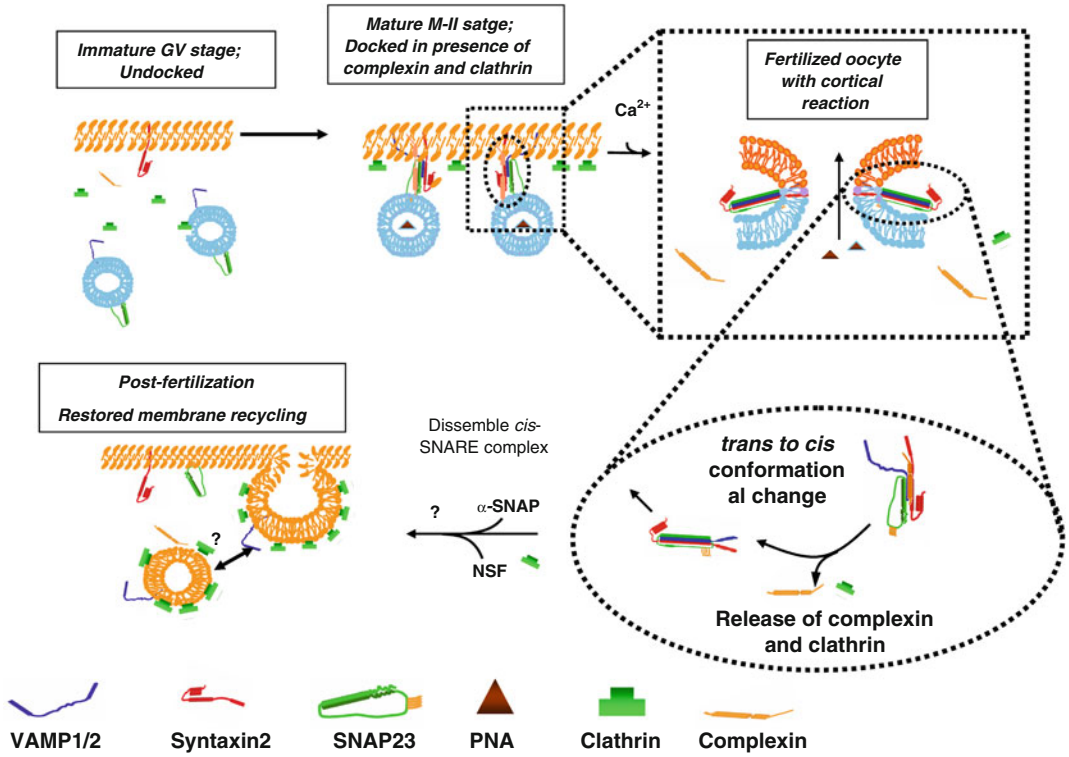

Fig. 5.4 Model for SNARE mediated cortical exocytosis of the fertilized oocyte. At the germinal vesicle stage (GV) the majority of the secretory granules (*blue* vesicles) are not residing in the cortex of the oocyte and are therefore do not interact with the oocyte plasma membrane (*yellow* membrane). During later meiotic maturation at the metaphase II stage (MII) about all granules have migrated towards the cortex region and strong co-localization of oocyte plasma membrane and cortical granule content has been demonstrated. Therefore, it is possible that the cortical granules are docked to the oocyte plasma membrane and that this interaction is stabilized in an analogous way to that of the acrosome. This would explain why the premature cortical exocytosis is not observed. Complexin and clathrin are at this stage exclusively present in the area where the cortical granules and the plasma membrane are interacting. It is noteworthy to mention that membrane recycling is silenced in MII oocytes and no exocytosis as well as endocytosis can be observed. The concentration of complexin and clathrin at the cortex may well have to do with this. Once the sperm has fertilized the oocyte, it will introduce oocyte activation factors into the oocyte that allow Ca^{2+} mobilization (see Section 5.7.1) and this results in at least the dissociation of clathrin and complexin. This dissociation has not been found in polyspermic fertilized oocytes that still retain membrane recycling blocked at the oocyte surface. Perhaps this explains why redundant sperm can cause polyspermic fertilization in those oocytes. In the monospermic oocytes the release of complexin and clathrin coincides with the onset of membrane recycling by means of endocytosis and exocytosis and thus to further embryo development. This figure summarizes the studies of Tsai et al. [107]

Taken together three membrane fusion events serve to achieve optimal monospermic fertilization of mammalian oocytes. However, it is surprising that, despite of decades of research, the actual molecular understanding of the fertilization of the mammalian oocyte is still relatively limited.

References

1. Wassarman PM, Litscher ES (2008) Mammalian fertilization is dependent on multiple membrane fusion events. Methods Mol Biol 475:99–113
2. Florman HM, Jungnickel MK, Sutton KA (2008) Regulating the acrosome reaction. Int J Dev Biol 52:503–510
3. Chiu PC, Wong BS, Chung MK et al (2008) Effects of native human zona pellucida glycoproteins 3 and 4 on acrosome reaction and zona pellucida binding of human spermatozoa. Biol Reprod 79:869–877

4. Buffone MG, Rodriguez-Miranda E, Storey BT et al (2009) Acrosomal exocytosis of mouse sperm progresses in a consistent direction in response to zona pellucida. J Cell Physiol 220:611–620
5. Gupta SK, Bansal P, Ganguly A et al (2009) Human zona pellucida glycoproteins: functional relevance during fertilization. J Reprod Immunol 83:50–55
6. Ganguly A, Bukovsky A, Sharma RK et al (2010) In humans, zona pellucida glycoprotein-1 binds to spermatozoa and induces acrosomal exocytosis. Hum Reprod 25:1643–1656
7. Gadella BM (2010) Interaction of sperm with the zona pellucida. Soc Reprod Fertil 67:267–287
8. Zanetti N, Mayorga LS (2009) Acrosomal swelling and membrane docking are required for hybrid vesicle formation during the human sperm acrosome reaction. Biol Reprod 81:396–405
9. Tsai PS, Garcia-Gil N, van Haeften T et al (2010) How pig sperm prepares to fertilize: stable acrosome docking to the plasma membrane. PLoS One 18:e11204
10. Kim KS, Gerton GL (2003) Differential release of soluble and matrix components: evidence for intermediate states of secretion during spontaneous acrosomal exocytosis in mouse sperm. Dev Biol 264:141–152
11. Kim E, Yamashita M, Kimura M et al (2008) Sperm penetration through cumulus mass and zona pellucida. Int J Dev Biol 52:677–682
12. Buffone MG, Foster JA, Gerton GL (2008) The role of the acrosomal matrix in fertilization. Int J Dev Biol 52:511–522
13. Suarez SS (2008) Regulation of sperm storage and movement in the mammalian oviduct. Int J Dev Biol 52: 455–462
14. Primakoff P, Myles DG (2007) Cell-cell membrane fusion during mammalian fertilization. FEBS Lett 581: 2174–2180
15. Vjugina U, Evans JP (2008) New insights into the molecular basis of mammalian sperm-egg membrane interactions. Front Biosci 13:462–476
16. Vigil P (1989) Gamete membrane fusion in hamster spermatozoa with reacted equatorial segment. Gamete Res 23:203–213
17. Gardner AJ, Williams CJ, Evans JP (2007) Establishment of the mammalian membrane block to polyspermy: Evidence for calcium-dependent and -independent regulation. Reproduction 133:383–393
18. McAvey BA, Wortzman GB, Williams CJ et al (2002) Involvement of calcium signaling and the actin cytoskeleton in the membrane block to polyspermy in mouse eggs. Biol Reprod 67:1342–1352
19. Gardner AJ, Evans JP (2006) Mammalian membrane block to polyspermy: new insights into how mammalian eggs prevent fertilisation by multiple sperm. Reprod Fertil Dev 18:53–61
20. Bleil JD, Wassarman PM (1983) Sperm-egg interactions in the mouse: sequence of events and induction of the acrosome reaction by a zona pellucida glycoprotein. Dev Biol 95:317–324
21. Schroeder AC, Schultz RM, Kopf GS et al (1990) Fetuin inhibits zona pellucida hardening and conversion of ZP2 to ZP2f during spontaneous mouse oocyte maturation in vitro in the absence of serum. Biol Reprod 43: 891–897
22. Kalab P, Kopf GS, Schultz RM (1991) Modifications of the mouse zona pellucida during oocyte maturation and egg activation: effects of newborn calf serum and fetuin. Biol Reprod 45:783–787
23. Gahlay G, Gauthier L, Baibakov B et al (2010) Gamete recognition in mice depends on the cleavage status of an egg's zona pellucida protein. Science 329:216–219
24. Coy P, Gadea J, Romar R et al (2002) Effect of in vitrofertilization medium on the acrosome reaction, cortical reaction, zona pellucida hardening and in vitro development in pigs. Reproduction 124:279–288
25. Coy P, Avilés M (2010) What controls polyspermy in mammals, the oviduct or the oocyte? Biol Rev Camb Philos Soc 85:593–605
26. Papi M, Brunelli R, Sylla L et al (2010) Mechanical properties of zona pellucida hardening. Eur Biophys J 39:987–92
27. Wassarman PM, Litscher ES (2009) The multifunctional zona pellucida and mammalian fertilization. J Reprod Immunol 83:45–49
28. Canovas S, Romar R, Grullon LA et al (2009) Pre-fertilization zona pellucida hardening by different cross-linkers affects IVF in pigs and cattle and improves embryo production in pigs. Reproduction 137:803–812
29. Avilés M, Gutiérrez-Adán A, Coy P (2010) Oviductal secretions: Will they be key factors for the future ARTs? Mol Hum Reprod 16:896–906
30. Tsai PS, Gadella BM (2009) Molecular kinetics of proteins at the surface of porcine sperm before and during fertilization. Soc Reprod Fertil Suppl 66:23–36
31. Boerke A, Dieleman SJ, Gadella BM (2007) A possible role for sperm RNA in early embryo development. Theriogenology 68:S147–S155
32. Cho C, Ge H, Branciforte D et al (2000) Analysis of mouse fertilin in wild-type and fertilin beta(-/-) sperm: evidence for C-terminal modification, alpha/beta dimerization, and lack of essential role of fertilin alpha in sperm-egg fusion. Dev Biol 222:289–295

33. Ghosh I, Datta K (2003)Sperm surface hyaluronan binding protein (HABP1) interacts with zona pellucida of water buffalo (Bubalus bubalis) through its clustered mannose residues. Mol Reprod Dev 64:235–244
34. Rodeheffer C, Shur BD (2004) Sperm from beta1,4-galactosyltransferase I-null mice exhibit precocious capacitation. Development 131:491–501
35. van Gestel RA, Brewis IA, Ashton PR et al (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
36. Montfort L, Frenette G, Sullivan R (2002) Sperm-zona pellucida interaction involves a carbonyl reductase activity in the hamster. Mol Reprod Dev 61:113–119
37. Shur BD, Rodeheffer C, Ensslin MA et al (2006) Identification of novel gamete receptors that mediate sperm adhesion to the egg coat. Mol Cell Endocrinol 250:137–148
38. Busso D, Cohen DJ, Maldera JA et al (2007) A novel function for CRISP1 in rodent fertilization: involvement in sperm-zona pellucida interaction. Biol Reprod 77:848–854
39. Copland SD, Murphy AA, Shur BD (2009) The mouse gamete adhesin, SED1, is expressed on the surface of acrosome-intact human sperm. Fertil Steril 92:2014–2019
40. Marín-Briggiler CI, González-Echeverría MF, Munuce MJ et al (2010) Glucose-regulated protein 78 (Grp78/BiP) is secreted by human oviduct epithelial cells and the recombinant protein modulates sperm-zona pellucida binding. Fertil Steril 93:1574–1584
41. Ensslin M, Calvete JJ, Thole HH et al (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
42. Caballero I, Vázquez JM, Rodríguez-Martínez H et al (2005) Influence of seminal plasma PSP-I/PSP-II spermadhesin on pig gamete interaction. Zygote 13:11–16
43. Nixon B, MacIntyre DA, Mitchell LA et al (2006) The identification of mouse sperm-surface-associated proteins and characterization of their ability to act as decapacitation factors. Biol Reprod 74:275–287
44. Manjunath P, Bergeron A, Lefebvre J et al (2007) Seminal plasma proteins: functions and interaction with protective agents during semen preservation. Soc Reprod Fertil Suppl 65:217–228
45. Vadnais ML, Roberts KP 2010 Seminal plasma proteins inhibit in vitro- and cooling-induced capacitation in boar spermatozoa. Reprod Fertil Dev 22:893–900
46. Gadella BM, Tsai PS, Boerke A et al (2008) Sperm head membrane reorganisation during capacitation. Int J Dev Biol 52:473–480
47. Gadella BM, Visconti PE (2006) Regulation of capacitation. In: de Jonge C, Barrett C (eds) The sperm cell, production maturation fertilization regeneration. Cambridge University Press, Cambridge
48. Parrish JJ, Susko-Parrish J, Winer MA et al (1988) Capacitation of bovine sperm by heparin. Biol Reprod 38:1171–1180
49. Hung PH, Miller MG, Meyers SA et al (2008) Sperm mitochondrial integrity is not required for hyperactivated motility, zona binding, or acrosome reaction in the rhesus macaque. Biol Reprod 79:367–375
50. Kumar V, Kota V, Shivaji S (2008) Hamster sperm capacitation: role of pyruvate dehydrogenase A and dihydrolipoamide dehydrogenase. Biol Reprod 79:190–199
51. Eddy EM (2006) The spermatozoon. In: Knobil E, Neill JD (eds) Physiology of reproduction, (3rd edn). Elsevier, Amsterdam, The Netherlands
52. van Gestel RA, Brewis IA, Ashton PR et al (2005) Capacitation-dependent concentration of lipid rafts in the apical ridge head area of porcine sperm cells. Mol Hum Reprod 11:583–590
53. Thaler CD, Thomas M, Ramalie JR (2006) Reorganization of mouse sperm lipid rafts by capacitation. Mol Reprod Dev 73:1541–1549
54. Selvaraj V, Buttke DE, Asano A et al (2007) GM1 dynamics as a marker for membrane changes associated with the process of capacitation in murine and bovine spermatozoa. J Androl 28:588–599
55. Wassarman PM (1988) Zona pellucida glycoproteins. Ann Rev Biochem 57:415–442
56. Benoff S, Chu CC, Marmar JL et al (2007) Voltage-dependent calcium channels in mammalian spermatozoa revisited. Front Biosci 12:1420–1449
57. Ramalho-Santos J, Moreno RD, Sutovsky P et al (2000) SNAREs in mammalian sperm: possible implications for fertilization. Dev Biol 223:54–69
58. De Blas GA, Roggero CM, Tomes CN et al (2005) Dynamics of SNARE assembly and disassembly during sperm acrosomal exocytosis. PLoS Biol 3:e323
59. Roggero CM, De Blas GA, Dai H et al (2007) Complexin/synaptotagmin interplay controls acrosomal exocytosis. J Biol Chem 282:26335–26343
60. Zhao L, Burkin HR, Shi X et al (2007) Complexin I is required for mammalian sperm acrosomal exocytosis. Dev Biol 309:236–244
61. Zhao L, Shi X, Li L, Miller DJ (2007) Dynamin 2 associates with complexins and is found in the acrosomal region of mammalian sperm. Mol Reprod Dev 74:750–757

62. Lopez CI, Belmonte SA, De Blas GA et al (2007) Membrane-permeant Rab3A triggers acrosomal exocytosis in living human sperm. FASEB J 21:4121–4130
63. Castillo Bennett J, Roggero CM, Mancifesta FE et al (2010) Calcineurin-mediated dephosphorylation of synaptotagmin VI is necessary for acrosomal exocytosis. J Biol Chem 285:26269–26278
64. Ackermann F, Zitranski N, Heydecke D et al (2008) The Multi-PDZ domain protein MUPP1 as a lipid raft-associated scaffolding protein controlling the acrosome reaction in mammalian spermatozoa. J Cell Physiol 214:757–768
65. Ackermann F, Zitranski N, Borth H et al (2009) CaMKIIalpha interacts with multi-PDZ domain protein MUPP1 in spermatozoa and prevents spontaneous acrosomal exocytosis. J Cell Sci 122:4547–4557
66. Dunbar BS, Dudkiewicz AB, Bundman DS (1985) Proteolysis of specific porcine zona pellucida glycoproteins by boar acrosin. Biol Reprod 32:619–630
67. Bleil JD, Greve JM, Wassarman PM (1988) Identification of a secondary sperm receptor in the mouse egg zona pellucida: role in maintenance of binding of acrosome-reacted sperm to eggs. Dev Biol 128:376–385
68. Howes E, Pascall JC, Engel W, Jones R (2001) Interactions between mouse ZP2 glycoprotein and proacrosin; a mechanism for secondary binding of sperm to the zona pellucida during fertilization. J Cell Sci 114:4127–4136
69. Miller DJ, Gong X, Shur BD (1993) Sperm require beta-N-acetylglucosaminidase to penetrate through the egg zona pellucida. Development 118:1279–1289
70. Hao Z, Wolkowicz MJ, Shetty J et al (2002) SAMP32, a testis-specific, isoantigenic sperm acrosomal membrane-associated protein. Biol Reprod 66:735–744
71. Bi M, Hickox JR, Winfrey VP et al (2003) Processing, localization and binding activity of zonadhesin suggest a function in sperm adhesion to the zona pellucida during exocytosis of the acrosome. Biochem J 375:477–488
72. Shetty J, Wolkowicz MJ, Digilio LC et al (2003) SAMP14, a novel, acrosomal membrane-associated, glycosylphosphatidylinositol-anchored member of the Ly-6/urokinase-type plasminogen activator receptor superfamily with a role in sperm-egg interaction. J Biol Chem 278:30506–30515
73. Hayashi M, Yonezawa N, Katsumata T et al (2004) Activity of exoglycosidases in ejaculated spermatozoa of boar and bull. Zygote12:105–109
74. Jagadish N, Rana R, Selvi R et al (2005) Characterization of a novel human sperm-associated antigen 9 (SPAG9) having structural homology with c-Jun N-terminal kinase-interacting protein. Biochem J 389:73–82
75. Yu Y, Xu W, Yi YJ et al (2006) The extracellular protein coat of the inner acrosomal membrane is involved in zona pellucida binding and penetration during fertilization: characterization of its most prominent polypeptide (IAM38) Dev Bio 290:32–43
76. Ziyyat A, Rubinstein E, Monier-Gavelle F et al (2006) CD9 controls the formation of clusters that contain tetraspanins and the integrin a_6b_1, which are involved in human and mouse gamete fusion. J Cell Sci 119: 416–424
77. Fujihara Y, Murakami M, Inoue N et al (2010) Sperm equatorial segment protein 1, SPESP1, is required for fully fertile sperm in the mouse. J Cell Si 123:531–1536
78. Kaji K, Oda S, Shikano T et al (2000) The gamete fusion process is defective in eggs of CD9-deficient mice. Nat Genet 24:279–282
79. Le Naour F, Rubinstein E, Jasmin C et al (2000) Severely reduced female fertility in CD9-deficient mice. Science 287:319–321
80. Maleszewski M, Kimura Y, Yanagimachi R (1996) Sperm membrane incorporation into oolemma contributes to the oolemma block to sperm penetration: evidence based on intracytoplasmic sperm injection experiments in the mouse. Mol Reprod Dev 44:256–259
81. Okabe M, Yagasaki M, Oda H et al (1988) Effect of a monoclonal anti-mouse sperm antibody (OBF13) on the interaction of mouse sperm with zona-free mouse and hamster eggs. J Reprod Immunol 13:211–219
82. Inoue N, Kasahara T, Ikawa M et al (2010) Identification and disruption of sperm-specific angiotensin converting enzyme-3 (ACE3) in mouse. PLoS ONE 5: e10301
83. Yáñez-Mó M, Barreiro O, Gordon-Alonso M et al (2009) Tetraspanin-enriched microdomains; a functional unit in cell plasma membranes. Trends Cell Biol 19:434–446
84. Rubinstein E, Ziyyat A, Prenant M et al (2006) Reduced fertility of female mice lacking CD81. Dev Biol 290: 351–358
85. Tiede A, Nischan C, Schubert J et al (2000) Characterisation of the enzymatic complex for the first step in glycosylphosphatidylinositol biosynthesis. Int J Biochem Cell Biol 32:339–350
86. Alfieri JA, Martin AD, Takeda J et al (2003) Infertility in female mice with an oocyte-specific knockout of GPI-anchored proteins. J Cell Sci 116:2149–2155
87. Ellerman DA, Pei J, Gupta S et al (2009) Izumo is part of a multiprotein family whose members form large complexes on mammalian sperm. Mol Reprod Dev 76:1188–1199
88. Inoue N, Ikawa M, Isotani A et al (2005) The immunoglobulin superfamily protein Izumo is required for sperm to fuse with eggs. Nature 434:234–238

89. Han C, Choi E, Park I et al (2009) Comprehensive analysis of reproductive ADAMs: Relationship of ADAM4 and ADAM6 with an ADAM complex required for fertilization in mice. Biol Reprod 80:1001–1008
90. Stein KK, Go JC, Primakoff P et al (2005) Defects in secretory pathway trafficking during sperm development in Adam2 knockout mice. Biol Reprod 73:1032–1038
91. Sosnik J, Miranda PV, Spiridonov NA et al (2009) Tssk6 is required for Izumo relocalization and gamete fusion in the mouse. J Cell Sci 122:2741–2749
92. Lewis WH, Wright ES (1935) On the early development of the mouse egg. Carnegie Inst Contrib Embryol 25:113–143
93. Odor DL, Blandau RJ (1949) The frequency of supernumerary sperm in rat ova. Anat Rec 104:1–11
94. Austin CR (1961) The mammalian egg. Charles C. Thomas, Springfield, IL
95. Hunter RH (1990) Fertilization of pig eggs in vivo and in vitro. J Reprod Fertil Suppl 40:211–226
96. Sengoku K, Tamate K, Horikawa M et al (1995) Plasma membrane block to polyspermy in human oocytes and preimplantation embryos. J Reprod Fertil 105:85–90
97. Hunter RH, Vajta G, Hyttel P (1998) Long-term stability of the bovine block to polyspermy. J Exp Zool 280: 182–188
98. Wolf DE, Edidin M, Handyside AH (1981) Changes in the organization of the mouse egg plasma membrane upon fertilization and first cleavage: Indications from the lateral diffusion rates of fluorescent lipid analogues. Dev Biol 85:195–198
99. Wolf DE, Ziomek CA (1983) Regionalization and lateral diffusion of membrane proteins in unfertilized and fertilized mouse eggs. J Cell Biol 96:1786–1790
100. Larson SM, Lee HJ, Hung PH et al (2010) Cortical mechanics and meiosis II completion in mammalian oocytes are mediated by myosin-II and Ezrin-Radixin-Moesin (ERM) proteins. Mol Biol Cell 21:3182–3192
101. Wolf DP, Nicosia SV, Hamada M (1979) Premature cortical granule loss does not prevent sperm penetration of mouse eggs. Dev Biol 71:22–32
102. Horvath PM, Kellom T, Caulfield J et al (1993) Mechanistic studies of the plasma membrane block to polyspermy in mouse eggs. Mol Reprod Dev 34:65–72
103. Maleszewski M, Kimura Y, Yanagimachi R (1996) Sperm membrane incorporation into oolemma contributes to the oolemma block to sperm penetration: evidence based on intracytoplasmic sperm injection experiments in the mouse. Mol Reprod Dev 44:256–259
104. Wortzman-Show GB, Kurokawa M et al (2007) Calcium and sperm components in the establishment of the membrane block to polyspermy: studies of ICSI and activation with sperm factor. Mol Human Reprod 13:557–565
105. Dale B, Wilding M, Coppola G, Tosti E (2010) How do spermatozoa activate oocytes? Reprod Biomed Online 21:1–3
106. Hoodbhoy T, Talbot P (2001) Characterization, fate, and function of hamster cortical granule components. Mol Reprod Dev 58:223–235
107. Tsai PS, van Haeften T, Gadella BM (2010) Preparation of the cortical reaction: maturation depdent migration of SNARE proteins, clathrin and complexin to the porcine oocyte's surface blocks membrane traffic until fertilization. Biol Reprod doi: 10.1095/biolreprod.110.085647
108. Gwatkin RB, Williams DT, Hartmann JF et al (1973) The zona reaction of hamster and mouse eggs: production in vitro by a trypsin-like protease from cortical granules. J Reprod Fertil 32:259–265
109. Wolf DP, Hamada M (1977) Induction of zonal and egg plasma membrane blocks to sperm penetration in mouse eggs with cortical granule exudate. Biol Reprod 17:350–354
110. Ducibella T (1996) The cortical reaction and development of activation competence in mammalian oocytes. Hum Reprod Update 2:29–42
111. Hoodbhoy T, Talbot P (1994) Mammalian cortical granules: contents, fate, and function. Mol Reprod Dev 39: 439–448
112. Izadyar F, Hage WJ, Colenbrander B et al (1988) The promotory effect of growth hormone on the developmental competence of in vitro matured bovine oocytes is due to improved cytoplasmic maturation. Mol Reprod Dev 49:444–453
113. Abbott AL, Fissore RA, Ducibella T (2001) Identification of a translocation deficiency in cortical granule secretion in preovulatory mouse oocytes. Biol Reprod 65:1640–1647
114. Tae JC, Kim EY, Jeon K et al (2008) A MAPK pathway is involved in the control of cortical granule reaction and mitosis during bovine fertilization. Mol Reprod Dev 75:1300–1306
115. Sun QY (2003) Cellular and molecular mechanisms leading to cortical reaction and polyspermy block in mammalian eggs. Microsc Res Tech 61:342–348
116. Florman HM, Ducibella T (2006) Fertilization in mammals. In: Knobil E, Neill JD (eds) Physiology of reproduction, 3rd edn. Elsevier, Amsterdam, The Netherlands
117. Zarelli VE, Ruete MC, Roggero CM et al (2009) PTP1B dephosphorylates N-ethylmaleimide-sensitive factor and elicits SNARE complex disassembly during human sperm exocytosis. J Biol Chem 284:10491–10503

118. Miyado K, Yamada G, Yamada S et al (2000) Requirement of CD9 on the egg plasma membrane for fertilization. Science 287:321–324
119. He ZY, Brakebusch C, Fassler R et al (2003) None of the integrins known to be present on the mouse egg or to be ADAM receptors are essential for sperm–egg binding and fusion. Dev Biol 254:226–237
120. Miller BJ, Georges-Labouesse E, Primakoff P et al (2000) Normal fertilization occurs with eggs lacking the integrin alpha$_6$beta$_1$ and is CD9-dependent. J Cell Biol 149:1289–1295
121. Baessler K, Lee Y, Sampson N (2009) Beta1 integrin is an adhesion protein for sperm binding to eggs. ACS Chem Biol 4:357–366
122. Evans JP (2009) Egg integrins: back in the game of mammalian fertilization. ACS Chem Biol 4:321–323
123. Vjugina U, Zhu X, Oh E et al (2009) Reduction of mouse egg surface integrin alpha9 subunit (ITGA9) reduces the egg's ability to support sperm-egg binding and fusion. Biol Reprod 80:833–841
124. Eto K, Huet C, Tarui T et al (2002) Functional classification of ADAMs based on a conserved motif for binding to integrin alpha$_9$beta$_1$: implications for sperm-egg binding and other cell interactions. J Biol Chem 277: 17804–17810
125. Inoue N, Ikawa M, Isotani A et al (2005) The immunoglobulin superfamily protein Izumo is required for sperm to fuse with eggs. Nature 434:234–238
126. Bigler D, Takahashi Y, Chen MS et al (2000) Sequence-specific interaction between the disintegrin domain of mouse ADAM2 (fertilin b) and murine eggs: Role of the a_6 integrin subunit. J Biol Chem 275:11576–11584
127. Takahashi Y, Bigler D, Ito Y et al (2001) Sequence-specific interaction between the disintegrin domain of mouse ADAM3 and murine eggs: Role of the beta$_1$ integrin-associated proteins CD9, CD81, and CD98. Mol Biol Cell 12:809–820
128. Zhu X, Bansal NP, Evans JP (2000) Identification of key functional amino acids of the mouse fertilin beta (ADAM2) disintegrin loop for cell-cell adhesion during fertilization. J Biol Chem 275:7677–7683
129. Cuasnicu PS, Conesa D, Rochwerger L (1990) Potential contraceptive use of an epididymal protein that participates in fertilization. In: Alexander NJ, Griffin D, Speiler JM, Waites GMH (eds) Gamete Interaction: Prospects for Immunocontraception. Wiley-Liss, New York, NY, pp. 143–153
130. Cuasnicu, PS, Gonzalez-Echeverria MF, Piazza AD et al (1984) Antibody against epididymal glycoprotein blocks fertilizing ability in rats. J Reprod Fert 72:467–471
131. Da Ros VG, Maldera JA, Willis WD et al (2008) Impaired sperm fertilizing ability in mice lacking Cysteine-RIch Secretory Protein 1 (CRISP1). Dev Biol 320:12–8
132. Wolkowicz MJ, Shetty J, Westbrook A (2003) Equatorial segment protein defines a discrete acrosomal subcompartment persisting throughout acrosomeal biogenesis. Biol Reprod 69:735–745
133. Toshimori K, Saxena DK, Tanii I et al (1998) An MN9 antigenic molecule, equatorin, is required for successful sperm-oocyte fusion in mice. Biol Reprod 59:22–29
134. Yamatoya K, Yoshida K, Ito C et al (2009) Equatorin: identification and characterization of the epitope of the MN9 antibody in mouse. Biol Reprod 81:889–897
135. Herrero MB, Mandal A, Digilio LC et al (2005) Mouse SLLP1, a sperm lysozyme-like protein involved in sperm-egg binding and fertilization. Dev Biol 284:126–42
136. Mandal A, Klotz KL, Shetty J et al (2003) SLLP1, A unique, intra-acrosomal, non-bacteriolytic, c lysozyme-like protein of human spermatozoa. Biol Reprod 68:1525–1537
137. Shetty J, Wolkowicz MJ, Digilio LC et al (2003) SAMP14, a novel, acrosomal membrane-associated, glycosylphosphatidylinositol-anchored member of the Ly-6/urokinase-type plasminogen activator receptor superfamily with a role in sperm-egg interaction. J Biol Chem 278:30506–30515
138. Correa LM, Cho C, Myles DG et al (2000) A role for a TIMP-3-sensitive Zn^{2+}-dependent metalloprotease in mammalian gamete membrane fusion. Dev Biol 225:124–134
139. Ellerman DA, Myles DG, Primakoff P (2006) A role for sperm surface protein disulfide isomerase activity in gamete fusion: evidence for the participation of ERp57. Dev Cell 10:831–837
140. Mammoto A, Masumoto N, Tahara M et al (1997) Involvement of a sperm protein sensitive to sulfhydry-depleting reagents in mouse sperm-egg fusion. J Exp Zool 278:178–188
141. Wilson DW, Wilcox CA, Flynn GC (1989) A fusion protein required for vesicle-mediated transport in both mammalian cells and yeast. Nature 339:355–359
142. Fenouillet E, Barbouche R, Courageot J et al (2001) The catalytic activity of protein disulfide isomerase is involved in human immunodeficiency virus envelope-mediated membrane fusion after CD4 cell binding. J Infect Dis 183:744–752
143. Ou W, Silver J (2006) Role of protein disulfide isomerase and other thiol-reactive proteins in HIV-1 envelope protein-mediated fusion. Virology 350:406–417