The Molecular Basis of Sperm – Oocyte Interactions

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Bachelor of Biotechnology Honours Class I

Doctor of Philosophy
Declaration

I hereby certify that this thesis is submitted in the form of a series of published papers of which I am the lead author. I have included as part of the thesis a written statement from each co-author; and endorsed by the Faculty Assistant Dean (Research Training), attesting to my contribution to the joint publications.

(Signed)……………………………

February 2012
ACKNOWLEDGEMENTS

“Success is the ability to go from one failure to another with no loss of enthusiasm”

“Out of intense complexities intense simplicities emerge”

Winston Church, 1874 – 1965

I would very much like to thank my supervisors Professor John Aitken and Associate Professor Brett Nixon for your guidance, patients, encouragement and friendship over the last 5 years. Your belief and trust in my abilities have provided me with the opportunity to express myself as a scientist, opening the door to a passion that I very much look forward to expressing over the next thirty or so years. I would also like to thank my step father Dr. Robert B.M. Dun OAM, whose passion for hard work coupled with clear thinking has encouraged me to pursue a life of discovery. My favourite memory from speaking to him during my studies was when he was describing his PhD to me, he said; “when I was doing my PhD I couldn’t believe that they were paying me to do something I considered a hobby”.

To my dear family I would very much like to say thank you to you all for believing in me and encouraging me to take on this challenging journey. It has been wonderful to grow up with each of you and I am extremely excited about sharing the next phase of our lives. I would especially like to thank my mother Sonia for all of her patients, generosity and love that she has shown to me over the last 34 years. No son could ask for any more. I would also like to thank my wifes family the Hindley’s, especially to Louise and Chris who have welcomed me into their family with open arms. I truly feel like a member of your wonderful clan, never more so than on Saturdays when I can hear Christopher yelling out from the sideline “come on Matthew”, it always makes me try harder. To my Western Australian friends, thanks for nurturing me into adulthood and then packaging me off to University. To the herd of special Seahorses that I love, you guys are the best friends to never win a premiership, no really thanks to you all for the good times and the friendship. Dearest Keiran thank you for everything, you are a wonderful friend. Smithy thank to you for keeping me sane, good luck with your own adventure.

My dearest Phoebe, without you this would never have happened. You are my inspiration, my muse and my love. I cherish every moment we spend together and am truly appreciative of your never wavering belief in me. I look forward to the next phase of our lives together and sincerely thank you for all that you have done during my studies. This thesis is dedicated Dr. Phoebe Anne Dun.
LIST OF PUBLICATIONS INCLUDED AS PART OF THE THESIS


   This project was led by Matthew, 70% contribution. Matthew conducted the literature search, compiled all data for tables, and contributed to the figures. He also took the lead role in manuscript preparation. Other authors contributed as follows: B. Nixon (20%), R.J. Aitken (5%) and L.A. Mitchell (5%).


   This project was led by Matthew, 75% contribution. Matthew led the study design collected all of the data and prepared the manuscript. Other authors contributed as follows: B. Nixon (10%), R.J. Aitken (5%), Nathan Smith (5%) and Baker and Lin contributed the remainder.


   This project was led by Matthew, 60% contribution. Matthew completed all data analyses took the lead role in manuscript preparation and collected most of the data that were analysed. Other authors contributed as follows: B. Nixon (10%), Amanda Anderson (10%), Kelly Asquith (10%), and McLaughlin, Aitken and Bromfield contributed the remainder.


   This project was led by Matthew, 90% contribution. Matthew conducted the literature search, compiled all data and tables, designed and produced all figures and also took
the lead role in manuscript preparation. Other authors contributed as follows: B. Nixon (5%) and R.J. Aitken (5%).

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ADDITIONAL PUBLICATION


As a result of the significant findings described in publication 1 and 2, these techniques have been employed in the above mentioned publication (Redgrove et al., 2011). Matthew contributed to the experimental design as well as performed a key figure of this manuscript.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>1-D</td>
<td>one-dimensional</td>
</tr>
<tr>
<td>1-D BN-PAGE</td>
<td>one-dimensional blue native polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>2-D</td>
<td>two-dimensional</td>
</tr>
<tr>
<td>ADAM</td>
<td>a disintegrin and a metalloprotease domain</td>
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<tr>
<td>ADAMTS10</td>
<td>a disintegrin and a metalloprotease domain with thrombospondin10</td>
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<td>ADAMTS10-A</td>
<td>ADAMTS10 active enzyme</td>
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<td>ADAMTS10 zymogen</td>
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<td>Ahal</td>
<td>Activator of HSP90 ATPase</td>
</tr>
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<td>AKAP</td>
<td>A kinase anchoring protein</td>
</tr>
<tr>
<td>AMP</td>
<td>ampicillin</td>
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<td>ANOVA</td>
<td>analysis of variance</td>
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<tr>
<td>apY</td>
<td>anti-phosphotyrosine</td>
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<td>AMP</td>
<td>adenosine monophosphate</td>
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<td>adenosine triphosphate</td>
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<td>bp</td>
<td>base pairs</td>
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<td>BN-PAGE</td>
<td>blue native polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>BWW</td>
<td>Biggers, Whitten and Whittingham medium</td>
</tr>
<tr>
<td>CABYR</td>
<td>calcium-binding tyrosine phosphorylation-regulating protein</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
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<td>cGMP</td>
<td>complementary guanosine monophosphate</td>
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<td>CHAPS</td>
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<td>carboxyl terminus of HSC-70 interaction protein (CHIP)</td>
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<tr>
<td>CRISP</td>
<td>cysteine-rich secretory protein</td>
</tr>
<tr>
<td>DABCO</td>
<td>1,4-diazobicyclo-[2.2.2]-octane</td>
</tr>
<tr>
<td>dATP</td>
<td>deoxyadenosine triphosphate</td>
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<td>dense bodies</td>
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<tr>
<td>dbcAMP</td>
<td>dibutyryl cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>dCTP</td>
<td>deoxyctydine triphosphate</td>
</tr>
<tr>
<td>DF</td>
<td>decapacitation factor</td>
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<td>deoxyguanosine triphosphate</td>
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<td>dimethyl sulfoxide</td>
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<td>deoxyribonucleic acid</td>
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<td>dNTP</td>
<td>deoxyribonucleotide</td>
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DSS  disuccinimidyl suberate
DTT  dithiothreitol
dTTP  deoxythymidine triphosphate
ECL  enhanced chemiluminescence
ECM  extracellular matrix
EMMPRIN  extracellular matrix metalloprotease inducer
ER  endoplasmic reticulum
ERK  extracellular-signal regulated kinase
ERP99  endoplasmin
FA-1  fertilisation antigen-1
FITC  fluorescein isothiocyanate
GalTase  galactosyltransferase
GCNA  germ cell nuclear antigen
GPI  glycosylphosphatidylinositol
GRP78  glucose regulated protein 78
GRP94  glucose regulated protein 94 (endoplasmin)
h  hours
HBSS  Hanks buffered salt solution
hCG  human chorionic gonadotropin
HDL  high density lipoprotein
HIP  HSP70-interacting protein
His  Histidine
HK-1  hexokinase type 1
HOP  HSP-organising protein
HOS  hyperosmotic swelling
HRP  horseradish peroxidase
HSP  heat shock protein
HSP10  heat shock protein 10
HSP60  heat shock protein 60
HSP70  heat shock protein 70
HSP90  heat shock protein 90
i.d.  inner diameter
IEF  isoelectric focusing
IgG  immunoglobulin G
IPTG  isopropylthio-β-D-galactoside
IU  international units
KAN  kanamycin
kDA  kilodalton
LB  Luria broth
M  molar
MALDI-TOF  matrix-assisted laser desorption ionisation – time of flight
MALDI-TOF/TOF  matrix-assisted laser desorption ionisation – time of flight/time of flight
MAPK  mitogen-activated protein kinase
Min  minutes
MMP  matrix metalloprotease
mRNA  messenger ribonucleic acid
MS/MS  tandem mass spectrometry
MW  molecular weight
NCBI  National Center for Biotechnology Information (USA)
o.d.  outer diameter
OD  optical density
ODF  outer dense fiber
OSP  oviductal secretory protein
PBS  phosphate buffered saline
PCR  polymerase chain reaction
The remarkable cellular communication events that characterise the highly species specific interactions observed during the ontogeny of mammalian fertilization, represent some of the most intriguing in all of biology. Given the 60 years or so of research conducted to elucidate the precise mechanisms that underpin these interactions, it is surprising that they still remain largely unknown. This can be mostly attributed to the unique luminal environment in which the sperm reside following insemination and the direct effects that these fluids have on their functionality. Although immense controversy surrounds the precise ligand responsible for the spermatozoa's binding to the oocyte’s zona pellucida, considerable contention is also afforded to the mechanism by which they bind. A number of landmark papers have recently emerged to suggest that these initial binding events may be facilitated by the formation and presentation of multimeric zona pellucida receptor complexes on the sperm surface during their terminal maturation, rather than the widely held paradigm that the zona pellucida receptor is a single molecular entity. During these studies the use of blue native polyacrylamide gel electrophoresis, for the first time in mammalian sperm, has provided direct evidence that a number of multimeric zona receptor complexes indeed reside on the apical plasma membrane of capacitated sperm and that two of these complexes have the ability to interact with the zona pellucida. Proteomic analysis of these two complexes has indicated that molecular chaperones (CCT/TRiC complex and HSPD1) are responsible for the formation of each complex, and individually, these complexes contain a number of receptor proteins (ZPBP2, ZP3R and ADAMTS10) that potentially provide the zona pellucida affinity. Collectively, these data provide an important biochemical insight into the molecular basis of sperm-zona pellucida interaction and a plausible explanation for how spermatozoa gain their ability to fertilize.
Sperm-Zona Pellucida Interaction: Molecular Mechanisms and the Potential for Contraceptive Intervention

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Accepted for publication by Handbook of Experimental Pharmacology
ABSTRACT

At the moment of insemination millions of mammalian sperm cells are released into the female reproductive tract with the single goal of finding the oocyte. The spermatozoa subsequently ignore the thousands of cells they make contact with during their journey to the site of fertilization, until they reach the surface of the oocyte. At this point, they bind tenaciously to the acellular coat, known as the zona pellucida, that surrounds the oocyte and orchestrate a cascade of cellular interactions that culminate in fertilization. These exquisitely cell- and species- specific recognition events are among the most strategically important cellular interactions in biology. Understanding the cellular and molecular mechanisms that underpin them has implications for the aetiology of human infertility and the development of novel targets for fertility regulation. Herein we describe our current understanding of the molecular basis of successful sperm-zona pellucida binding.
INTRODUCTION

The continuance of all mammalian species relies on the cellular interactions that occur between gametes during the process of fertilization. These remarkable cell- and species-specific interactions are initiated by recognition and binding of a spermatozoon to the zona pellucida (ZP), a thick extracellular matrix that surrounds and protects the ovulated oocyte. Understanding the basic biology of this interaction has profound implications for the diagnosis of human infertility and the development of novel targets for fertility regulation. Accordingly, considerable research effort has been devoted to investigating the molecular mechanisms that underpin sperm-oocyte interaction. Nonetheless, this fundamental interaction remains poorly understood and is the subject of considerable controversy.

Studies of sperm-zona pellucida adhesion conducted during the last 60 years have led to the advancement of a widely accepted paradigm that sperm-ZP interaction is mediated by a single sperm receptor that engages with a complementary ligand within the ZP. While such a model holds obvious appeal, it fails to account for the fact that targeting of individual sperm proteins through inhibition studies (e.g. competitive substrates and mono-specific antibodies) and / or genetic deletion has failed to elicit the anticipated block to sperm-ZP interaction. Against this background we have introduced a novel hypothesis which states that sperm-ZP interaction requires the coordinated action of several sperm proteins, each of which contributes to the high affinity and specificity of this fundamental cellular interaction. Furthermore, we have also suggested that these discrete zona recognition proteins are assembled into a multimeric receptor complex during sperm capacitation. Throughout this review we have chosen to focus on the mouse model as it represents the most widely studied of all laboratory animals with respect to mammalian fertilization. However, the authors encourage caution in extrapolating these data for cross-species comparison.

Sperm-zona pellucida interaction

The zona pellucida

The zona pellucida (ZP) is synthesized during oogenesis and is located between the oocyte and the innermost layer of granulosa cells (Wassarman and Albertini, 1994). The mature ZP is a porous matrix whose functions include the mediation of species-specificity in gamete interaction (Vieira and Miller, 2006), prevention of polyspermy, and protection of the developing embryo prior to implantation (McLeskey et al., 1998a). The importance of these functions has been underscored by studies involving the targeted deletion of the genes encoding ZP proteins (Rankin and Dean, 2000). Such studies have shown that the ZP is essential in maintaining the physiological status of the oocyte and in regulating successful growth and
development of the embryo. Furthermore, it has been demonstrated that oocytes failing to correctly translate and assemble a ZP are unable to be fertilized (Liu et al., 1996; Rankin et al., 1996). Indeed, this structure represents the first barrier that mammalian sperm must encounter and breach in order to achieve fertilization.

In the mouse, the ZP is assembled as a trimeric protein matrix composed of long ZP2 and ZP3 heterodimer filaments that are cross-linked by homodimers of the third zona protein, ZP1 (Bleil and Wassarman, 1980c; Greve and Wassarman, 1985; Wassarman and Mortillo, 1991) in a molar ratio of 4:4:1, respectively (Green, 1997) (Fig. 1). The three mouse ZP proteins were initially characterized by Bleil and Wassarman (Bleil and Wassarman, 1980c; Bleil and Wassarman, 1980d; Bleil and Wassarman, 1980a) and have subsequently been shown to be encoded by single-copy genes located on chromosomes 19, 7, and 5 (Epifano et al., 1995). Determination of the primary structure of the ZP proteins revealed considerable divergence between their predicted molecular weight and that determined experimentally via SDS-PAGE (ZP1, ~200 kDa; ZP2, ~120 kDa; and ZP3 ~83 kDa). Such differences are accounted for by dramatic post-translational modification of the mature proteins, primarily in the form of glycosylation (Ringuette et al., 1986; Kinloch et al., 1988; Liang et al., 1990; Epifano et al., 1995), a feature which appears critical for their biological activity. The oligosaccharides are in turn modified by sulfation, sialylation, and the addition / removal of other moieties (Liu et al., 1997).

As well as being essential structural components of the zona pellucida, ZP2 and ZP3 possess specific functions during the sequence of sperm-oocyte interactions that culminate in fertilization. The balance of evidence indicates that mouse ZP3 functions as both the primary sperm receptor, preferentially binding the plasma membrane region overlying the acrosome of acrosome-intact sperm, and as an inducer of the acrosome reaction following recognition of the zona matrix (Bleil and Wassarman, 1986; Vazquez et al., 1989). For instance, it has been demonstrated that solubilized ZP3 is able to competitively inhibit sperm-ZP binding, whereas ZP1 and ZP2 do not elicit a similar response (Bleil and Wassarman, 1980a). Similarly, female mice bearing a null mutation for ZP3 are infertile (Liu et al., 1996). It is noteworthy however, that ZP3 is not uniquely responsible for facilitating sperm interaction in all mammalian species. In the pig for instance, sperm-binding activity resides in a heterodimer formed between the ZP1 and the ZP3 orthologues (Yurewicz et al., 1998). Similarly, the ZP1 orthologue, ZPB, has been shown to play a major role in sperm binding to the bovine zona (Yonezawa et al., 2001), while in humans and the bonnet monkey there is also compelling evidence that a fourth zona glycoprotein, ZP4, which is thought to be dysfunctional in the mouse (Lefievre et al., 2004), participates in primary sperm-adhesion (and the induction of acrosomal exocytosis) (Gupta et
al., 2007b). The biological significance of this interspecies complexity in ZP structure and function is presently unknown. Nonetheless, given that the mouse remains the most widely studied model for understanding sperm-ZP interaction, this species will serve as the focus for the following discussion.

**Figure. 1. The mouse zona pellucida.** The ovulated oocyte is surrounded by the ZP, an acellular matrix whose functions include the mediation of species-specificity in gamete interaction, prevention of polyspermy, and protection of the developing embryo prior to implantation. The mouse zona pellucida is a fibrillar structure, the major strands of which are composed of repeating dimers of ZP2 and ZP3 glycoproteins. These strands are crosslinked by ZP1 to form a meshlike network.

The mouse ZP3 glycoprotein comprises a number of domains including an N-terminal signal sequence, a large ZP domain, a consensus furin cleavage site (CFCS) and a hydrophobic transmembrane region located near the C-terminus. The ZP domain is in fact common to all ZP proteins and consists of a 260 amino acid sequence with 8 conserved cysteine residues. This domain is believed to be responsible for the polymerization of the ZP proteins into the extensive lattice-like network that enables it to surround the oocyte (Jovine et al., 2002). Mouse ZP3 is initially synthesized as a 424 amino acid polypeptide, but is subject to dramatic post-translation modification resulting in the addition of complex N- (asparagine) and O-linked (serine/threonine) carbohydrates. The sperm-binding domain of the ZP3 glycoprotein has been mapped to the C-terminus of the protein and encompasses both the ZP and CFCS domains (Litscher et al., 2009). This region of polypeptide is commonly referred to as the sperm combining site and contains O-linked sugar residues that putatively interact with
complementary receptors on the surface of acrosome intact spermatozoa (Wassarman et al., 2004b). However, as discussed below, this model of sperm-ZP interaction is not universally accepted.

**The role of O-linked ZP3 sugars in mouse sperm-ZP interaction**

Prevailing evidence indicates that primary sperm-oocyte interaction is mediated by binding between ZP3 carbohydrates (Gwatkin et al., 1977; Hoodbhoy and Dean, 2004) and complementary lectin-like proteins located on the surface of the sperm (see Section 2.2 and Table 1). The most widely accepted model of this interaction, emphasizes the importance of O-linked carbohydrate moieties (Florman and Wassarman, 1985; Litscher et al., 1995; Wassarman et al., 1999). For instance, it has been demonstrated that small glycopeptides derived from mouse ZP3 retain full sperm receptor activity (Florman et al., 1984). Conversely, the enzymatic and chemical removal or modification of O-linked oligosaccharides from ZP3 abolishes its sperm receptor activity whereas the removal of N-linked oligosaccharides elicits only negligible effects (Florman and Wassarman, 1985). The importance of O-linked glycans has been further advanced by the demonstration that genetically engineered chimeric mouse oocytes expressing human ZP3, acquire the same O-linked glycans as mouse ZP3 and bind mouse, rather than human, spermatozoa (Rankin et al., 1996; Hoodbhoy and Dean, 2004). Furthermore, the results of targeted mutagenesis studies indicate that the key O-linked carbohydrates responsible for sperm-binding activity most likely reside within the C-terminal portion of the ZP3 polypeptide chain (Kinloch et al., 1995).

However, while it is widely accepted that the principal bioactive component of ZP3 is associated with its O-linked carbohydrate moieties, the relative importance of the different oligosaccharide ligand(s) remains to be unequivocally established (Easton et al., 2000; Diekman, 2003). This situation is due in part to the complexity of O-linked glycans, with recent mass spectrometry analysis of mouse ZP3 glycosylation revealing that the predominant core type 2 sequences are terminated with sialic acid, lacNac (Galβ1-4GlcNAc), lacdiNac (GalNAcβ1-4GlcNAc), Galα1-3Gal, and NeuAcα2-3[GalNAcβ1→4]Galβ1→4 (Sda antigen) (Dell et al., 2003). Early studies suggested that galactose, located in an α-linkage at the non-reducing terminus of O-linked oligosaccharides served as a critical determinant of sperm binding to ZP3 (Florman and Wassarman, 1985; Bleil and Wassarman, 1988b; Litscher et al., 1995). However, such claims have since been refuted (Nagdas et al., 1994; Thall et al., 1995b) in favor of terminal β-linked galactose (Yonezawa et al., 2005) in addition to N-acetylglucosamine (Miller et al., 1992), mannose (Tulsiani et al., 1989; Cornwall et al., 1991), N-acetylgalactosamine and fucose residues (Johnston et al., 1998; Kerr et al., 2004), each of which have been demonstrated to inhibit sperm-zona binding (reviewed in (Benoff, 1997). Arguments against the involvement...
of α-linked galactose residues include the demonstration that sperm-ZP interaction is inhibited by pre-treatment of oocytes with β-galactosidase but not α-galactosidase (Mori et al., 1997b). Furthermore, female transgenic mice bearing a null mutation for α1→3 galactosyltransferase (and therefore terminal Galα1→3Gal residues) produce oocytes that display normal sperm binding characteristics (Thall et al., 1995b). These data are further supported by evidence from a novel heterologous cell-adhesion assay between mouse spermatozoa and rabbit erythrocytes. The precocious binding of these two cell types appears to be attributed to the presence of multiple branches of Galα1→3Galβ1→4GlcNAcβ1→6 linked to a linear polyolactosamine backbone in the erythrocytes (Clark et al., 1996; Clark and Dell, 2006b; Sutton-Smith et al., 2007). However, pre-treatment of the erythrocytes with α-galactosidase fails to elicit the anticipated reduction in sperm adhesion (Clark and Dell, 2006b) thereby suggesting sperm instead interact with β1→4-linked glycans. These results indicate that sperm can recognize terminal Galβ1→4GlcNAc sugars (Mori et al., 1997b) which interestingly, are essentially the same structures in both ZP3 and ZP2 (Noguchi and Nakano, 1993).

The role of N-linked ZP3 sugars in sperm-ZP interaction

In addition to the classes of O-linked oligosaccharides described above, murine ZP3 is also known to be furnished with both high mannose and complex-type N-glycans (Easton et al., 2000). The predominant high mannose-type glycan is composed of Man5GlcNAc2, whereas the array of biantennary, triantennary, and tetraantennary complex-type N-glycans have been shown to be terminated with the following antennae: Galβ1→4GlcNAc, NeuAcα2→3Galβ1→4GlcNAc, NeuGcα2→3Galβ1→4GlcNAc, the Sda antigen, and terminal GlcNAc (Easton et al., 2000). Interestingly, with the exception of the latter sugar, these N-glycan sequences resemble those that terminate the β1→6-linked branches of ZP3 O-glycans. Such findings, raise the prospect that N-linked glycans may also contribute to sperm adhesion. Indeed, in species such as the pig, N-linked and not the O-linked carbohydrates appear to mediate sperm-ZP interaction (Yonezawa et al., 1995; Nakano et al., 1996). It may therefore be argued that carbohydrate moieties of the ZP glycoproteins may be underpinning the species-specificity associated with sperm-ZP interaction.

Carbohydrate-independent models of sperm-ZP interaction

Notwithstanding the compelling evidence in favor of carbohydrate residues as the main determinant in mediating sperm-ZP interaction, the production of transgenic mice bearing null mutations for key glycosyltransferases have also raised some doubt regarding the overall necessity of ZP carbohydrates for binding sperm. For instance, female mice singly deficient in any one of the three known glycosyltransferases that generate core 2 O-glycans (C2GnT1, C2GnT2, and C2GnT3), and therefore many of the O-glycans normally found in the zona, are
fertile (Ellies et al., 1998; Stone et al., 2009). Remarkably, elimination of all three C2GnTs is also permissive of fertility (Ellies et al., 1998). Similarly, mice that do not possess MGAT-I, the enzyme that initiates complex and hybrid-type glycan synthesis, produce oocytes that retain the ability to be fertilized (Shi et al., 2004).

Among the carbohydrate-independent models that have been proposed, recent analyzes conducted by Tanphaichitr and colleagues have raised the interesting prospect that the sulfation of ZP glycans may play a key role in sperm adhesion (Tanphaichitr et al., 2007a). Specifically, it has been postulated that sulfated sugar residues of ZP3 serve as a ligand for a sulfatase enzyme, arylsulfatase-A (ARSA), that is added to the sperm surface during post-testicular maturation and becomes annexed within the apical region of the sperm head following capacitation (Tanphaichitr et al., 1993; White et al., 2000; Weerachatyanukul et al., 2001; Carmona et al., 2002; Tantibhedhyangkul et al., 2002; Weerachatyanukul et al., 2003) (see Tab. 1).
<table>
<thead>
<tr>
<th>Name (synonyms)</th>
<th>Species</th>
<th>Evidence</th>
<th>References</th>
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<tr>
<td>α-D-mannosidase</td>
<td>Mouse, Rat,</td>
<td>Integral plasma membrane protein that may participate in sperm-ZP interaction by binding to mannose-containing ZP saccharides. Treatment of sperm with either D-mannose or an antibody raised against the protein elicit a dose-dependent inhibition of sperm-ZP binding.</td>
<td>(Akama et al., 2002; Cornwell et al., 1991; Pereira et al., 1998; Tulsi et al., 1993; Tulsi et al., 1989; Yoshida-Komiya et al., 1999)</td>
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<td>(MAN2B2)</td>
<td>Hamster, Human</td>
<td></td>
<td></td>
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<tr>
<td>Arylsulfatase A</td>
<td>Mouse, Human</td>
<td>Acquired from epididymal fluid via interaction with SGG and localizes to the membrane overlying the acrosome. A dose-dependent decrease in sperm-ZP binding is observed following pretreatment of sperm with ARSA antibodies. ARSA null males are fertile.</td>
<td>(Hess et al., 1996; Tantibhedhyangkul et al., 2002; Weerachatyanukul et al., 2003)</td>
</tr>
<tr>
<td>(AS-A; ARSA)</td>
<td>Boar</td>
<td></td>
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<tr>
<td>β-1,4-galactosyltransferase</td>
<td>Mouse, Rat, Human, Guinea pig, Rabbit, Bull, Boar, Stallion</td>
<td>Transmembrane protein located on dorsal surface of the anterior sperm head overlying the intact acrosome. Acts as a receptor for terminal GlcNAc residues on ZP3. ZP3-induced GalTase aggregation triggers a pertussis toxin-sensitive G-protein cascade leading to induction of acrosomal exocytosis. Transgenic mice overexpressing GalTase are hypersensitive to ZP3 and undergo precocious acrosome reactions. Sperm from mice bearing targeted deletions in GalTase are unable to bind ZP3 nor undergo ZP3-dependent acrosomal exocytosis. However, GalTase-null sperm retain the ability to bind to the egg coat.</td>
<td>(Lopez and Shur, 1987; Lu and Shur, 1997; Shi et al., 2001; Shur and Hall, 1982)</td>
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<tr>
<td><strong>Fertilization antigen 1</strong> (FA1)</td>
<td><strong>Mouse</strong></td>
<td>Localized to postacrosomal region of sperm head. Immunization of mice with recombinant FA-1 antigen induces a strong immunoconceptive response. Anti-FA-1 antibodies have been implicated in immune infertility in humans. No recorded knockout mouse.</td>
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<td><strong>Human</strong></td>
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<td><strong>Bull</strong></td>
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<tr>
<td>Glutathione S-transferase (GST)</td>
<td><strong>Mouse</strong></td>
<td>Two isoforms (Pi and Mu) localized to periphery of plasma membrane over anterior head, postacrosomal region and principal piece. Selective inhibition of Pi, but not Mu, isoform with specific antisera leads to a reduction in fertilization rate. Both isoforms bind to ZP3.</td>
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<td></td>
<td><strong>Rat</strong></td>
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<td></td>
<td><strong>Human</strong></td>
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<td><strong>Goat</strong></td>
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<tr>
<td>Milk fat globule-EGF factor 8 (MFGES; P47; SED1)</td>
<td><strong>Mouse</strong></td>
<td>An EGF repeat and discoidin domain protein that coats sperm (plasma membrane overlying the acrosome) within the epididymis. Binds specifically to the ZP of unfertilized but not fertilized eggs. Recombinant MFGES and anti-MFGES antibodies competitively inhibit sperm-egg binding. MFGES null males are subfertile and their sperm are unable to bind to the ZP in vitro.</td>
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<td><strong>Boar</strong></td>
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<tr>
<td>Proacrosin (acrosin)</td>
<td><strong>Mouse</strong></td>
<td>Localizes to acrosome and inner acrosomal membrane from where it is thought to mediate secondary binding to ZP2 following acrosomal exocytosis. Binding to ZP is non-enzymatic and thought to involve recognition of polysulphate groups on ZP glycoproteins. Acrosin null males are fertile but show compromised ZP penetration.</td>
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<td><strong>Boar</strong></td>
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(References: Coonrod et al., 1994; Menge et al., 1999; Naz et al., 1984; Naz et al., 1992; Naz and Zhu, 1998; Aravinda et al., 1995; Gopalakrishnan et al., 1998; Hemachand et al., 2002; Shaha et al., 1988; Ensslin et al., 1995; Ensslin et al., 1998; Ensslin and Shur, 2003; Baba et al., 1994; Howes et al., 2001; Howes and Jones, 2002; Urch and Patel, 1991)
<table>
<thead>
<tr>
<th>Protein Type</th>
<th>Species</th>
<th>Description</th>
<th>References</th>
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<tr>
<td>Sperm adhesion molecule 1 (SPAM1; PH-20)</td>
<td>All mammals</td>
<td>Widely conserved sperm surface protein. Localized to plasma membrane over anterior head of mouse sperm. Possesses hyaluronidase activity that assists in the digestion of cumulus cells. Relocalizes to inner acrosomal membrane following acrosome reaction where it is putatively involved in secondary ZP binding. SPAM1 null males are fertile but their sperm are less efficient in dispersal of cumulus cells.</td>
<td>(Baba et al., 2002; Hunnicutt et al., 1996; Lin et al., 1994; Morales et al., 2004; Myles and Primakoff, 1997)</td>
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<td>Sperm autoantigenic protein 17 (SPA17, SP17)</td>
<td>Mouse, Rabbit Human, Primates</td>
<td>SPA17 is conserved, highly antigenic protein variously localized to acrosome and flagellar fibrous sheath. Has been implicated in regulation of sperm maturation, capacitation, acrosome reaction and ZP binding. Shown to bind specific carbohydrate components (mannose) of the ZP.</td>
<td>(Chiriva-Internati et al., 2009; Grizzi et al., 2003; Yamasaki et al., 1995)</td>
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<tr>
<td>Spermadhesins (AWN; AQN-1; AQN-3)</td>
<td>Boar, Stallion Bull</td>
<td>Spermadhesin represents a major component of seminal plasma and is deposited over the sperm head via interaction with plasma membrane phospholipids. Multifunctional lectins able to bind to carbohydrates, sulfated glycosaminoglycans, phospholipids and protease inhibitors. May therefore participate in several sequential steps of the fertilization process.</td>
<td>(Petrunkina et al., 2000; Sanz et al., 1993; Sinowatz et al., 1995; Topfer-Petersen et al., 1998)</td>
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<tr>
<td>Sulfogalactosylglycerolipid (SGG)</td>
<td>Mouse, Rat Human, Boar</td>
<td>Localizes to plasma membrane overlying acrosome and postacrosomal regions of mouse and human spermatozoa. Restricted to anterior head plasma membrane of boar sperm. SGG is a major sperm sulfoglycolipid that putatively facilitates the uptake of sulfolipid-immobilizing protein-1 (SLIP1) and ARSA. Following capacitation, SGG is predominantly found in membrane rafts, microdomains that possess ZP affinity. Pretreatment of sperm with monovalent anti-SGG Fab fragments markedly inhibits sperm binding to the ZP.</td>
<td>(Bou Khalil et al., 2006; Kornblatt, 1979; Tannahachitirat et al., 1990; Tannahachitirat et al., 1993; Weerachatyanukul et al., 2001; White et al., 2000)</td>
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<td>Protein Type</td>
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<td>Zonadhesin (ZAN)</td>
<td>Mouse</td>
<td>ZAN localizes to the apical head overlying the acrosome following spermatogenesis and epididymal maturation.</td>
<td>(Bi et al., 2003; Gasper and Swanson, 2006)</td>
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<td></td>
<td>Hamster</td>
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<td>(Hardy and Garbers, 1994)</td>
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<td></td>
<td>Rabbit</td>
<td>The protein displays testis-specific expression.</td>
<td>(Hardy and Garbers, 1995)</td>
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<td>Boar</td>
<td>Zan features a mosaic protein architecture with several domains (MAM, mucin, D- and EGF) that putatively enable the protein to participate in multiple cell adhesion processes including ZP binding.</td>
<td>(Herlyn and Zischler, 2008; Hickox et al., 2001; Olson et al., 2004)</td>
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<td>Bull</td>
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<td>Horse</td>
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<td>Primates</td>
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<td>Human</td>
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<td>Zona pellucida binding proteins 1 &amp; 2 (ZPBP1, Sp38; IAM38; ZPBP2)</td>
<td>Mouse</td>
<td>Both ZPBP1 and its paralog, ZPBP2, localize to the sperm acrosome and are expressed exclusively in the testes of mice and humans.</td>
<td>(Dun et al., 2011; Lin et al., 2007; Mori et al., 1993; Mori et al., 1995; Redgrove et al., 2011; Yu et al., 2006)</td>
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<td>Human</td>
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<td></td>
<td>Boar</td>
<td>Both proteins have been implicated in secondary ZP binding. Preincubation of boars spermatozoa with anti-ZPBP1 antibodies block <em>in vitro</em> fertilization.</td>
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<td>ZPBP1 null males produce sperm with abnormal head morphology and no forward motility and are consequently sterile.</td>
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<td>ZPBP2 null males produce dysmorphic sperm with reduced ability to penetrate zona pellucida.</td>
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<td>ZP3 receptor protein (ZP3R; SP56; AM67)</td>
<td>Mouse</td>
<td>ZP3R is an intra-acrosomal protein but may presented to surface of the apical region of the sperm head region following capacitation.</td>
<td>(Bleich and Wassarman, 1990; Buffone et al., 2008; Cheng et al., 1994; Cohen and Wassarman, 2001; Foster et al., 1997; Kim et al., 2001a; Kim et al., 2001b; Kim and Gerton, 2003)</td>
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<td></td>
<td>Rat</td>
<td>Evidence that the ZP3R may be involved in either primary and/or secondary ZP interactions.</td>
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<td></td>
<td>Guinea pig</td>
<td>Oocytes pretreated with recombinant ZP3R show a dose dependent decrease in sperm affinity.</td>
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<td>No recorded knockout mouse</td>
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Support for this model rests with the demonstration that the components of the zona pellucida (Prasad et al., 2000), as well as the sperm surface (Murray et al., 1980), are highly sulfated in nature. Furthermore, it has been shown that a range of synthetic sulfated substrates (such including arylsulfates, sulphated monosaccharides and ascorbate 2-sulphate), are capable of competitively inhibiting the fertilization of hamster oocytes in vitro at the level of sperm-zona binding (Ahuja and Gilburt, 1985). In addition, the exposure of spermatozoa to exogenous enzymes capable of desulfating biological macromolecules (such as cerebrosides, glycosaminoglycans and glycoproteins), significantly inhibits their zona binding affinity (Ahuja and Gilburt, 1985). We have recently observed comparable levels of inhibition following treatment of mouse spermatozoa and oocytes with a similar range of reagents (Nixon et al., unpublished).

At present, the nature of sulfated zona binding sites on spermatozoa remain to be fully elucidated, however these findings take on added significance in light of the recent report that male mice bearing a targeted deletion of the gene for protein-tyrosine sulfotransferase 2 (TPST2) are infertile (Borghei et al., 2006). TPST2 is one of two closely related isoenzymes that mediate the tyrosine O-sulfation of a myriad of substrates such as adhesion molecules, G-protein-coupled receptors, coagulation factors, serpins, extracellular matrix proteins, and hormones, in both mice and humans (Beisswanger et al., 1998). TPST2 null mice have normal spermatogenesis and produce normal numbers of epididymal sperm that appear indistinct from their wild type counterparts in terms of their morphology, motility, ability to capacitate in vitro and undergo acrosome exocytosis in response to an agonist (Borghei et al., 2006). However, they are severely defective in terms of their ability to fertilize ZP-intact eggs. The substrates for tyrosine O-sulfation in spermatozoa await further investigation.

Interestingly, in addition to the carbohydrate and sulfate residues that adorn the mature ZP proteins, a small number of studies have also suggested that sperm-ZP interaction may be facilitated, at least in part, by the core polypeptide backbone of ZP3 (Florman et al., 1984; Chapman et al., 1998; Hinsch et al., 2005). Specifically, the polypeptide backbone has been implicated in the induction of acrosomal exocytosis (Chapman et al., 1998; Hinsch et al., 2005). This notion is consistent with the demonstration that although sperm are able to bind to the glycocalyx of rabbit erythrocytes in a manner that appears analogous to that of ZP binding, this interaction fails to elicit the signalling cascades required to induce an acrosome reaction (Clark et al., 1996; Clark and Dell, 2006b). Notably, phenotypic analysis of a number of transgenic mouse models have also raised the prospect that the three-dimensional structure of the zona matrix, specifically the cleavage status of ZP2, rather than a single protein (or carbohydrate),
may be central to mediation of sperm binding (Dean, 2004; Hoodbhoy and Dean, 2004; Dean, 2005).

Models of ZP3 independent sperm-ZP interaction
The debate regarding the nature of the ZP3 ligand(s) responsible for initiating sperm-egg interaction has been overshadowed by recent evidence that sperm may be able to resolve gamete recognition into at least two distinct binding events. In this context, the work of Shur and colleagues have raised the interesting prospect that prior to engaging in interaction with ZP3 ligands, sperm are able to be tethered to the oocyte via adhesion to an oviduct-derived glycoprotein (oviduct-specific glycoprotein, OGP) (Rodeheffer and Shur, 2004; Lyng and Shur, 2009a). OGP is a high molecular weight glycoprotein synthesized and secreted by oviductal cells within the fimbriae and infundibulum and apparently coats the periphery of the ZP in addition to permeating the perivitelline space of ovulated mouse oocytes (Rodeheffer and Shur, 2004; Lyng and Shur, 2009a). Support for this ZP3 independent model has been advanced by the demonstration that both immunoprecipitated and natively purified OGP are able to competitively inhibit sperm-egg binding (Lyng and Shur, 2009). The sperm-binding activity of OGP appears to be carbohydrate-dependent since denatured OGP retains the ability to inhibit binding, and interestingly is restricted to a relatively minor peanut agglutinin (PNA)-binding glycoform (Lyng and Shur, 2009). While such findings may initially appear to be at odds with data from competitive inhibition assays indicating that mouse sperm-oocyte interaction is potently inhibited by preincubation of the sperm with either solubilized ZP or purified mouse ZP3 (Bleil and Wassarman, 1980a), it must be remembered, that these latter experiments were conducted in vitro and therefore may not be entirely physiologically relevant. It is also noteworthy however, that ectopic ovarian pregnancies, although rare, have been recorded in humans (Cabero et al., 1989). These pregnancies take place without the oocyte ever reaching the oviduct and therefore would be unlikely to have been exposed to OGP.

The role of ZP2 in sperm-ZP interactions
The role of ZP2 during gamete interaction has traditionally be viewed as that of a secondary ligand that possesses the ability to bind to the inner acrosomal membrane of acrosome-reacted sperm, thus ensuring close contact between the penetrating spermatozoon and the zona matrix (Bleil et al., 1988). This sperm-binding affinity of ZP2 is abrogated by the proteolytic modification of the protein that accompanies cortical granule exocytosis at the moment of fertilization (Moller and Wassarman, 1989). The modification of ZP2 facilitates an increase in the interaction between the ZP filaments, in turn promoting the hardening of the ZP (Moller and Wassarman, 1989) and thus producing one of two blocks to polyspermy. An interesting caveat to this model has recently been advanced by the work of Dean and colleagues using transgenic
mice expressing a chimeric zona pellucida, containing human ZP2 (Dean, 2004). The oocytes of these mice bind mouse but not human spermatozoa. Surprisingly however, this sperm binding activity persists even after fertilization of the oocytes. This phenomenon is not completely understood, since the ZP2 proteolytic cleavage domain is conserved between species and the human ZP2 would therefore have been expected to be digested following cortical granule exocytosis (Dean, 2004; Dean, 2005). Interestingly, similar results were also observed in mice expressing human ZP3. One possible explanation for these anomalous results is that sperm binding is modulated by the overall supramolecular structure of the zona pellucida rather than relying on individual proteins and/or oligosaccharides (Dean, 2004).

**Sperm receptor molecules involved in zona pellucida interaction**

In accordance with the complexity of the various models for sperm-zona interaction, it has proven difficult to identify definitively the corresponding sperm surface molecules that mediate primary recognition and adhesion to the ZP. A multiplicity of putative ZP receptors have been postulated on the basis of a range of experimental techniques including analysis of mutations influencing fertility, development of inhibitory monoclonal antibodies, analysis of sperm autoantigens, ZP affinity columns, photoaffinity crosslinking and binding of radiolabeled ZP to sperm lysates (reviewed by (McLeskey et al., 1998b); Table 1). Consistent with the model of primary sperm-egg interaction being initiated by defined carbohydrate structures on ZP3 (Section 2.1.1), a number of these putative receptors possess lectin-like affinity for specific sugar residues (Table 1). However, notwithstanding compelling in vitro data implicating these molecules in zona adhesion, no single candidate has been identified that is uniquely responsible for directing the interaction between sperm and the ZP. Such findings fuel speculation that this fundamental cellular interaction may require the coordinated action of several sperm proteins. Indeed, emerging evidence supports the concept that sperm-zona interaction is mediated by a multimeric complex incorporating several discrete molecular entities, each of which may have a specific role at different stages of the recognition process (see Section 3.2.2). Furthermore, in recognition of the fact that gamete interaction is predicated on spermatozoa acquiring a state of functional maturation during their post-testicular development, it has been suggested that the assembly of this complex may be causally linked to membrane remodeling events associated with epididymal maturation and/or capacitation.

**Acquisition of the ability to engage in sperm-ZP interaction**

Mammalian spermatozoa are produced by spermatogenesis, a prolonged, inordinately complex process that culminates in the generation of a morphologically mature, yet functionally incompetent sperm cell (reviewed by (Eddy and O’Brien, 1994). During this process, spermatids undergo a dramatic metamorphosis from a rounded shape into an elongated cell
consisting of a number of highly specialized regions: a head comprising the acrosomal vesicle, nucleus, cytoskeletal structures and cytoplasm; a midpiece which houses the mitochondria; and a flagellum that is used for locomotion. The final phase of cytodifferentiation, spermiogenesis, is also characterized by the repackaging of the chromosomes in preparation for their delivery to the oocyte (Eddy and O'Brien, 1994). As a consequence of these events, it is widely held that spermatozoa leave the testes in a transcriptionally silent state and similarly lack the capacity for de novo protein synthesis (Engel et al., 1973). The post-testicular functional transformation of these cells that ensues is therefore reliant upon protein changes (loss, acquisition and post translational modification) driven by changes in the external environment, as these cells move through the male and female reproductive tracts (Fig. 2).

Epididymal Maturation

Notwithstanding the high degree of morphological specialization that is achieved during spermatogenesis, spermatozoa enter the epididymis without the capacity to exhibit forward progressively motility, nor to recognize and engage in interaction with the ZP (reviewed by Yanagimachi, 1994b). Spermatozoa acquire the potential to express these functional attributes during their transit of the male reproductive tract, particularly the epididymis (reviewed by Cooper, 1986). Elegant ligation and epididymostomy studies have provided compelling evidence that the accompanying changes are not intrinsic to spermatozoa, but rather appear to be driven by dynamic changes in the ambient intraluminal milieu as they pass along the length of the epididymal tubule (Cooper, 1986). Indeed, the exposure of spermatozoa to the microenvironment created by the combined secretory and resorptive functions of the epididymal epithelial cells, has been variously correlated with the addition, re-positioning, removal and / or modification of specific proteins and lipids within the sperm membrane (Jones, 1998; Jones, 1999; Jervis and Robaire, 2001; Chaurand et al., 2003; Johnston et al., 2005; Turner et al., 2006).
Figure 2. Phases of sperm maturation required for successful sperm-oocyte interaction. Following their production in the testes (spermatogenesis), mammalian spermatozoa enter the male reproductive tract (epididymis) as functionally incompetent cells. The exposure to the intraluminal milieu of the epididymis results in the acquisition of the potential for forward progressively motility and engage in interaction with the ZP. However these functional attributes are only expressed after a final phase of maturation (capacitation) as the spermatozoa ascend the female reproductive tract.

The ability of epididymal spermatozoa to bind to the ZP is first observed in the proximal corpus epididymis and achieves maximal levels in the caudal region in virtually all species studied to date (Cooper, 1986). Interestingly, the acquisition of zona binding competence coincides with the attainment of the potential for movement (Aitken et al., 2007b). However, it is considered unlikely that these two events are causally related since, unlike motility, sperm-zona interaction is dependent on the ability of the spermatozoa to undergo capacitation, with non-capacitated cells proving largely refractory to zona adhesion (Asquith et al., 2004a). Additionally, zona binding ability is retained in immobilized caudal epididymal spermatozoa (Saling, 1982). The acquisition of zona binding also appears temporally associated...
with the exposure of spermatozoa to two distinct subsets of macromolecular structures in the epididymal lumen: the first being amorphous, chaperone laden ‘dense bodies’ (Asquith et al., 2005b) and the second being membrane bound prostasome-like particles known as epididymosomes (Saez et al., 2003a). It has been hypothesized that together, these epididymal granules facilitate the bulk transfer of proteins to the sperm surface during their transit of the organ. This idea is consistent with the demonstration that biotinylated proteins are able to be transferred between epididymosomes and the acrosomal cap and midpiece of spermatozoa (Saez et al., 2003a). However at present, neither the molecular mechanisms that underpin protein transfer nor the identity of the transferred protein(s) have been fully elucidated. Similarly, the causative nature of this relationship remains the subject of ongoing investigation.

Sperm capacitation
Following their passage through the epididymis spermatozoa must complete an additional phase of maturation, termed capacitation, before realizing their full potential for fertilization. Capacitation occurs in vivo as spermatozoa ascend the female reproductive tract and encompasses a series of elaborate cellular modifications. Indeed, in the sixty years that have elapsed since capacitation was first described (Austin, 1951b; Chang, 1951a), a number of changes have been correlated with this process, including extensive remodelling of the sperm plasma membrane and the post-translational modification of intrinsic sperm proteins (Visconti et al., 1995a; Visconti et al., 1995b; Gadella and Van Gestel, 2004; Boerke et al., 2008; Gadella, 2008b; Gadella et al., 2008b). Among the posttranslational modifications that have been documented to date, a global upregulation of phosphotyrosine expression has emerged as a critical factor in regulating the ability of spermatozoa to hyperactivate, bind to the zona pellucida, undergo an acrosome reaction and ultimately fertilize the oocyte (Visconti et al., 1995a; Visconti et al., 1995b; Luconi et al., 1998; Urner et al., 2001; Visconti et al., 2002b; Sakkas et al., 2003; Asquith et al., 2004a; O'Flaherty et al., 2005; Baker et al., 2006b; Mitchell et al., 2007a).

The induction of tyrosine phosphorylation appears to be modulated predominantly by a unique soluble adenylyl cyclase / cAMP / PKA axis (Visconti et al., 1995a; Visconti et al., 1995b; Aitken et al., 1998). However, in addition to PKA-dependent phosphorylation of targets which for the most part appear to reside within the sperm flagellum (Visconti et al., 1997; O'Flaherty et al., 2005; Baker et al., 2006b; Mitchell et al., 2007a) an alternative subset of tyrosine phosphorylated proteins have been detected on the surface of live, capacitated mouse spermatozoa (Asquith et al., 2004a; Piehler et al., 2006). Furthermore, the expression of these proteins appears confined to the plasma membrane overlying the acrosomal domain of the sperm head - an ideal position from which to orchestrate the membrane remodeling events
associated with sperm-ZP recognition. In contrast to the aforementioned flagellar proteins, we have recently secured evidence that the phosphorylation of these sperm surface proteins is largely insensitive to inhibition with specific antagonists of the canonical PKA pathway (Nixon et al., 2010a). Rather our findings suggest that an alternative signaling pathway involving the classical MAP kinases may underpin this capacitation-associated surface exposure of phosphotyrosine residues in mouse spermatozoa (Nixon et al., 2010a). These findings take on added significance in light of the demonstration that the inhibition of the MAP kinase pathway, and hence sperm surface phosphotyrosine expression, induces a concomitant reduction in sperm-zona pellucida interaction (Nixon et al., 2010a).

Although surface phosphorysine expression does not appear to be a universal correlate of capacitation in all species (Liu et al., 2006), it is not unique to mouse spermatozoa. For instance, recent quantitative studies of surface phosphotyrosine expression in boar spermatozoa have revealed a significant increase in phosphotyrosine associated fluorescence following capacitation (Piehler et al., 2006). This increase coincides with the exposure of several tyrosine phosphorylated proteins on the outer leaflet of the boar sperm plasma membrane, at least two of which possess high affinity for the ZP (Flesch et al., 1999; Flesch et al., 2001). In contrast, plasma membrane proteins isolated from freshly ejaculated boar spermatozoa did not exhibit any ZP binding proteins, likely because these proteins were not tyrosine phosphorylated (Flesch et al., 1999; Flesch et al., 2001). Unfortunately however, the identity of these proteins remains to be elucidated.

Interestingly, our own analysis of the repertoire of phosphoproteins that are uniquely expressed on the surface of capacitated mouse spermatozoa, identified a subset of molecular chaperone proteins including heat shock 60kDa protein 1 (HSPD1) and heat shock protein 90, beta 1 (HSP90B1) (Ecroyd et al., 2003b; Asquith et al., 2004a). Both of these proteins have in turn been localized to dense bodies within the proximal corpus epididymis (see Section 3.2.1.1) and to the sperm surface overlying the anterior acrosome, the precise location where sperm-ZP interaction is initiated (Asquith et al., 2004a; Asquith et al., 2005b). Although such findings invite speculation that these chaperones may directly mediate sperm-egg interaction, our cumulative evidence argues against such a conclusion (Walsh et al., 2008a). Rather, it is suggested that these proteins are responsible for chaperoning key recognition molecules to the site of sperm-oocyte interaction and / or orchestrating their assembly into a multimeric zona-receptor complex on the sperm surface (Nixon et al., 2005b) (see Section 2.3.1; Fig. 3). In agreement with this model we have recently employed the novel technique of blue native polyacrylamide gel electrophoresis (BN-PAGE) to provide the first direct evidence for the expression of chaperone laden complexes on the surface of capacitated mouse spermatozoa.
Interestingly, a subset of these complexes also harbor putative ZP receptor proteins and possess strong affinity for solubilized zonae (Dun et al., unpublished).

**Zona pellucida receptor candidates**

Considerable research has been devoted to investigating the identity of the individual proteins in spermatozoa that facilitate the binding of this specialized cell to the ZP (reviewed by Nixon et al., 2007b). On the basis of varying degrees of circumstantial and direct evidence in excess of ten different candidate proteins have been proposed to participate in different aspects of this interaction in the mouse model alone (Table 1). Consistent with the notion that primary sperm-zona pellucida binding is a carbohydrate-mediated event, a number of these candidate sperm proteins are either glycoenzymes or possess the requisite lectin-like affinity for ZP3 sugars.

Perhaps the most widely studied of the putative ZP3 receptor candidates is mouse β-1,4-galactosyltransferase (GalTase). This enzyme normally resides within the Golgi apparatus, where it functions in the biosynthesis of complex glycoconjugates on secretory and membrane-bound glycoproteins (Nixon et al., 2001). However a novel, functionally distinct, isoform of GalTase has been shown to be expressed during spermatogenesis and localized to the dorsal, anterior aspect of the membrane overlying the intact acrosome (Shur and Neely, 1988). From this position, GalTase is thought to function as a gamete receptor by binding to complementary terminal N-acetylglucosamine (GlcNAc) residues that furnish the Sperm Combining Site of the ZP3 protein (Shur and Hall, 1982; Shur and Neely, 1988; Shur, 1989; Miller et al., 1992). Furthermore, aggregation of GalTase by ZP3 oligosaccharides activates a heterotrimeric G-protein coupled signaling cascade that culminates in the induction of the acrosome reaction (Macek et al., 1991; Miller et al., 1993). Accordingly, overexpression of GalTase on the sperm surface leads to increased ZP3 binding, accelerated G-protein activation and precocious acrosome reactions (Youakim et al., 1994).

The expression of GalTase isoforms in the anterior portion of the sperm head of a variety of mammalian species raises the possibility that the zona receptor activity of the protein may be widely distributed (Humphreys-Beher and Blackwell, 1989; Sullivan et al., 1989; Larson and Miller, 1997). Surprisingly, however, targeted mutations of mouse GalTase do not induce the anticipated infertility phenotype (Lu and Shur, 1997). Rather, spermatozoa from GalTase null males retain their fertility despite a marked reduction in their ZP3 binding affinity and an inability to undergo a ZP3 induced acrosome reaction (Lu and Shur, 1997). Thus, although the ZP3-GalTase receptor-ligand complex may confer a physiological advantage on fertilizing spermatozoa, its expression is dispensable for fertilization. Similarly, definitive studies examining the effects of null mutations on additional sperm surface components that
have been implicated in ZP adhesion have shown that the majority are also superfluous (see Table 1). Collectively these findings raise the intriguing possibility, discussed below, that the zona receptor is in fact a multimeric complex incorporating several discrete molecular entities.

**Molecular basis for multiple sperm-ZP receptor candidates**

Despite the significant advances in our understanding of the initial interaction between sperm and the oocyte, it is clear that this fundamental recognition event remains largely enigmatic. The large number of sperm molecules that possess affinity for the ZP (Table 1) challenges the concept of a simple lock and key mechanism to account for gamete interaction. Indeed, despite the wealth of in vitro data implicating various ZP receptor candidates, the prevailing evidence now indicates that none are uniquely responsible for directing the interaction between sperm and the ZP (Nixon et al., 2007b). The fact that spermatozoa are adorned with a multiplicity of ZP receptor candidates could afford the cells with a level of functional redundancy commensurate with the overall importance of this fundamental cellular interaction. However, it is also possible that the individual receptors function in a coordinated fashion, each with unique role(s) during the multifaceted ZP recognition process. The latter model is consistent with biochemical and biophysical studies of sperm-ZP binding that indicate it comprises both low and high affinity interactions (Thaler and Cardullo, 1996c; Thaler and Cardullo, 2002b). Indeed, prior to penetration of the ZP, spermatozoa first adhere loosely to the zona matrix in a manner that is easily disrupted by repetitive pipetting or density gradient centrifugation (Bleil and Wassarman, 1980a). The promiscuous nature of this initial binding event contrasts with the high affinity, comparatively species-specific, interaction that follows. The former adhesion event is therefore likely to employ sperm surface molecules that are conserved across species, while those involved in the latter binding event are, instead, expected to be species- and / or order-specific (Tanphaichitr et al., 2007a). In addition to those sperm proteins required for zona adhesion, alternative candidates may be engaged in the activation of ZP-induced sperm signaling events that culminate in the acrosome reaction. If this model holds true, it inevitably raises questions regarding how the presentation of such a large number of putative ZP receptor and signal transduction candidates is coordinated. As discussed below, recent analyses have led to the proposal of at least two complementary mechanisms involving molecular chaperones and membrane rafts, the relative contribution of which may vary depending on the species.

**Towards an integrated model of sperm-zona interaction**

*The role of molecular chaperones in sperm-zona pellucida interaction*

The term molecular chaperone denotes a large family of highly conserved proteins that form a ubiquitous defense system within cells. However, in addition to their archetypal role of protecting cells from the adverse effects of stress, it has become increasingly apparent that
chaperones play additional roles in diverse cellular phenomena under normal physiological conditions. Of particular note is the recognized ability of molecular chaperones to direct the assembly of oligomeric protein complexes and mediate their transport across the plasma membrane (Voos and Rottgers, 2002). In addition, emerging evidence indicates that certain members of the chaperone family exert a necessary, but still poorly understood, role in the recruitment and clustering of specific receptors on the cell surface and in signal transduction (Triantafilou et al., 2001; Triantafilou and Triantafilou, 2003; Triantafilou and Triantafilou, 2004). Consistent with such roles, chaperones have been identified within a number of divergent subcellular compartments including the plasma membrane of a wide variety of cell types (Soltys and Gupta, 1996; Soltys and Gupta, 1997; Soltys, 1999; Shin et al., 2003).

Interestingly, the chaperoning activity of the plasma membrane resident chaperones appears to be regulated by their phosphorylation status (Khan et al., 1998). The significance of this finding is underscored by the demonstration that at least two key chaperone proteins are phosphorylated during the capacitation of mouse spermatozoa (Asquith et al., 2004a) (see Section 2.2.1.2). Although several molecular chaperones, including calmegin, calnexin and members of the HSP60, 70 and 90 families, have been identified in spermatogenic cells (Tanaka et al., 1997; Zhu et al., 1997b; Eddy, 1998; Ohsako et al., 1998; Ogi et al., 1999; Yoshinaga et al., 1999a), the functional significance of many of these proteins remains unclear. However, at least one member of this family, calmegin, has been implicated in sperm-ZP interaction (Ikawa et al., 1997; Ikawa et al., 2001; Yamagata et al., 2002). Despite the fact that calmegin is not expressed in mature spermatozoa, it has been identified as a critical determinate in the functioning of these cells on the basis of its role in ensuring the correct folding of endoplasmic reticulum glycoproteins destined for the acrosomal matrix and the plasma membrane. Targeted disruption of the calmegin gene compromises male fertility due to impaired sperm transport in the female reproductive tract in vivo (Ikawa et al., 2001) and the loss of sperm-zona binding ability (Ikawa et al., 1997). An absence of signaling proteins or antigenic determinants from the surface of sperm has been proposed as the mechanism to explain these defects in sperm function. Interestingly, in this regard, sperm from calmegin<sup>−/−</sup> mice also lack fertilin beta (ADAM2), a protein implicated in sperm-egg plasma membrane binding and fusion (Ikawa et al., 2001). Thus, the chaperone function of calmegin may regulate the correct processing of a variety of sperm molecules. Collectively, such observations invite speculation that chaperones direct the assembly of key recognition molecules on the sperm surface.

Support for this hypothesis, rests with the demonstration that mouse spermatozoa express a subset of molecular chaperones (including: HSPE1, HSPD1, HSP90 and HSP90B1) within the peri-acrosomal region of their head (Ecroyd et al., 2003b; Asquith et al., 2004a;
Walsh et al., 2008a). Interestingly, the surface expression of these proteins increases dramatically in populations of sperm in which capacitation has been actively driven. Nonetheless, a direct role for the chaperones in sperm-oocyte interaction has been discounted on the basis that incubation of sperm with anti-chaperone antibodies does not significantly compromise their ability to bind to the ZP (Walsh et al., 2008a). Rather it appears that chaperones play an indirect role possibly in the assembly of multiple zona adhesion molecules into a functional receptor complex (Fig. 3). An alternative possibility is that molecular chaperones participate in the active translocation of sperm proteins to their site of action.

Since spermatozoa lack the molecular machinery for protein synthesis these proteins must be either unmasked or held cryptic within the cell prior to their surface presentation. A growing body of evidence favors the latter interpretation, indeed many of the putative ZP receptors are proteins one would normally associate with the sperm acrosome (Tulsiani and Abou-Haila, 2001; Tulsiani and Abou-Haila, 2004b). The zona pellucida 3 receptor (ZP3R; formerly sperm protein 56 or SP56) provides an interesting example of one such protein. ZP3R was originally identified on the basis of elegant photoaffinity crosslinking studies as a primary receptor for ZP3 (Bleil and Wassarman, 1990a; Cheng et al., 1994). This role was subsequently discounted on the basis of immunoelectron microscopy evidence that revealed the protein was enclosed acrosomal matrix (Foster et al., 1997; Kim et al., 2001a). Since such a location is incompatible with the mediation of ZP3 binding in acrosome intact spermatozoa, it was postulated that the ZP3R was likely to participate in secondary sperm-ZP interactions. Resolution of this apparent discrepancy has recently been afforded by the demonstration that ZP3R, in addition to other acrosomal matrix proteins, are progressively released to the sperm surface during capacitation through the formation of small fusion pores (Fig. 3) (Kim et al., 2001c; Kim and Gerton, 2003b; Buffone et al., 2008d). This evidence not only challenges the widely held view of acrosomal exocytosis as an all or none reaction but also raises the intriguing possibility that the acrosome may fulfill a secondary role as a reservoir for key ZP recognition molecules (Buffone et al., 2008b). This interpretation may explain why uncapacitated mammalian sperm are unable to engage in high affinity interaction with the ZP. However, while it is tempting to speculate that chaperones mediate the relocalization of these proteins and hence prime the sperm surface for ZP adhesion, direct evidence in support of this model has yet to be furnished.

Among the main challenges that remain in establishing the definitive role of molecular chaperones in mature spermatozoa is the characterization of the client proteins with which they associate in both non-capacitated and capacitated spermatozoa (Nixon et al., 2007b). Unfortunately, the use of conventional techniques such as affinity purification and
immunoprecipitation has proven largely unsuccessful in this regard (Walsh et al., 2008a). This lack of success may reflect the fact that chaperones generally form only weak, transient interactions with their client proteins. Among the alternative strategies that could prove informative in this regard are the isolation and detailed proteomic characterization of the repertoire of membrane-associated proteins that populate the region of the sperm head that interacts with the oocyte. Such an approach has been published by Myles and colleagues (Stein et al., 2006). Following vectorial labeling of the mouse sperm surface, the authors conducted a comparative analysis of the profile of surface exposed proteins with that of proteins recovered in hybrid membrane vesicles released from the anterior sperm head following the acrosome reaction (Stein et al., 2006). This approach has helped define the basic proteomic inventory of the anterior sperm head, the significance of which is highlighted by the fact that among the 85 proteins identified were at least three molecular chaperones (including HSP90B1) in addition to nine proteins that have been implicated in fertilization in vivo on the basis of gene knockout studies (Stein et al., 2006). One limitation of this approach however, was that it did not address the important question of the temporal and spatial organization of membrane-associated proteins in relation to the dynamic cellular changes that accompany capacitation.

A complementary strategy has recently been published by Gadella and colleagues (van Gestel et al., 2007a). In this study, the authors isolated the apical plasma membrane from porcine sperm by nitrogen cavitation, achieving an approximate 20 fold enrichment in plasma membrane markers compared to that of contaminating membrane markers. These membrane preparations were then co-incubated with isolated zona ghosts and sperm–ZP binding proteins were identified by tandem mass spectrometry (van Gestel et al., 2007a). This study confirmed the involvement of multiple sperm proteins in ZP binding, with 24 sperm proteins reproducibly remaining associated with zona ghosts under conditions of low stringency. As anticipated, a subset of these proteins were identified as previously characterized ZP-binding receptors including: spermadhesin (AQN-3), P47 (SED1) and fertilin beta (ADAM2). Remarkably, the majority of the zona ghost-binding proteins were also been detected in lipid ordered membrane microdomains (membrane rafts) that are assembled in the apical ridge area of the sperm head plasma membrane during in vitro capacitation (Boerke et al., 2008). On the basis of such evidence, it has been postulated that the study of membrane rafts may provide novel insights into the molecular mechanisms that underpin sperm-ZP interaction (Tanphaichitr et al., 2007a; Gadella, 2008b; Gadella et al., 2008b; Nixon and Aitken, 2009).

The role of membrane rafts in sperm-zona pellucida interaction

Membrane rafts (formerly lipid rafts) are generally defined as small, heterogeneous domains that serve to compartmentalize cellular processes (Pike, 2006). The unique, ordered properties
of these domains reflect the stabilizing influence of hydrogen bonds and hydrophobic interactions between their resident saturated fatty acids and the rigid structure of intercalated cholesterol. These properties also result in the resistance of lipid rafts to solubilization by a number of non-ionic detergents (Schuck et al., 2003) and hence they are often referred to as detergent resistant membranes (DRMs). Despite their stability, rafts remain highly dynamic and have been observed to display considerable lateral movement in various cell types in response to appropriate physiological stimuli or cellular activation events (Simons and Vaz, 2004). The significance of these structures is highlighted by the myriad of cell adhesion, signaling and trafficking molecules that have been found to preferentially associate with isolated membrane rafts (Foster et al., 2003). Indeed membrane rafts are now considered as platforms for mediating membrane trafficking, cellular signal transduction and cellular adhesion events as diverse as viral entry and fertilization (Nixon and Aitken, 2009).

It has been demonstrated that liquid-ordered domains analogous to the membrane rafts observed in somatic cells are present in the spermatozoa of all mammalian species studied to date, albeit at a larger scale (Cross, 2004; Shadan et al., 2004; Sleight et al., 2005; Bou Khalil et al., 2006b; Selvaraj et al., 2006; Weerachatyanukul et al., 2007; Boerke et al., 2008; Asano et al., 2009a; Nixon et al., 2009a; Selvaraj et al., 2009a). Indeed, the size and stability of sperm membrane rafts appear quite excessive, raising the possibility that they may represent “superrafts” consisting of stably segregated smaller subdomains (Selvaraj et al., 2006; Selvaraj et al., 2009a). This is consistent with a recent demonstration that a number of subtypes of membrane raft domains are likely to exist in these cells (Asano et al., 2009a). With the recognition that mammalian spermatozoa possess membrane rafts, two lines of enquiry have predominated. Firstly, whether the physical and biochemical properties of the membrane rafts are influenced by the capacitation status of spermatozoa, and secondly whether the rafts modulate important aspects of sperm function (Tanphaichitr et al., 2007a). Paradoxically, two conflicting views have emerged from the former studies, with evidence suggesting that these domains may either be compromised by the capacitation-associated loss of cholesterol (Sleight et al., 2005), or alternatively may cluster within the sperm head and coalesce to form larger ordered membrane microdomains during capacitation (Shadan et al., 2004; Bou Khalil et al., 2006b; Boerke et al., 2008; Nixon et al., 2009a). It is still unclear what functions might underlie such distinct membrane remodeling, however the focal enrichment of membrane rafts within the sperm head encourages speculation that they may serve as platforms for modulating oocyte interaction (Fig. 3) (Tanphaichitr et al., 2007a). This hypothesis is commensurate with the demonstration that DRMs isolated from both boar and mouse spermatozoa possess the ability to bind with high affinity and specificity to the zona pellucidae of homologous oocytes (Bou Khalil et al., 2006b; Boerke et al., 2008; Nixon et al., 2009a).
Consistent with these findings, comprehensive proteomic profiling of isolated sperm DRMs has confirmed the anticipated presence of the majority of molecules that have been implicated in sperm-zona pellucida binding (Table 1) in addition to many of those involved in downstream interaction with the oolemma (Sleight et al., 2005; Nixon et al., 2009a). Although caution is required in equating DRM association with a protein’s residence in membrane raft domains in situ (Foster et al., 2003; Munro, 2003), such findings encourage speculation that sperm membrane rafts serve as constitutive platforms for the spatial constraint of key recognition molecules and that the remodeling events associated with capacitation lead to their assembly and presentation on the outer leaflet of the sperm plasma membrane (Nixon et al., 2005b; Nixon et al., 2007b).

Such a conclusion is supported by the demonstration that the proteomic composition of membrane rafts undergoes substantial changes in response to the induction of capacitation (Thaler et al., 2006). Among the various models that could account for changes, Tulsiani and colleagues (Abou-Haila and Tulsiani, 2003) have postulated that capacitating spermatozoa undergo a progressive priming that results in the exposure of intra-acrosomal enzymes. Their model is based on the precept that as capacitation proceeds, the outer acrosomal membrane evaginates, forming a vesicle that enlarges and becomes tethered to the plasma membrane through complementary vesicle-associated (v-) SNARE and target membrane (t-) SNARE proteins residing within the two membranes (Fig. 3). Although the movement of vesicles between the sperm acrosome and plasma membrane has not previously been documented during capacitation, many of the components of the molecular machinery necessary for coordinating the assembly and trafficking of exocytotic vesicles are present in spermatozoa. For instance, dynamin, an enzyme that forms restriction collars around budding vesicles and promotes their release has recently been identified within acrosome of mouse spermatozoa (Zhao et al., 2007). Furthermore, the complementary SNARE proteins, VAMP, SNAP and syntaxin have been localized to the outer acrosomal membrane and apical sperm head plasma membrane respectively, of mouse spermatozoa (Brahmaraju et al., 2004; Tsai et al., 2007) in addition to that of other species (Schulz et al., 1998; Tomes et al., 2002; De Blas et al., 2005). Notably these SNARE proteins share the lateral redistribution properties of both ZP-binding proteins and raft marker proteins, each of which are able to be recovered within DRMs prepared from capacitated spermatozoa (Boerke et al., 2008). Collectively these data invite speculation that key components of a zona adhesion complex are conveyed from the acrosomal vesicle into membrane rafts during capacitation. This notion is supported by the demonstration that antibodies to VAMP and SNAP inhibited mouse sperm-zona pellucida interaction (Brahmaraju et al., 2004).
It is also noteworthy, that sperm membrane rafts are laden with a subset of molecular chaperone proteins (Nixon et al., 2009a), at least two of which, HSP90B1 and HSPD1, have previously been implicated in remodeling the sperm surface and enhancing oocyte interaction (Asquith et al., 2004a; Asquith et al., 2005a); see Section 2.2.1.2). The identification of a number of constitutively expressed molecular chaperones (HSP90B1, HSPA8, HSPD1, and DNAJB1) as integral components of membrane rafts in other cell types (Broquet et al., 2003; Chen et al., 2005b) suggests that these proteins may fulfill an important general mechanism operating at the level of the plasma membrane through which cellular signaling / adhesion complexes are sorted and assembled. In this regard, previous studies have demonstrated that chaperones play important roles in maintaining the stability of lipid raft-associated signal transduction complexes (Chen et al., 2005b). Conversely, it has also been demonstrated that lipid rafts regulate the functions of resident chaperones through the spatial constraint of their substrates (Elhyany et al., 2004 ). Taken together these results suggest that sperm membrane rafts provide a favorable environment for chaperones to mediate the conformational conversion and assembly of functional zona receptor complexes. Furthermore, the aggregation of such microdomains during capacitation may facilitate the recruitment of these complexes to the site of engagement with the ZP.
Figure 3. Model for mouse sperm-zona pellucida interaction. We propose that sperm membrane rafts serve as a platform for the recruitment of key zona adhesion molecules and promote their delivery to the sperm head. This process coincides with the capacitation-associated phosphorylation of a subset of molecular chaperones and their exposure on the sperm surface. The chaperones subsequently provide the molecular machinery to assemble a functional receptor complex, rendering the sperm competent to bind to the glycans that furnish the zona pellucida. Based on emerging evidence we also propose that capacitation-associated sperm surface remodeling may be underpinned by the translocation of a subset of ZP receptors from the acrosomal vesicle to the sperm membrane. At least two mechanisms, i.e. vesicle mediated transport and the formation of small fusion pores, have been postulated to account for the incremental exposure of these proteins.

Potential for contraceptive intervention

In addition to fundamental benefits in terms of understanding causes of male infertility, the molecular dissection of sperm-ZP interaction also promises to inform the development of novel approaches for contraceptive intervention. Indeed, it has long been held that the identification of sperm proteins involved in ZP recognition and binding events could provide a range of candidates that, by virtue of their specificity, location and susceptibility to suppression, would exhibit potential as contraceptive targets with equal effectiveness for both male and females. Such contraceptives would be of considerable benefit for the control of both captive and feral animal species and thus contribute to ameliorating global problems associated with a lack of habitat, overcrowding and disease (Hardy and Braid, 2007; Kirkpatrick, 2007; Fayrer-Hosken, 2008). The realization of such technology may also contribute to the development of novel, safe, effective measures to fill the void in the current contraceptive armoury for our own species, the population of which continues to grow at an alarming rate (McLaughlin and Aitken, 2010). Contraceptive vaccines for example, have the potential to provide safe, effective, prolonged, reversible protection against pregnancy in a form that can be easily administered in the Third World. However, in order to meet the above criteria, the target antigen must be an essential component of fertility and must be drugable.

Target antigens of the zona pellucida

The zona pellucida glycoproteins are among the most widely investigated candidate targets for immunocontraceptive vaccines. The ZP proteins afford the advantage of being a female organ-specific antigen and an immune response elicited against these proteins could in principle block sperm-ZP interaction. In practice however, the reduction in fertility achieved following immunisation with homologous or heterologous zona proteins appears to be primarily attributed
to either the loss of endogenous antigen and / or the induction of ovarian specific autoimmune disease (Paterson et al., 2000). This is highlighted by fertility trials involving whole native porcine zona pellucida (pZP), a popular heterologous antigen in current use for feral and exotic animal fertility control (Hardy and Braid, 2007). Such studies have demonstrated that pZP is a potent heteroimmunogen in most species and is efficacious in the control of fertility in horses, white tailed deer, bonnet monkeys, wallaby, bears and elephants (Bagavant et al., 1994; Fayrer-Hosken et al., 1999; Miller et al., 2000; Kitchener et al., 2002; Turner et al., 2002; Delsink et al., 2007; Lane et al., 2007; Locke et al., 2007; Kitchener et al., 2009). However, the use of this material as an immunogen is problematical as it has been shown to induce ovarian pathology, the loss of hormone dependent behavior and permanent sterility (Dietl et al., 1982; Drell et al., 1984; Bhatnagar et al., 1992). The isolation of a consistent native pZP product, free of viral contamination, for immunisation purposes has also proven technically challenging (Kaul et al., 1996). While the latter problem may be alleviated by the production of glycosylated porcine ZP recombinant protein in a defined mammalian cell line, the permanent ovarian pathology that accompanies the active immunity against ZP antigens represents a significant barrier to their clinical use (McLaughlin and Aitken, 2010). Until researchers separate the immunocontraceptive effect from the unwanted pathology induced by immunodominant epitopes, ZP proteins will remain unlikely target antigens for a human immunocontraceptive vaccine. Furthermore, the fact that ZP immunogens lack species-specificity imposes restraints on their mode of delivery and hence overall applicability in free-ranging wildlife species. Other antigenic targets are clearly required.

**Target antigens of spermatozoa**

The demonstration that spermatozoa are highly immunogenic in both females and males presents a strong rationale for the development of contraceptive technologies centered on a defined sperm specific antigen. This is emphasised by the fact that the presence of anti-sperm antibodies in the male or female partner have been identified as a causative agent in the infertility associated with a relatively large number (9-36%) of couples seeking recourse to assisted conception (Menge et al., 1982; Collins et al., 1993; Ohl and Naz, 1995). Furthermore, the development of anti-sperm antibodies that occurs in over 70% of vasectomized men limits the potential for recovery of fertility even after successful vaso-vasostomy surgery (Hull et al., 1985).

Notwithstanding the award of a U.S. patent for a spermatoxic vaccine based around the injection of whole semen (Baskin, 1932), this approach has limited utility. Among the obvious problems is the fact that spermatozoa express numerous antigens that are shared with somatic cells, thus raising the prospect of potentially severe immunopathological side effects. Attention
has instead focused on the identification of individual sperm proteins capable of eliciting a contraceptive response. The appropriate sperm antigen should display sperm-specific expression, surface accessibility and have a pivotal role in fertilization. In principle, sperm proteins involved in ZP interaction therefore represent ideal candidates. Accordingly a myriad of these proteins, including: SP17 (O'Rand and Widgren, 1994), SPAM1 and ADAM1/2/3 (Primakoff et al., 1987b; Primakoff et al., 1988; McLaughlin et al., 1997; McLaughlin et al., 2001), LDHC4 (Goldberg, 1973; Goldberg et al., 1981; Chen et al., 2008), SP10 (Srinivasan et al., 1995), ZPR3 (Hardy and Mobbs, 1999), FA1 (Naz and Wolf, 1994; Naz and Zhu, 1998), SOB2 (Lefevre et al., 1997), a novel form of CD52 (Diekman et al., 1999), human sperm associated antigen 9 (hSPAG9) (Jagadish et al., 2006) and nuclear autoantigenic sperm protein (tNASP) (Wang et al., 2009), have been investigated as the basis for a fertility-regulating vaccine. Nevertheless, while some of these antigens have shown promise in animal trials with notable inhibition of sperm-ZP interaction and concomitant subfertility, such studies have failed to deliver on the objective of identifying a single, suitable target that induces a 100% block to fertility.

Collectively this lack of success highlights the naiveté of the paradigm that sperm-ZP interaction is regulated by a single molecular entity that is constitutively expressed on the cell surface. Rather, it is likely that multiple sperm receptors are required to achieve high affinity binding to the complex multivalent polysaccharide ligands present within the ZP (see Section 2.2.2.1). The growing acceptance of this model is demonstrated by the fact that researchers have recently opted for construction of multi-antigen vaccines. Examples include multi-antigen recombinant polypeptides comprising the mouse reproductive antigens SP56, ZP3, ZP2, and ZP1 administered to female mice (Hardy et al., 2008). This resulted in significantly reduced fertility without significant ovarian pathology (Hardy et al., 2008). Using six sperm specific antigens (mFA-12,19, mFA-1117136, YLP12, P10G, A9D and SP56) also resulted in reduced fertility in multipeptide vaccination studies (Naz and Aleem, 2007). A third vaccine formulation comprised of five recombinant human intra-acrosomal sperm proteins (ESP, SLLP1, SAMP32, SP10 and SAMP14) was used to immunise female cynomolgus monkeys, all of which developed IgG and IgA serum responses to each immunogen, indicating that a multivalent contraceptive vaccine may be a viable alternative in primates (Kurth et al., 2008).
SUMMARY

Despite the myriad of putative sperm-zona pellucida adhesion molecules that have been reported, no single candidate appears uniquely responsible for mediating this important interaction. Rather than this simple lock and key mechanism, the balance of evidence favors the novel hypothesis that sperm-egg interaction is mediated by the coordinated action of several sperm receptors, each of which contribute to the high affinity and specificity of the recognition process. Furthermore, it appears that these discrete receptors are either constitutively or inducibly associated with lipid rafts following the process of sperm capacitation. The fact that these specialized membrane microdomains also accommodate a family of molecular chaperones raises the intriguing possibility that spermatozoa express a multimeric zona receptor complex that is assembled into a functional unit during capacitation. The examination of this hypothesis will provide informed insights into the molecular basis of sperm–zona pellucida interaction and may pave the way for the development of novel contraceptives for feral animals and humans and the diagnosis and treatment of infertility.
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AIMS AND HYPOTHESES

The studies presented in this thesis were targeted towards elucidation of the molecular basis of the initial recognition and adhesion events between the mammalian spermatozoon and oocyte. Using the mouse as a model species, key biochemical properties of fertilisation - competent sperm were determined. This information was then be used as a foundation for investigating the nature of the zona pellucida receptor on the surface of spermatozoa. The raison d'être for the experiments presented in each paper of this thesis is described below.

The Chaperonin Containing TCP1 Complex (CCT/TRiC) is involved in mediating Sperm-Oocyte Interaction – The recent emergence of molecular chaperones on the surface of functionally mature mammalian spermatozoa has variously been reported, but as yet no report until now describes the interacting proteins of these multivalent complexes, nor delineates their functional role. Therefore the identification and application of BN-PAGE for use in membrane multiprotein complex analysis, represents a novel biochemical method, allowing for the maintenance of the critical protein – protein interactions formerly only conceived on the surface of functional spermatozoa. This report identifies a number of multimeric zona pellucida receptor complexes, focusing on a chaperonin multiprotein complex that had not previously been discovered on the surface of functional sperm. This large multiprotein complex interacts with a known zona pellucida binding protein during capacitation, which is suggested to facilitate its zona binding affinity. This paper paves-the-way for future investigations unlocking previously unknown protein interactions that are now known to populate the membrane of function sperm and give reason why single gene deletion strategies have failed to reveal the mechanism of sperm – zona pellucida interactions.

Investigation of the Expression and Functional Significance of the Novel Mouse Sperm Protein, A Disintegrin and Metalloprotease with Thrombospondin Type 1 Motifs Number 10 (ADAMTS10) – The paper presented in this thesis propose that putative receptor complexes in the sperm plasma membrane may be responsible for recognition and binding to the zona pellucida. Further it is anticipated that this complex
comprises molecular chaperones that become activated during the cell signalling events associated with capacitation to facilitate the exposure of functional zona recognition moieties to the cell surface. The culmination of these studies was to investigate the identities of other members of the second putative receptor complex shown to interact with isolated zona pellucida proteins during Far Western blotting. As a result of this research the protein a disintegrin and a metalloprotease domain with thrombospondin motifs 10 (ADAMTS10) was putatively identified as a member of this sperm surface complex. Members of this protein family have previously been implicated in cell-cell interaction. Further characterisation of the chaperone/ADAMTS10 interaction in spermatozoa will inform future studies on sperm-zona interaction and will have applications in the treatment of male infertility and the development of novel methods of male-oriented fertility control.
The Chaperonin Containing TCP1 Complex (CCT/TRiC) is Involved in Mediating Sperm-Oocyte Interaction

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Accepted for publication by Journal of Biological Chemistry
ABSTRACT

Sperm-oocyte interactions are among the most remarkable processes in cell biology. These cellular recognition events are initiated by an exquisitely specific adhesion of free-swimming spermatozoa to the zona pellucida, an acellular matrix that surrounds the ovulated oocyte. Decades of research focusing on this interaction have led to the establishment of a widely held paradigm that the zona pellucida receptor is a single molecular entity that is constitutively expressed on the sperm cell surface. In contrast, we have employed the techniques of blue native polyacrylamide gel electrophoresis, far-Western blotting and proximity ligation to secure the first direct evidence in support of a novel hypothesis that zona binding is mediated by multimeric sperm receptor complex(es). Furthermore, we show that one such multimeric association, comprising the chaperonin-containing TCP1 complex (CCT/TRiC) and a zona binding protein, zona pellucida binding protein 2, is present on the surface of capacitated spermatozoa and could account for the zona binding activity of these cells. Collectively, these data provide an important biochemical insight into the molecular basis of sperm-zona pellucida interaction and a plausible explanation for how spermatozoa gain their ability to fertilize.
INTRODUCTION
Sperm-oocyte recognition not only initiates fertilization but also represents a key process in the aetiology of male infertility and a strategic target for fertility regulation. When they are released from the testes, spermatozoa are completely lacking in any capacity to recognize the surface of the oocyte. They only acquire this competence as a consequence of a post-testicular maturation process, which proceeds in two major phases. The first of these phases occurs in the epididymis and bestows upon spermatozoa the potential to exhibit progressive motility and interact with the oocyte (Cooper, 1998). However, it is not until these cells enter the female reproductive tract that this functional potential is expressed as the spermatozoa complete the second phase of maturation, known as capacitation (Austin, 1951a; Chang, 1951b).

Since spermatozoa are transcriptionally inactive, their functional transformation during capacitation is reliant upon post-translational modifications and surface remodeling events. These processes are associated with the activation of complex, capacitation-associated signal transduction pathways, one of the most significant of which leads to a dramatic increase in protein tyrosine phosphorylation (Visconti et al., 1995a; Visconti et al., 1995b; Salicioni et al., 2007). Interestingly, these intracellular events also have a profound influence on the sperm surface architecture, rendering these cells competent to bind to the zona pellucida (ZP) and to respond to this cell recognition event with the initiation of acrosomal exocytosis (Harrison and Gadella, 2005; Nixon et al., 2007a).

The molecular mechanisms that direct sperm-ZP interactions remain controversial and are the subject of ongoing debate (Dean, 2004; Hoodbhoy and Dean, 2004; Rodeheffer and Shur, 2004; Dean, 2005; Lyng and Shur, 2009b). The most widely accepted paradigm holds that spermatozoa interact in a relatively species-specific manner with O-linked carbohydrate ligands that furnish the ZP3 glycoprotein (Bleil and Wassarman, 1980b; Bleil et al., 1981; Bleil and Wassarman, 1983; Florman and Wassarman, 1985; Bleil and Wassarman, 1988a; Mori et al., 1993; Litscher et al., 1995; Thall et al., 1995a; Wassarman et al., 2004a; Clark and Dell, 2006a). Consistent with this notion, a number of candidate ZP3 receptors have been identified (Nixon et al., 2007a). However, it has become increasingly apparent that no single receptor candidate is uniquely responsible for mediating ZP adhesion (Lu and Shur, 1997; Ensslin and Shur, 2003). Rather, the work of a number of independent laboratories has raised the intriguing prospect that individual receptor candidates may be presented to the ZP in the form a functional multimeric complex. Moreover, there is strong correlative evidence to suggest that the assembly of such a complex may be choreographed by the concerted action of a family of molecular chaperones that are themselves activated during capacitation (Ikawa et al., 1997; Asquith et al., 2004b; Walsh et al., 2008b; Nixon et al., 2009b).
While such a model of sperm-ZP interaction would account for both the diversity of candidate receptors and the dependence of sperm-zona binding on capacitation (Austin, 1951a; Chang, 1951b), at present there is no direct evidence that spermatozoa possess the proposed multimeric zona receptor complexes nor express them in a subcellular region compatible with zona binding. In the studies described herein we have employed blue native polyacrylamide gel electrophoresis (BN-PAGE) as a means of providing direct evidence for the presence and functional significance of multimeric sperm surface complexes. BN-PAGE is an electrophoretic technique for high-resolution separation of biologically active, native protein complexes. The isolation of such complexes is facilitated by solubilization of cellular membranes in a weak, nonionic detergent that maintains protein-protein interactions (Krause, 2006). Although originally developed for the analysis of the large multienzyme complexes comprising the mitochondrial electron transport chain (Schagger and von Jagow, 1991; Schagger et al., 1994), BN-PAGE has since been successfully adapted for the isolation and functional characterization of protein complexes embedded within the plasma membrane of a variety of cell types (Schagger and von Jagow, 1991; Neff and Dencher, 1999; Aivaliotis et al., 2006). Utilizing this technique, we have been able to substantiate the presence of chaperone laden protein complexes on the surface of capacitating mouse spermatozoa and demonstrate that a subset of these complexes display high affinity binding to the zona pellucida.
**EXPERIMENTAL PROCEDURES**

*Reagent*

Unless otherwise specified, chemicals and antibodies were purchased from Sigma Chemical Co. (St Louis, MO) and were of molecular biology or research grade. BSA and CHAPS detergent were purchased from Research Organics (Cleveland, OH). HEPES, penicillin and streptomycin were obtained from Gibco (Paisley, UK). Protease inhibitor tablets were obtained from Roche (Mannheim, Germany). Nitrocellulose membranes were from Amersham (Buckinghamshire, UK). Coomassie brilliant blue G250 was from Serva (Heidelberg, Germany). Anti-CCT2 (rat monoclonal, c/n sc-58864), anti-CCT6A (goat polyclonal, c/n sc-13897), anti-CCT8 (goat polyclonal, c/n sc-13891) and anti-PRM2 (rabbit polyclonal, c/n sc-30172) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-CD59 (mouse monoclonal, c/n ab9182) and anti-ZP3R (mouse monoclonal, c/n ab17358) were purchased from Abcam (Cambridge, UK). Anti-ZPBP2 (mouse polyclonal, c/n H00124625-B01) was purchased from Abnova (Taipei, Taiwan). Anti-OxPhos complex IV subunit 1 (mouse monoclonal, c/n 459600) was purchased from Invitrogen (Camarillo, CA). Fluorescein isothiocyanate (FITC) conjugated secondary antibodies and Alexa Fluor 594-conjugated Arachis hypogaea lectin (PNA) were purchased from Sigma. Proximity ligation reagents were all acquired from Olink Bioscience (Uppsala, Sweden). Horseradish peroxidase (HRP) conjugated streptavidin was purchased from Chemicon (Temecula, CA). Protein G Magnetic Beads were obtained from Millipore (Billerica, MA). 3,3’-dithiobis[sulfosuccinimidylpropionate] (DTSSP) was purchased from Pierce Biotechnology (Rockford, IL). NativePAGE Novex 4-16% Bis-Tris gels and Native-Mark unstained protein standards were bought from Invitrogen (Carlsbad, CA).

*Preparation of mouse spermatozoa*

All animal procedures described in this article received approval from our institutional animal ethics board. Spermatozoa were isolated from the caudae epididymides of euthanized adult Swiss mice by retrograde perfusion. Both negative control (non-capacitated) and positive control (capacitated) spermatozoa were prepared as previously described (Asquith et al., 2004b). Spermatozoa were isolated from caput epididymides following gentle homogenization of the tubules in BWW. The sperm suspension was then overlaid above a 27% Percoll gradient and centrifuged at 600 × g for 15 min at 37°C (Nixon et al., 2006).
**Biotinylation of sperm surface proteins**

To investigate the surface orientation of the native protein complexes isolated in this study, spermatozoa were vectorially labeled with sulfo-NHS-LC-biotin (Pierce), a membrane impermeable derivative of biotin as previously described (Nixon et al., 2006).

**Blue Native polyacrylamide gel electrophoresis**

Following incubation under either capacitating or non-capacitating conditions (Asquith et al., 2004b), suspensions of $2 \times 10^6$ sperm/ml were lightly pelleted ($300 \times g$ for 5 min) and resuspended in two volumes of native protein lysis buffer consisting of: 1% n-dodecyl-β-D-maltoside (a weak, nonionic detergent; adjusted to a final concentration below that of the critical micelle concentration; Sigma), 0.5% Coomassie Blue G250n (Serva) and a cocktail of protease inhibitors (Roche). The samples were gently mixed using a slow revolution vortex and then incubated at 4°C on an orbital rotator for 30 minutes. Following incubation, the samples were again lightly vortexed, then centrifuged at 14,000 $\times g$ at 4°C for 15 min to remove cellular debris. The solubilized protein suspension was then dialyzed against BWW (Biggers et al., 1971) overnight at 4°C to remove excess salts and detergent. In experiments designed to examine the ability of these native protein preparations to adhere to the zona pellucida, sperm were extracted using native protein lysis buffer prepared without Coomassie blue G250.

For the purpose of one dimensional blue native PAGE (1D BN-PAGE), native protein lysates were loaded onto pre-cast BN-PAGE gels (NativePAGE Novex 4-16%, Bis-Tris; Invitrogen, Carlsbad, CA), and resolved using the NativePAGE cathode and anode buffer system (Invitrogen). The gels were then removed from the electrophoresis apparatus and stained with Coomassie G250. Where necessary, this was followed by silver staining to detect less abundant material. Alternatively, the gels were prepared for either Western blot analysis or two dimensional BN-PAGE (2D BN-PAGE).

2D BN-PAGE was conducted in order to separate native protein complexes into their individual components. Preparation for this analysis involved excising a single lane of a 1D BN-PAGE gel and subjecting it to reduction and alkylation in 0.5% w/v DTT and 4% w/v iodoacetamide respectively. The lane was then loaded above a 10% SDS PAGE gel and secured in place with molten agarose. The second dimension gel was resolved at 100V for 2 h as described (Schagger and von Jagow, 1991). Gels were then removed from their cassette and either stained with colloidal Coomassie G-250 or prepared for Western blotting.
Western blot and far-Western blot analyze

Proteins resolved by either 1D, 2D BN-PAGE or SDS-PAGE were transferred onto nitrocellulose membranes using conventional Western blotting techniques. The primary antibody was diluted in TBS containing 1% w/v BSA and 0.1% v/v polyoxymethylene sorbitan monolaurate (Tween-20; TBS-T) (anti-CCT2, 1:1000; anti-CCT6A, 1:1000; anti-CCT8, 1:1000; anti-CD59, 1:4000; anti-COX, 1:1000; anti-PRM2, 1:2000; anti-α-tubulin, 1:4000; anti-ZP3R, 1:1000; and for anti-ZPB2, 1:1000) and incubations were conducted for 1 h at room temperature. Labeled proteins were detected using an enhanced chemiluminescence (ECL) kit (Amersham).

To identify native protein complexes with affinity for zonae pellucidae, 1D BN-PAGE gels were transferred to nitrocellulose membranes and prepared for far-Western blotting with solubilized, biotin-labeled preparations of mouse zonae pellucidae. These zonae pellucidae were prepared by incubation of oocytes (approximately 200/experiment) in 1 mg/ml sulfo-NHS-LC-biotin at 37°C for 30 min. The biotin reaction was quenched by the addition of Tris (pH 7.4) to a final concentration of 1 mM. Oocytes were washed 3 × to remove unbound biotin and the zonae pellucidae solubilized by incubation in acidified HBSS/PVA (pH adjusted to 2.0 with 1 M HCl) for 15 min at 37°C. Solubilized zona proteins were removed from insoluble oocyte material by aspiration with a fine bore micropipette, and then the pH readjusted to 7.4 with 1 M NaOH. This preparation was then incubated with 1D BN-PAGE Western blots overnight at 4°C on an orbital rotator. Membranes were then washed 3 × in TBS-T before incubation with HRP-conjugated streptavidin (diluted 1:3000 in 1% w/v BSA/TBS-T). Labeled complexes were then detected using ECL as described above.

Immunolocalization of proteins of interest
Following isolation, spermatozoa were diluted to 1 × 10⁶ cells/ml, and incubated in either non-capacitating or capacitating medium (Asquith et al., 2004b). The cells were then fixed in 1% paraformaldehyde, washed 3 × with 0.05 M glycine in phosphate-buffered saline (PBS), plated onto poly-L-lysine coated glass slides and allowed to settle overnight at 4°C. Unless indicated all incubations were performed in a humid chamber at 37°C. The cells were blocked with 10% serum/3% BSA for 1 h. Slides were washed 3 × with PBS for 5 min and incubated in a 1:100 dilution of primary antibody at 4°C overnight. Slides were then subjected to 3 × 5 min washes with PBS and incubated in a 1:300 dilution of the appropriate FITC-conjugated secondary antibody for 1 h at 37°C. Slides were again washed and mounted in 10% mowiol 4-88 (Calbiochem) with 30% glycerol in 0.2 M Tris (pH 8.5) with 2.5% 1,4-diazobicyclo-(2.2.2)-octane (DABCO).
**In Situ Proximity Ligation Assay**

Sperm cells were prepared as described for the immunolocalization procedure above until the addition of primary antibody step. At this point, two individual primary antibodies from different host species [mouse anti-ZPBP2 and anti-α-tubulin antibodies (1:100 dilution) and goat anti-CCT6A and anti-CCT8 (1:100 dilution)] were incubated with the cells simultaneously overnight at 4°C, and were then washed 3 × with PBST. To detect primary antibodies with the *in situ* proximity ligation assay (PLA), the PLA probes mouse PLUS and goat or rabbit MINUS (Olink Biosciences, Sweden) were added at a 1:5 dilution in antibody dilution buffer (Olink Biosciences, Sweden) for 60 min at 37 °C. After washing the coverslips with PBST 3 ×, the probe was detected using *in situ* PLA detection kit 594 (Olink Biosciences) according to the manufacturer's instructions. Immunolocalization on sperm cells was examined using either a Zeiss Axioplan 2 fluorescence microscope or an LSM510 laser scanning confocal microscope equipped with argon and helium/neon lasers (Carl Zeiss Pty, Sydney, Australia).

In order to assess whether protein localization was influenced by the acrosomal status of spermatozoa, acrosomal exocytosis was induced by incubation of capacitated cells with 5 µM calcium ionophore A23187 for 30 min as previously described (Bleil and Wassarman, 1983). Following incubation, the cells were sequentially labeled with the appropriate primary and FITC-conjugated secondary antibody as indicated above. Spermatozoa were then dual labeled with PNA conjugated to Alexa Fluor 594. The slides were then washed, mounted in antifade medium as described above, and assessed by fluorescence microscopy.

**Flow cytometry**

The surface localization of proteins in live spermatozoa was assessed by flow cytometry following labeling of these cells with the appropriate primary and secondary antibodies as previously described. The spermatozoa were then counterstained for 15 min with propidium iodide (0.5 µM) and after washing, were analyzed on a fluorescence-activated cell sorting (FACS) Vantage flow cytometer (Becton Dickinson, San Jose, CA, USA). This system collects fluorescence data in logarithmic mode and light-scatter data in linear mode. Ten thousand cells were counted in each sample at a rate of 50–500 events per second. Data were analyzed using the Cell Quest package.

**Immunobead detection of proteins on live spermatozoa**

To confirm the surface expression of the chaperonin proteins, 5 µg of each chaperonin antibody was conjugated to protein G magnetic beads (Millipore) overnight at 4°C. Controls included beads conjugated to an irrelevant, antibody (anti-α-tubulin) and a sample of non-conjugated
beads that were incubated with PBS only. The antibody-bead complexes were washed twice in PBS and then incubated with populations of non-capacitated and capacitated spermatozoa. The sperm-bead suspensions were plated onto pre-warmed microscope slides and recorded using an Axiovert S100 phase contrast microscope with a JVC digital CCD color video camera attached. Following a 15 min co-incubation with beads, the percentage of live motile spermatozoa with bound beads was determined for each treatment.

**Sperm-zona pellucida binding assay**

To examine the physiological importance of the proteins identified by ESI-MS in relation to sperm-zona pellucida interaction, capacitated and non-capacitated spermatozoa were prepared as described and incubated with the appropriate antibodies (diluted 1:100) for 30 min at 37°C as described (Asquith et al., 2004b) in the presence of 10 to 12 cumulus free mouse oocytes per experiment. At the end of this incubation period the eggs were removed, washed 3 × to remove loosely adherent spermatozoa and mounted in 5 µl of warm BWW. The number of sperm bound to each zona pellucida was counted using phase contrast microscopy.

**Co-immunoprecipitation of chaperonin interacting proteins**

Approximately 60 µl (per treatment) of protein G magnetic beads (Millipore) were washed 3 × in PBS. This was followed by conjugation with 5 µg of either anti-CCT6A antibody or an irrelevant, isotype matched antibody (anti-β-tubulin) at 4°C overnight with constant mixing. Following conjugation, the antibody-bead complexes were washed 2 × and then covalently cross-linked by incubation in 15 mM DTSSP (Pierce) for 2 h at 4°C. The cross-linking reaction was quenched using 1 M Tris and the conjugated beads were washed as above. A control sample of beads was also left non-conjugated and was incubated with PBS only. Each of the conjugated bead preparations were then incubated with approximately 100 µg of native sperm lysates (prepared as described above) that had been pre-cleared against unconjugated beads to limit non-specific interactions. After an overnight incubation at 4°C with constant mixing, the beads were washed 3 × prior to elution of bound proteins with 0.2 M glycine (pH 2.5) for 2 min at room temperature. The beads were finally boiled in SDS running buffer at 100°C for 5 min. Precipitated proteins were resolved on 10% polyacrylamide gels and silver stained.

**Mass spectrometry identification of proteins of interest**

Proteins of interest were carefully excised and separation of tryptic peptide mixtures was achieved by nanoscale reversed phase high pressure liquid chromatography (HPLC), in combination with online electrospray ionization (ESI)-MS. The mass spectrometric analysis was performed on an LTQ-linear ion trap system (Thermo Scientific, West Palm Beach, FL,
USA). Prior to ion trap analysis, an HPLC-separation was performed using a nano-AQUITY system (Waters, Rydalmer, Australia), employing a linear gradient of 2-40% buffer B (100% ACN, 0.1% formic acid) over 60 min. The C18 column system consisted of a trapping column (5 μM bead, 180 μm inner diameter × 20 mm) and a separation column (1.7 μM bead, 75 μm inner diameter × 150 mm length). For online coupling, a nano ion spray source was used, equipped with an ESI needle (10 μm silica tip). The needle voltage was 2.1 kV in positive ion mode. The scan cycle consisted of a survey scan (mass range 500-2000 a.m.u.) followed by MS/MS of the 4 most intense signals in the spectrum with an exclusion list for ion signals set to 25 sec after one occurrence. For CID analysis, we used normalized collision energies set to 26 or 35; q = 0.18 with an activation time set to 30 milliseconds and the isolation width set to m/z 1.0. Analysis was performed in CID only mode as previously described (Baker et al., 2010).

**Bioinformatics**

The derived mass spectrometry datasets were converted to generic format (*.dta) files using the Bioworks Browser (v3.3.1). These files were then searched against the mouse IPI database (v3.44 containing 55,078 proteins) using the Bioworks (v3.3.1) search algorithm, TurboSequest™ (v3.3.1; Thermo-Finnigan). The species subset was set to *Mus musculus*, the number of allowed trypsin missed cleavages was set to 2.0. Carbamidomethylation of cysteine was set as a fixed modification, while oxidation of methionine and phosphorylation of serine, threonine and tyrosine were set as differential modifications. The parent ion selection was set to 1.4 Da with fragment ion set to 0.7 Da. Peptides failing the preliminary filters set for the charge states +1 to +4 respectively with xcorr values lower than 2.5, 2.9, 3.1 and 3.2 were automatically rejected. Those spectra meeting these criteria were manually inspected on a residue by residue basis to ensure accurate y and b-ion detection with overlapping sequence coverage.

LTQ RAW files were also processed by the in house licensed Mascot database search program (version 2.2; Matrix Science) utilizing the LCQ_DTA script to convert the RAW files into peak lists prior to MS/MS database searching. Peak lists were searched against the SwissProt Database (version 51.6). The taxonomy, missed cleavage sites, modifications and mass tolerances were kept the same as the TurboSequest searches. Results were filtered to a confidence level of 0.001 and the peptides were manually inspected and validated.

**Statistics**

All experiments were replicated a minimum of 3 × with pooled sperm samples obtained from at least 3 mice. Graphical data are presented as mean values ± standard error of the mean (s.e.m),
being calculated from the variance between samples. Statistical significance was determined using an analysis of variance (ANOVA).
RESULTS

Mouse spermatozoa express a number of surface-oriented multimeric protein complexes

The separation of native multimeric protein complexes from mouse spermatozoa was achieved using BN-PAGE techniques optimized from the methodology originally described by Schagger and von Jagow (31). This system consistently resolved in excess of ten, high molecular weight (100 to 1100 kDa) entities, which we cautiously define as multi-protein complexes (MPCs), from populations of both caput and caudal epididymal spermatozoa (Fig. 1A and B). Interestingly, the profile of MPCs showed several qualitative changes during epididymal maturation, with caput cells being characterized by a number of bands that appeared to be absent in the later stages of their development. However, the subsequent capacitation of these spermatozoa did not appear to be associated with further overt changes in the MPC profile (Fig. 1C).

To explore the possibility that the putative protein complexes reside on the surface of spermatozoa, a position compatible with a role in sperm-zona pellucida interaction, populations of capacitated sperm were surface labeled with a membrane-impermeable derivative of biotin. These cells were then lysed under native conditions, the proteins resolved by BN-PAGE, transferred to nitrocellulose membranes and affinity labeled with HRP-conjugated streptavidin. As depicted in Fig. 1D, this approach revealed at least eight prominent biotinylated protein bands ranging in molecular mass from 60 to >800 kDa. Importantly, a number of the putative protein complexes identified by 1D BN-PAGE (Fig. 1C) appeared to co-migrate with those detected by biotinylation, suggesting that at least some of the components of these complexes are surface expressed. Support for this conclusion is also afforded by the specificity of surface biotinylation. In this context, representative proteins that are abundantly expressed in the cytosol (α-tubulin), mitochondria (Complex IV Subunit 1) or nucleus (protamine) were not detected among any of the biotinylated complexes (Fig. S1A-E). To the best of our knowledge, these results represent the first reported example of the isolation of native protein complexes from the surface of mouse spermatozoa.

Mouse sperm MPCs adhere to solubilized zonae pellucidae

To investigate the functional significance of the putative sperm protein complexes their affinity for solubilized zona pellucida was assessed under conditions of high stringency using the technique of far-Western blotting. This revealed at least two relatively large protein complexes, of molecular weight ~820 and ~220 kDa (hereafter referred to as Complex I and Complex II, respectively) (Fig. 1E), that bound to the zona pellucida proteins. It was of considerable interest that the migration of both of these complexes corresponded with bands that featured prominently among those displaying surface expression (Fig. 1D).
Figure 1. Separation and functional analysis of multiprotein complexes isolated from mouse spermatozoa. Native lysis prepared from A: caput and B-C: cauda (non-capacitated and capacitated) epididymal sperm were resolved by BN-PAGE and stained with Coomassie G250. The numbers on the top of panel A, correspond to the molecular weight (kDa) of BN-PAGE protein markers. D: Capacitated spermatozoa were surface labeled with biotin prior to lysis of the cells under native conditions. The lysate was then prepared for BN-PAGE and affinity labeling with HRP-conjugated streptavidin. E: Following separation of capacitated sperm proteins by BN-PAGE, the gel was transferred to a nitrocellulose membrane and incubated with
solubilized, biotinylated zona pellucida recovered from approximately 200 oocytes. The blot was then washed thoroughly prior to detection of bound zonae with HRP-conjugated streptavidin. Two predominant bands (I and II) were detected that possessed ZP affinity. F: A single lane of a 1D BN-PAGE gel comprising capacitated sperm protein complexes was excised and individual proteins resolved on a 10% SDS PAGE gel. These experiments were each replicated 3 times and representative gels and Western blots are shown.

Identification and characterization of the individual proteins that comprise sperm protein Complex I (820 kDa)

Two-dimensional BN-PAGE was used to confirm that the putative MPCs recovered from spermatozoa were indeed composite structures compromising multiple proteins. As anticipated, this technique was effective in resolving the majority of the MPCs into a number of discrete proteins (Fig. 1F). It was of particular interest that the large, surface expressed complex of approximately 820 kDa, Complex I, resolved into several individual proteins with molecular weights ranging from 55 to 65 kDa (Fig. 1F). Given that this complex was demonstrated to possess an affinity for the zona pellucida, these individual proteins were excised and prepared for identification using an electrospray ionization mass spectrometry interface. This sequencing strategy identified all 8 members of the eukaryotic cytosolic chaperonin containing TCP-1 (CCT), also known as the TCP1 ring complex (TRiC) (i.e. CCT1-CCT8) (Tab. S. 1). In light of the novelty of this finding, studies were undertaken to confirm both the validity of our sequencing data and the putative surface orientation of this complex. For this purpose, commercial antibodies against three of the 8 CCT proteins (CCT2, CCT6A and CCT8) were sourced and validated (Fig. S2) prior to use in immunoblotting and immunofluorescence analyses.

As demonstrated in Fig. 2A-C, each of the three CCT proteins demonstrated cross-reactivity with a single band in 1D BN-PAGE blots corresponding to Complex I. Following resolution of this complex by 2D BN-PAGE, a single cross-reactive protein of the appropriate molecular weight of approximately 60 kDa was detected using anti-CCT2 and anti-CCT8 antibodies (Fig. 2D and F). Anti-CCT6A generated similar results however in addition to the expected band of 58 kDa it also labeled a second protein of approximately 60 kDa (Fig. 2E). Our preliminary analysis of this additional protein indicates that it may originate from a post-translational modification of the parent protein during capacitation (Fig. S2). However we are yet to explore the nature of this potential modification.
### Western Blot Analysis

**1D BN-PAGE Western Blot**

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**Antibodies Used:**
- anti-CCT2
- anti-CCT6A
- anti-CCT8

**2D BN-PAGE Western Blot**

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Figure 2. Western blot analysis of the composition of sperm membrane complex I. A-C: Native sperm lysates were resolved by 1D BN-PAGE and transferred to nitrocellulose membranes. The membranes were then probed sequentially with either A: anti-CCT2, B: anti-CCT6A or C: anti-CCT8 followed by an appropriate HRP-conjugated secondary antibody. D-F: Two dimensional BN-PAGE Western blots were probed with a similar panel of antibodies, D: anti-CCT2, E: anti-CCT6A and F: anti-CCT8. Each experiment was replicated 3 × and representative Western blots are shown.

Having confirmed that the chaperonin proteins were constituents of the approximate 820 kDa multimeric zona-binding Complex I, we sought to localize the proteins on populations of fixed but non-permeabilized spermatozoa (Fig. 3A). Three predominant labeling patterns: (i) mid- and principal- piece of the tail, (ii) periacrosomal region combined with the mid- and principal-piece of the tail, and (iii) periacrosomal region of the head alone, were observed in these cells (Fig. 3A). However, the percentage of cells in each category differed among the chaperonin subunits examined and was also influenced by the capacitation status of the sperm population. Interestingly in this regard, the portion of cells displaying periacrosomal labeling for the CCT2 and CCT6A proteins increased following the induction of capacitation (Fig. 3B). In contrast, CCT8 was consistently observed in the periacrosomal region of the sperm head, regardless of the capacitation status of the cell (Fig 3B).
Figure 3. Immunolocalization of the subunits of the CCT/TRiC in mouse spermatozoa. Fixed populations of non-capacitated and capacitated epididymal spermatozoa were sequentially labeled with anti-CCT2, anti-CCT6A or anti-CCT8 and the appropriate FITC-conjugated secondary antibody. A: Representative images of the patterns of (i) midpiece and tail, (ii) periacrosomal region, midpiece and tail, and (iii) periacrosomal region only labeling obtained for CCT2, CCT6A and CCT8 are depicted. B: Since the labeling patterns differed with each antibody and with the capacitation status of the spermatozoa, a quantitative assessment was performed of the labeling patterns observed across a minimum of 300 cells. These data are expressed as the mean ± s.e.m from 3 replicates. ** P<0.01 and *** P<0.001. C: The interaction between CCT subunits and CTB (a high affinity label of the membrane raft marker, G_{M1} gangliosides) was investigated using standard colocalization techniques. Merged images of
spermatozoa dual labeled with antibodies against CCT subunits (green) and CTB (red), co-localization results in a yellow fluorescent signal. D: The expression of each of the chaperonin subunits (CCT2, CCT6A & CCT8) was examined on the surface of live populations of non-capacitated and capacitated spermatozoa via flow cytometry. Importantly, no labeling was observed in the absence of primary antibody. In contrast, cells labeled with the anti-CD59 as a positive control revealed surface expression on more than 70% of the viable sperm population. This experiment was repeated 3 × with a minimum of 10,000 viable cells scored for each experiment; data expressed as the mean ± s.e.m. * P<0.05 and ** P<0.01 compared with non-capacitated sample.

Given the resemblance between the spatial and temporal patterns of CCT labeling and that previously recorded for the membrane raft marker, G_M1 ganglioside (Bou Khalil et al., 2006a; Nixon et al., 2009b), we conducted co-localization studies using cholera toxin B subunit (CTB) to determine if the CCT/TRiC complex resides within membrane rafts. As illustrated in Fig. 3C, the most substantive CTB/CCT co-localization was confined to the periacrosomal region of the head of capacitated sperm, the exact location where sperm-zona interaction occurs. The surface orientation of the CCT/TRiC complex was examined through the use of a flow cytometry assay, which demonstrated that the capacitation-associated increase in the intensity of head labeling was complemented by a significant increase in the number of spermatozoa expressing CCT2 and CCT6A on their surface after this event (Fig. 3D; Fig. S3A-C). These findings were further confirmed through the application of an immunobead assay (24), the results of which demonstrated that a significantly higher percentage of capacitated spermatozoa bound to protein G beads conjugated with anti-CCT2 and anti-CCT6A antibodies compared to that of non-capacitated cells (Fig. S4B).

The CCT/TRiC complex participates indirectly in sperm-zona pellucida interaction

On the basis of the evidence presented above, experiments were conducted to determine if the CCT/TRiC complex participates either directly or indirectly in gamete interactions. For the former studies, capacitated spermatozoa were preincubated in antibodies directed against the subunits of the CCT/TRiC complex. Irrespective, of whether these antibodies were presented to sperm individually or as a cocktail of 3 antibodies (i.e. anti-CCT2, anti-CCT6A and anti-CCT8) they failed to disrupt significantly the efficacy of sperm-zona adhesion (Fig. 4A). From these results we infer that the CCT/TRiC complex does not participate directly in sperm-zona pellucida binding.
Figure 4. Investigation of the role of the CCT/TRiC in sperm-zona pellucida interaction. A: Capacitated sperm were incubated with anti-CCT2, anti-CCT6A, anti-CCT8 antibodies (diluted 1:100) individually or in the presence of a cocktail of antibodies against the CCT/TRiC (anti-TCP-1; i.e. anti-CCT2, anti-CCT6A and anti-CCT8 each diluted 1:100) for 30 min at 37°C.
The sperm suspensions were then washed and approximately $5 \times 10^5$ cells were placed in a droplet containing 10-12 salt stored oocytes. The latter were then washed to remove loosely adherent spermatozoa and the number of cells remaining bound to each zona was scored and expressed as a percentage of the positive control (i.e. capacitated sperm sample). An antibody directed against the sperm surface marker CD59 (anti-CD59) was included as a control. This experiment was replicated $3 \times$ and the data are expressed as the mean ± s.e.m, as a percentage change from the capacitated control *** $P<0.001$. B: Caudal epididymal spermatozoa were capacitated for 45 min prior to exposure to exogenous ATP (20-2000 μM) or GTP (2000 μM) for 15 min at 37°C. The sperm were assessed for their affinity for the zona pellucida as described above. This experiment was replicated $3 \times$ and the data are expressed as the mean ± s.e.m as a percentage change from the capacitated control, * $P<0.05$, ** $P<0.01$ or *** $P<0.001$. C-F: Native lysates were prepared from capacitated spermatozoa as described and then incubated with zona intact oocytes. C: After stringent washing, the oocytes were probed with anti-CCT2 and FITC conjugated secondary antibody. Controls consisted of D: 2-cell embryos (which have lost their sperm-binding properties) probed with anti-CCT2 and secondary antibody, E: oocytes incubated with secondary antibody only. F: In addition, oocytes were incubated with native lysate prepared from sperm pre-treated with 2 mM ATP and then probed as outlined above. This experiment was replicated 3 times with representative images of oocytes presented. Scale bar represents 20 μm.

In order to determine whether the CCT/TRiC complex instead participates indirectly in zona adhesion, possibly through the presentation of zona receptors, we exploited the fact that ATP binding and hydrolysis stimulates the CCT/TRiC complex to release bound substrates (Yaffe et al., 1992; Dekker et al., 2008). Capacitating populations of spermatozoa were therefore incubated with varying concentrations of exogenous adenosine 5′-triphosphate magnesium salt (ATP; 20 - 2000 μM) and examined for their zona binding affinity (Fig. 4B). Consistent with our hypothesis, ATP elicited a significant, dose-dependent inhibition of sperm-zona pellucida interactions (Fig. 4B). To control for the possibility that this inhibition was attributed to the exogenous ATP stimulating plasma membrane depolarization and acrosomal exocytosis (Rossato et al., 2005), the integrity of the acrosome was examined in each of the treatment groups (Fig. S5). Importantly, under the conditions employed in the present study, ATP did not elicit an increase in acrosomal exocytosis with at least 75% of the cells remaining acrosome intact across all treatment groups. It is also noteworthy that, in contrast to ATP, the exposure of mouse spermatozoa to GTP, incorporated as a purine nucleoside control in this experiment, led to a significant increase in the number of sperm that bound to the zona pellucida (Fig. 4B).
### Table B

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As an additional strategy for confirming the physiological relevance of the CCT/TRiC complex, native lysates were prepared from capacitated spermatozoa and incubated with zona pellucida intact mouse oocytes. The oocytes were then subjected to stringent washing before being probed with anti-chaperonin antibodies. This assay demonstrated that chaperonin proteins were indeed present within a complex that possessed an affinity for the zona pellucida (Fig. 4C). The specificity of this interaction and the requirement for the native conformation of this complex was demonstrated by the fact that anti-chaperonin antibodies failed to label the zona pellucida of 2-cell embryos (Fig. 4D) which have lost their sperm-binding properties. Similarly, the anti-chaperonin antibodies also failed to label oocytes that were incubated in denatured sperm lysates (results not shown). Secondary antibody only controls showed modest staining of the oocyte but consistently failed to label the zona pellucida (Fig. 4E). Commensurate with the results of the previous study, chaperonin binding to the zona pellucida was eliminated if the native lysates were prepared from sperm that were pre-treated with 2 mM ATP (Fig. 4F).

The CCT/TRiC complex interacts with putative zona pellucida binding proteins

The cumulative results presented above raise the possibility that the CCT/TRiC complex mediates the presentation of zona pellucida receptor(s) in capacitated spermatozoa. To test this possibility we employed a co-immunoprecipitation strategy to identify the cohort of potential receptors contributing to the zona-binding complexes following capacitation. As demonstrated in Fig. 5A, at least three predominant protein bands appeared to be precipitated with anti-CCT6A when incubated with native lysate and eluted under conditions of low pH. These bands did not appear to co-migrate with proteins precipitated with an alternative, isotype matched antibody, nor were they detected in either the bead only or anti-CCT6A antibody only controls. The analysis of these bands by LCMS (Fig. 5B) revealed the anticipated presence of members of the CCT/TRiC complex in addition to a number of putatively interacting molecular chaperones including HSPD1, a protein that has previously been implicated in the formation of multimeric zona receptor complex (26) (Fig. 5B, II). The lower band (Fig. 5B, III) yielded one of the most...
interesting findings with the identification of zona pellucida binding protein 2 (ZPBP2), one of several proteins implicated in sperm-zona pellucida interactions (Lin et al., 2007).

**Figure 6. Characterization of CCT/TRiC associated zona pellucida receptor protein ZPBP2.**

A: Western blots of SDS-PAGE, 1D and 2D BN-PAGE gels containing lysates prepared from non-capacitated and capacitated spermatozoa were probed with anti-ZPBP2 antibodies. B: Fixed populations of non-capacitated and capacitated spermatozoa were sequentially labeled with anti-ZPBP2 and an appropriate FITC-conjugated secondary antibody (green). They were then counterstained with DAPI (blue) C: Detection of the interactions of CCT6A and ZPBP2 was achieved using in situ PLA in spermatozoa capacitated under either normal conditions or in the presence of 2 mM ATP. D: The expression of ZPBP2 was examined on the surface of live populations of non-capacitated and capacitated spermatozoa via flow cytometry analysis. The percentage of live spermatozoa expressing surface immunofluorescence specific for ZPBP2 was
The role of ZPBP2 in zona pellucida binding was assessed by examining the ability of either anti-CD59 (control) or anti-ZPBP2 (diluted 1:100) to inhibit sperm binding to zona-intact oocytes. The number of spermatozoa bound to each zona was scored and expressed as a percentage of the positive control (i.e. capacitated sperm sample). Each experiment was replicated 3 × and representative Western blots and immunocytochemical pictures are shown. Graphical data expressed as the mean ± s.e.m., * P<0.05, *** P<0.001.

**Confirmation of the presence of ZPBP2 in a zona pellucida receptor complex**

Consistent with our sequencing data, antibodies directed against ZPBP2 labeled a single protein band that co-migrated with Complex I in 1D and 2D BN-PAGE gels (Fig. 6A). Furthermore, the use of electrospray ionization (ESI)-MS confirmed that ZPBP2 peptides were indeed present within Complex I (Tab. S2). Interestingly, this sequencing strategy revealed the presence of two additional proteins with previously reported zona affinity, ZP3R (formerly SP56) (Buffone et al., 2008c) and ZPBP1 (formerly SP38) (Lin et al., 2007), that also putatively reside within Complex I (Tab. S2).

Immunolocalization of ZPBP2 demonstrated that this molecule resides within the acrosomal domain of acrosome-intact (Fig. 6B), but not acrosome-reacted spermatozoa (results not shown). *In situ* proximity ligation revealed a striking co-localization of ZPBP2 and CCT6A in the acrosomal region of the head of non-permeabilized capacitated spermatozoa. In contrast, sperm cells co-incubated with 2 mM ATP during capacitation did not show co-localization within the periacrosomal region (Fig. 6C).

As previously noted for CCT2 and CCT6A, the surface exposure of ZPBP2 increased significantly in cells in which capacitation was actively driven (Fig. 6D). Moreover, the anti-ZPBP2 antibodies were able to reduce sperm-ZP interactions (Fig. 6E) emphasizing the significance of this protein in the molecular mechanisms regulating sperm-oocyte recognition.

In view of these data, additional studies were undertaken to determine whether the presence of ZPBP2 accounts, at least in part, for the zona affinity displayed by the CCT/TRiC complex. For this purpose, native lysates were prepared from capacitated spermatozoa, incubated with zona pellucida intact mouse oocytes and probed with anti-chaperonin antibodies as previously described (Fig. 4C). This assay revealed that pre-incubation of the lysates with anti-ZPBP2 antibodies led a marked reduction in the zona binding affinity of the CCT/TRiC complex (Fig. 7B). Since binding was not eliminated we conducted similar studies in the presence of antibodies against ZP3R, an additional zona adhesion candidate identified as a putative client protein of the CCT/TRiC complex (Tab. S2). The inclusion of these antibodies
also reduced CCT/TRiC binding albeit to a lesser degree. Interestingly however, the combined use of anti-ZPBP2 and anti-ZP3R antibodies virtually eliminated binding of the CCT/TRiC complex (Fig. 7D). As anticipated, an irrelevant antibody control (anti-CD59) had no effect on binding of the CCT/TRiC complex. Taken together these data highlight the importance of the CCT/TRiC complex and its client proteins in the mediation of sperm-zona pellucida adhesion.

**Figure 7.** Investigation of the role of ZPBP2 in mediating CCT/TRiC - zona pellucida interaction. A: Native lysates were prepared from capacitated spermatozoa and then incubated with zona-intact oocytes (positive control). The oocytes were then sequentially probed with anti-CCT2 and FITC conjugated secondary antibody. Alternatively, the native lysate was pre-treated with B: anti-ZPBP2 antibodies, C: anti-ZP3R antibodies or D: a cocktail of these two
antibodies prior to incubation with oocytes. Negative controls for this experiment consisted of 
E: lysates pretreated with antibodies against CD59 (a sperm surface protein not involved in zona 
adhesion) and F: oocytes incubated with secondary antibody only. This experiment was 
replicated 3 × with representative images of oocytes presented. Scale bar represents 20 µm.
DISCUSSION

Recent research into the mechanisms that underpin sperm-zona pellucida interaction have highlighted the potential importance of molecular chaperone proteins in this process (Ikawa et al., 1997; Asquith et al., 2004b). Specifically, it has been suggested that a subset of molecular chaperones are responsible for the assembly of key recognition molecules into functional zona-pellucida receptor complexes that are expressed on the sperm surface during capacitation (Asquith et al., 2004b). This novel hypothesis draws on the well characterized roles of molecular chaperones in mediating the folding of polypeptides and their assembly into mult-subunit structures. It also accounts for the myriad of candidate zona pellucida receptors that have been identified in the literature and the apparent redundancy evident from studies in which these individual candidates have been eliminated through the creation of knockout models (Adham et al., 1997; Ikawa et al., 1997; Lu and Shur, 1997; Mbikay et al., 1997; Ormandy et al., 1997; Couldrey et al., 1999; Nishimura et al., 2001; Ren et al., 2001; Luong et al., 2002). However to date, no studies have formally demonstrated the existence of chaperone containing multimeric protein complexes on the sperm surface.

In order to directly investigate whether spermatozoa express multimeric protein complexes, we employed the technique of BN-PAGE. This native gel system was selected as a separation platform since it represents a robust and versatile technique, suitable for the analysis of interacting proteins on both an analytical and preparative scale (Schagger and von Jagow, 1991; Schagger et al., 1994; Krause, 2006). This technique also affords greater resolution than gel filtration or sucrose density ultracentrifugation and, in contrast to immunoprecipitation and two-hybrid approaches, it allows the determination of the size, relative abundance and subunit composition of multiprotein complexes. To the best of our knowledge, the studies described herein represent the first recorded use of BN-PAGE for the analysis of mouse sperm proteins and provide compelling evidence that sperm do indeed express a number of large multiprotein complexes. By coupling BN-PAGE with Far-Western immunoblot analyzes it was further demonstrated that the isolated complexes retain their biological activity. In this context, at least two of the high molecular weight sperm surface complexes demonstrated an affinity for solubilized zona pellucida. Proteomic analysis of one such complex identified the 8 unique subunits of the CCT/TRiC complex (i.e. CCT1-CCT8). The original TCP-1 polypeptide was identified as a highly expressed mouse testicular protein (Silver et al., 1979) encoded by a gene located within the t-complex on chromosome 17 (Silver, 1985). The t-complex is of interest as it is known to harbor genes that influence mouse development and male, but not female, fertility (Bennett et al., 1975; Silver et al., 1979). For instance, it has been variously reported that t-specified differences exist in sperm antigenic properties (Artzt et al., 1979; Cheng and Bennett,
1980) metabolic levels (Ginsberg and Hillman, 1975; Wudl and Sherman, 1978) and, significantly, sperm-zona pellucida receptor activity (Shur, 1981).

Similar to other molecular chaperones, CCTs form a high molecular weight complex, with subunits arranged into two superimposed multimeric rings, each enclosing a central cavity that facilitates substrate binding (Gao et al., 1992; Lewis et al., 1992; Marco et al., 1994). However, unlike other chaperone complexes which are generally assembled from one or two different types of subunit, the CCT/TRiC complex is a hetero-oligomeric complex comprising eight different subunits. The CCT subunits share only 22.5-36.2% amino acid sequence identity but possess several conserved motifs (Kubota et al., 1994a) that may contribute to their function. The presence of non-conserved regions, particularly within the putative CCT substrate binding domains, suggests that each subunit may possess differing affinities for target sequences or structures within substrate proteins.

The CCT/TRiC complex is believed to function mainly as folding machinery for cytosolic proteins. While little is known regarding the mechanisms that determine its substrate specificity, recent reports suggest that this may be influenced by interaction of the complex with other molecular chaperones, including HSPD1 (Valpuesta et al., 2002; Dekker et al., 2008), a finding consistent with the results of the present study (Fig. 5B). Alternatively, it has also been suggested that the phosphorylation status of the CCT subunits may determine which specific proteins are accepted into the substrate binding pocket (Abe et al., 2009). This is of significance as our proteomic analyses indicated that a number of the identified CCT peptides displayed charge shifts consistent with those expected of phosphorylation (Tab. S1).

The major CCT/TRiC complex substrates identified to date appear to be the cytoskeletal proteins of tubulin and actin. However, the fact that these proteins show no significant amino-acid sequence similarity and represent some of the most abundant proteins within the cell (and are thus the easiest proteins to detect as substrates), raises the possibility that the CCT/TRiC complex may interact with additional protein substrates. Indeed, a number of studies have provided evidence that the CCT/TRiC complex is in fact relatively promiscuous, suggesting that ~5-10% of newly synthesized proteins flow through the CCT/TRiC complex (Kubota et al., 1994a; Thulasiraman et al., 1999a; Yam et al., 2008).

The highest expression of the CCT/TRiC complex is observed within the mammalian testis (Soues et al., 2003) where it appears to play a key role in the cytodifferentiation of spermatids, including nuclear shaping and reorganization of the cytoplasmic organelles, two processes that rely on extensive remodeling of the microtubule cytoskeleton (Soues et al., 2003).
However, the presence and function of the CCT/TRiC complex has not previously been investigated in mature spermatozoa. Our finding that components of the CCT/TRiC complex are expressed on the surface of capacitated spermatozoa and comprise part of a complex that displays affinity for the zona pellucida therefore extends the known localization and potential functions of this intriguing multimeric structure. Interestingly, we documented marked changes in the subcellular localization of the different CCT subunits during capacitation, characterized by a striking increase in the surface labeling of at least two CCT subunits. At present it is not known whether such changes reflect the structural assembly or rearrangement of each subunit leading to the novel exposure of certain epitopes and the masking of others (Pappenberger et al., 2006), or if this represents a genuine relocation of the CCT/TRiC complex as has been suggested for other molecular chaperones.

In addition to Far-Western blotting, the notion that the CCT/TRiC complex participates in zona pellucida interactions is supported by the demonstration that the native complex shows selective adherence to intact zona pellucida. Notwithstanding these data, it is considered unlikely that the CCT/TRiC complex serves as a primary receptor for zona proteins. Rather, our collective evidence suggests that it plays an intermediary role in the presentation of the appropriate adhesion molecules. Such a conclusion is consistent with the proposed role of alternative chaperone proteins (Ikawa et al., 1997; Asquith et al., 2004b; Yamaguchi et al., 2006; Walsh et al., 2008b; Nixon et al., 2009b). Calmegin, for instance fulfils a critical role in spermatogenesis by ensuring the correct folding of nascent glycoproteins destined for the acrosomal matrix and the plasma membrane of mature spermatozoa. Targeted disruption of the calmegin gene compromises the delivery of several proteins to the sperm surface and results in a concomitant reduction in male fertility attributable to the loss of sperm-zona binding ability (Ikawa et al., 1997). Our model for chaperone mediated sperm-zona pellucida interaction also aligns with the role of surface expressed molecular chaperones in other cell types. For example, HSP90 has been shown to direct the presentation of matrix metalloproteinases on the surface of fibrosarcoma cells and thus play an important role in promoting cancer invasiveness (Eustace et al., 2004).

Among the potential CCT/TRiC interacting proteins that could account for its zona affinity, we identified ZPBP2 as a compelling candidate. ZPBP2 is a paralog of the acrosomal matrix protein, zona pellucida binding protein 1 (ZPBP1), which has been implicated in secondary sperm-zona pellucida interactions (Yu et al., 2006; Lin et al., 2007; Yu et al., 2009). However, in contrast to ZPBP1 which is distributed throughout the acrosome, ZPBP2 is characterized by a discrete localization along the rostral ridge of the acrosome (Lin et al., 2007). Furthermore, while male mice lacking ZPBP1 bear substantial morphological abnormalities
arising as a result of incomplete acrosome compaction, males null for ZPBP2 display subfertility primarily associated with defects in zona pellucida interaction (Lin et al., 2007). It is also of interest that although pre-incubation of spermatozoa with an anti-ZPBP2 antibody significantly suppressed sperm-zona binding, the inhibition was incomplete and did not reach the low levels typical of non-capacitated spermatozoa (Fig. 6E). Such incomplete inhibition is consistent with our hypothesis that the multimeric zona-binding complexes expressed on the sperm surface during capacitation contain more than one type of zona receptor. For example both ZPBP1 and ZP3R (SP56) were found in Complex I (Tab. S2) and additional receptors may well be present in Complex II. At present it is not known how ZPBP2 or any of the other potential zona binding proteins interact with the CCT/TRiC complex, nor the mechanism by which this protein is presented to zona ligands. It is of interest however that some acrosomal matrix proteins, including ZP3R, are released to the sperm surface during capacitation (Wassarman, 2009). Additional work is now required to determine if this relocation is influenced by their interaction with the CCT/TRiC complex.

One particularly interesting finding was that the zona binding affinity of the CCT/TRiC complex is greatly reduced by the exposure of sperm to exogenous ATP, a result that suggests this treatment induces the release of ZPBP2 and potentially other ZP binding proteins. This interpretation is consistent with former accounts that ATP induces sequential conformational changes in the CCT/TRiC complex that facilitate the release of its bound substrates (Yaffe et al., 1992; Rivenzon-Segal et al., 2005; Dekker et al., 2008). Further compositional analysis of released CCT/TRiC substrates will aid in elucidating the full complement of sperm surface proteins with which the complex interacts.

In summary, these studies have demonstrated that capacitated spermatozoa contain chaperone-laden multiprotein complexes that display affinity for the zona pellucida and are presented at the sperm surface during capacitation. These observations encourage a complete reappraisal of the molecular mechanisms underpinning sperm-oocyte interaction; away from the single ZP3 sperm receptor model that has dominated thinking in this area for the past half century and towards a novel construct involving the dynamic presentation of zona–binding complexes on the sperm surface during capacitation. This model will open up new avenues of research into areas such as male infertility, which frequently involve failures of sperm-zona recognition (Franken et al., 1989), and contraception, where the inhibition of this process is an important strategic aim (Aitken et al., 2008).
ACKNOWLEDGMENTS

All mass spectrometry conducted during this investigation was performed by the University of Newcastle ABRF facility.
### SUPPLEMENTAL DATA

#### Supplemental Table 1. Identification of the individual proteins comprising complex I by reverse-phase high-performance liquid-chromatography (RP-HPLC) coupled with electrospray ionization mass spectrometry (ESI-MS).†

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Five proteins resolved by 2D BN-PAGE that aligned with complex I were excised and subjected to tandem mass spectrometry analysis by RP-HPLC-ESI-MS. IPI database (version 3.23) and search algorithm TurboSequest™ (v3.2; ThermoFinnigan) was used to identify peptide sequences. In each case, 3 or more unique validated peptides were used to assign protein identity. Actual mass represents the actual mass of the matched peptide compared to the \( \Delta M \) which is the difference between the actual mass and the observed mass. \( X_c \) represents the raw cross-correlation score of the top candidate peptide for a given input data file. The \( \Delta C_n \) column represents the difference in cross-correlation score (Xcorr) between the top candidate and the current candidate for a given input data file. The \( S_p \) corresponds to the preliminary score of the top candidate peptide or protein for a given input data file while the \( R_Sp \) is the ranking of the preliminary raw score \( S_p \) among the candidate peptides or proteins for a given input file. Ions represent the number of \( b \) and \( y \) fragment ions that were matched for a given peptide. \#phosp is the number of predicted phosphorylation events that were observed for each detected peptide.

Supplementary Table 2. Identification of proteins comprising complex I by reverse-phase high-performance liquid-chromatography (RP-HPLC) coupled with electrospray ionization mass spectrometry (ESI-MS)\(^1\).

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\(^1\)Identifies proteins resolved by 2D BN-PAGE that aligned with complex I and were excised and subjected to tandem mass spectrometry analysis by RP-HPLC-ESI-MS. IPI database (version 3.23) and search algorithm TurboSequest™ (v3.2; ThermoFinnigan) was used to identify peptide sequences. In each case, 3 or more unique validated peptides were used to assign protein identity. Actual mass represents the actual mass of the matched peptide compared to the \( \Delta M \) which is the difference between the actual mass and the observed mass. \( X_c \) represents the raw cross-correlation score of the top candidate peptide for a given input data file. The \( \Delta C_n \) column represents the difference in cross-correlation score (Xcorr) between the top candidate and the current candidate for a given input data file. The \( S_p \) corresponds to the preliminary score of the top candidate peptide or protein for a given input data file while the \( R_Sp \) is the ranking of the preliminary raw score \( S_p \) among the candidate peptides or proteins for a given input file. Ions represent the number of \( b \) and \( y \) fragment ions that were matched for a given peptide. \#phosp is the number of predicted phosphorylation events that were observed for each detected peptide.
null
Complex I was excised from 1D BN-PAGE gels and digested with Trypsin and subjected to tandem mass spectrometry analysis by RP-HPLC-ESI-MS (Waters nanoAcquity and Thermo LTQ XL). SwissProt Database (version 51.6) and the MASCOT search algorithm (version 2.2; Matrix Science) was used to identify peptide sequences. Peptide thresholds were set at a confidence interval of 0.001 and the MS/MS of all peptides was manually inspected and validated. The ‘observed’ mass is the actual measured experimental m/z, ‘Mr(expt)’ is the observed mass transformed to a relative molecular mass, ‘Mr(calc)’ is the calculated relative molecular mass of the matched peptide, the ‘delta’ value is the difference between the Mr(expt) and Mr(calc), ‘Miss’ represents the number of missed cleavage sites in the matched peptide, ‘Score’ is the ion score, ‘Expect’ is based on the number of times you could expect to get the Score or better by chance and Rank is the rank of the ions match, where 1 is the best match).
Supplemental Figure 1. Investigation of the specificity of surface biotinylation of native sperm complexes. A: In order to secure evidence in support of the specificity of the surface biotinylation used in this study, native sperm lysates were resolved by 1D BN-PAGE and transferred to nitrocellulose membranes. The membranes were then probed sequentially with either anti-COX1 (mitochondrial Complex IV marker), anti-protamine 2 (PRM2, nuclear marker) or anti-α-tubulin (cytosolic marker) followed by an appropriate HRP-conjugated secondary antibody. As anticipated, neither protamine 2 nor α-tubulin were detected in the native sperm lysates. However, the absence of positive labeling for the mitochondrial marker (COX1) prompted us to perform additional experiments to ensure the specificity of the anti-COX1 antibody and investigate the possibility that the cognate antigens remain attached to spermatozoa following extraction with native lysis buffer. B: For this purpose, Western blots were prepared using cell lysates isolated under reducing or native conditions and resolved on SDS-PAGE gels. The membranes were then probed with anti-COX1 antibodies before being stripped and reprobed with anti-ZPBP2 (to demonstrate appropriate protein loading). This
analysis revealed that the anti-COX1 antibodies recognized a single predominant protein of the correct molecular weight in protein extracts prepared by stringent solubilization of spermatozoa in SDS detergent. The protein was however absent in native lysates suggesting that it did remain bound to spermatozoa following this treatment. C: This notion was supported by the demonstration that the anti-COX1 antibodies retained the ability to label populations of spermatozoa that had been pre-treated with native lysis buffer. D: Furthermore, SDS protein extracts prepared from spermatozoa, after pre-treatment of the cells with native lysis buffer, did contain the COX1 protein and E: accordingly the protein was not able to be detected in populations of spermatozoa subjected to these treatments.

Supplemental Figure 2. Validation of CCT/TRiC subunit antibodies. The specificity of the anti-CCT2, CCT6A and CCT8 antibodies sourced for use in this study was assessed by immunoblotting against sperm cell lysates prepared with 10% SDS extraction buffer. As illustrated, anti-CCT2 and CCT8 antibodies labeled a single predominant band of the appropriate molecular weight (~60kDa) in lysates prepared from both non-capacitated and capacitated spermatozoa. In contrast, proteins prepared from capacitated sperm labeled two proteins of ~58 and 60 kDa when probed with anti-CCT6A antibodies. Each experiment was replicated 3 times and representative Western blots and immunofluorescence images are shown.
Supplemental Figure 3. Flow cytometric analysis of the surface expression of the CCT/TRiC subunits in mouse spermatozoa. The expression of A: CCT2, B: CCT6A and C: CCT8 was
examined on the surface of live populations of non-capacitated and capacitated spermatozoa. For this purpose, cells were labeled with anti-CCT2, CCT6A and CCT8 and an appropriate FITC conjugated secondary antibody. They were then counterstained with propidium iodide to enable determination of the viable cells in each population. This experiment was repeated three times with a minimum of 10,000 viable cells scored for each experiment and the data are expressed as the mean ± s.e.m. * P<0.05 or ** P<0.01 compared with non-capacitated sample.

**Supplemental Figure 4.** Immunodetection of chaperonins on the surface of live spermatozoa using an immunobead assay. The presence of the chaperonins on the surface of live non-capacitated and capacitated spermatozoa was assessed with anti-CCT2, anti-CCT6A and anti-CCT8 conjugated protein G magnetic beads respectively. Non-conjugated beads (beads only) and an irrelevant antibody anti-α-tubulin (α-TUB) were used as negative controls. A: Representative images of live non-bead bound (bead only control) and live bead-bound
capacitated spermatozoa are presented. **B**: The percentage of motile spermatozoa expressing the chaperonin proteins on their surface as assessed by immunobead assay. This experiment was replicated 3 times with a minimum of 100 motile cells scored for each experiment and the data are expressed as the mean ± s.e.m. *P<0.05 and **P<0.01.

Supplemental Figure 5. Investigation of the acrosomal integrity of sperm incubated with exogenous ATP. Cauda epididymal spermatozoa were capacitated for 45 min prior to exposure to exogenous ATP (20-2000 µM) or GTP (2000 µM) for 15 min at 37°C. The sperm suspensions were then assessed for their acrosome integrity by incubating fixed sperm with FITC conjugated PNA. This experiment was repeated three times with cells were scored for each experiment and the data are expressed as the mean ± s.e.m.
REFERENCES


Lu Q & Shur B D. (1997) Sperm from beta 1,4-galactosyltransferase-null mice are refractory to ZP3-induced acrosome reactions and penetrate the zona pellucida poorly. *Development* **124**, 4121-4131.


Investigation of the Expression and Functional Significance of The Novel Mouse Sperm Protein, A Disintegrin And Metalloprotease with Thrombospondin Type 1 Motifs Number 10 (ADAMTS10)

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Accepted for publication by the International Journal of Andrology
ABSTRACT

Fertilization represents the culmination of a series of complex interactions between male and female gametes. Despite advances in our understanding, the precise molecular mechanisms underlying these fundamental interactions remain largely uncharacterized. There is however growing recognition that this process requires the concerted action of multiple sperm receptors that possess affinity for complementary zona pellucida ligands and those that reside on the surface of the oolemma. Among the candidate sperm proteins that have been implicated in fertilization, those belonging to the ADAM (a disintegrin and metalloprotease) family of proteases have received considerable attention. The focus of the studies described herein has been the characterization of a closely related member of this protease family, ADAMTS10 (a disintegrin and metalloprotease with thrombospondin type 1 motifs number 10). We have demonstrated that ADAMTS10 is expressed during the later stages of mouse spermatogenesis and incorporated into the acrosomal domain of developing spermatids. During sperm maturation, the protein appears to be processed before being expressed on the surface of the peri-acrosomal region of the head. Our collective data suggests that from this position ADAMTS10 participates in sperm adhesion to the zona pellucida. Indeed, preincubation of capacitated spermatozoa with either galardin, a broad spectrum inhibitor of metalloprotease activity, or anti-ADAMTS10 antisera elicited a significant reduction in their ability to engage in zona adhesion. Overall, these studies support the notion that sperm-oocyte interactions involve considerable functional redundancy and identify ADAMTS10 as a novel candidate in the mediation of these fundamentally important events.
INTRODUCTION

Mammalian fertilization is a highly complex process, the molecular basis of which has proven extremely difficult to resolve. Despite decades of research, the paucity of our knowledge in this field continues to overshadow attempts to resolve the aetiology of defective sperm function observed in a significant proportion of infertile males and to progress the development of novel, male-oriented methods for fertility regulation (McLaughlin and Aitken, 2011). This problem stems in part from widely accepted models that depict sperm-oocyte recognition as being attributed to a single receptor-ligand interaction. Notwithstanding the appeal of this simple lock and key mechanism, it fails to account for both the myriad of putative receptors and their complementary ligands that have been identified to date and mounting evidence that gamete adhesion can be resolved into multiple interactions of both low and high affinity (Thaler and Cardullo, 1996b; Thaler and Cardullo, 2002a; Dun et al., 2010). Against this background, it has been argued that the sperm receptor may in fact be a composite structure containing multiple recognition molecules that are assembled into a functional complex during the latter stages of sperm maturation (Nixon et al., 2005a; Nixon et al., 2007a; Tanphaichitr et al., 2007b; van Gestel et al., 2007b; Gadella, 2008a; Gadella et al., 2008a). Among the challenges posed by this new model are the mechanisms by which the activity of such a large cohort of proteins is coordinated to ensure both productive interaction with the egg and integration with downstream cell signaling events. An intriguing possibility is that specialized membrane microdomains, or rafts, may serve as platforms to sequester and/or mediate the assembly of, multimeric zona receptor complexes on the outer leaflet of the sperm surface (van Gestel et al., 2005; Bou Khalil et al., 2006a; Tanphaichitr et al., 2007b; Boerke et al., 2008; Gadella et al., 2008a; Nixon et al., 2009b; Nixon et al., 2010b).

Taking advantage of unprecedented technological developments in high-throughput mass spectrometry and enhanced methods for protein pre-fractionation, we have begun to explore the proteomic profile of sperm membrane rafts. Such studies have generated extensive protein inventories, revealing the anticipated presence of a number of proteins that have been implicated in sperm-zona pellucida binding, in addition to those involved in downstream interaction with the oolemma (Nixon et al., 2009b; Nixon et al., 2010b). In addition they have identified a number of previously unknown and/or uncharacterized sperm proteins and provided the impetus for defining which specific elements of the raft proteome are of functional significance to the fertilizing spermatozoon. With this goal in mind we have focused on the characterization of one such novel protein, the metalloprotease ADAMTS10 (a disintegrin and metalloprotease with thrombospondin type 1 motifs number 10).
The rationale for targeting such a protein stems in part from pilot studies in which ADAMTS10 was identified as a putative client protein of heat shock protein 1 (HSPD1, formerly HSP60; Nixon, unpublished), a molecular chaperone protein implicated in assembly of multimeric sperm receptor complexes (Asquith et al., 2004b; Asquith et al., 2005b). Furthermore, ADAMTS10 shares a similar modular architecture (Somerville et al., 2004) and a high degree of sequence identity to the ADAM family of metalloproteases that are known to mediate diverse cell and extracellular matrix adhesion events across a variety of cell types (Primakoff and Myles, 2000; van Goor et al., 2009). A gamut of ADAM family proteins are expressed in the male reproductive tract, many of which represent isoforms unique to the male germ line (Evans, 2001). Perhaps the best characterized of these are fertilin α (ADAM1b) and β (ADAM2), a sperm membrane heterodimer originally implicated in sperm-oolemmal binding and fusion (Primakoff et al., 1987a; Wolfsberg et al., 1995). Several lines of evidence suggest that fertilin binds to integrin(s) on the oocyte plasma membrane and it has been suggested that a short hydrophobic amino acid sequence reminiscent of viral fusion proteins is responsible for its proposed role in sperm-oocyte fusion (Blobel et al., 1992; Almeida et al., 1995). However, knockout studies failed to confirm these assertions in an in vivo system (Cho et al., 1998). Somewhat surprisingly, the sperm from ADAM2 null mice showed only modest reductions in their ability to engage in oolemmal binding and fusion but a far more prominent defect in their ability to bind to the zona pellucida and navigate through the uterotubal junction (Cho et al., 1998). A similar phenotype has also been documented following targeted deletion of other sperm ADAMs (ADAM1a and ADAM3) (Shamsadin et al., 1999; Nishimura et al., 2004b) in addition to the testis specific molecular chaperones, calmegin and calsperin (Ikawa et al., 2001; Ikawa et al., 2010b), that appear necessary for ADAM protein assembly and/or presentation on the sperm surface. These data highlight a fascinating causal link between ADAM protein expression and fertilization (Ikawa et al., 2010a). It is therefore of considerable interest to determine if the closely related family of ADAMTS proteins are also critical determinants of sperm function.
MATERIALS AND METHODS

Chemicals and Reagents

Unless otherwise specified, chemical reagents were obtained from Sigma (St. Louis, MO) and were of molecular or research grade. Polyclonal antibodies raised against a synthetic peptide based on the catalytic domain of ADAMTS10 were purchased from Abcam (catalogue # ab59813; Cambridge, MA). Monoclonal anti-CD59 (cat # MCA1927) and polyclonal anti-HSPD1 (cat # sc1052) antibodies were purchased from AbD Serotec (Raleigh, NC) and Santa Cruz Biotechnology (Santa Cruz, CA), respectively. Alexa Fluor 555-labeled cholera toxin B subunit (CTB-AF555), Alexa Fluor 594-labeled *Arachis hypogaea* lectin (PNA), appropriate Alexa Fluor 488 (green) and 594 (red) conjugated secondary antibodies, and SYTOX green were all obtained from Invitrogen (Carlsbad, CA). Horseradish peroxidase (HRP) conjugated secondary antibodies were from Upstate Biotechnology (Lake Placid, NY).

Preparation of polyclonal antisera to mouse ADAMTS10

Total RNA was isolated from adult mouse testes using Trizol (Invitrogen, Carlsbad, CA) and reverse transcribed with reverse transcriptase III (Invitrogen). *Adamts10* cDNA incorporating the metalloprotease, disintegrin, cysteine-rich and spacer domains (amino acids 257–886, Fig. S1) was amplified using Taq polymerase with proofreading activity (Advantage 2 PCR Enzyme System, BD Biosciences, Sparks, MD) with synthetic oligonucleotide primers (forward: 5’-CAGAGAGATGTGGAGCAGTATGT-3’; reverse: 5’-TGGTGGACATGGTTCTGTGTTGC-3’) using standard procedures to generate an expected product size of 1890 bp. Amplified PCR products were gel purified and sequenced using standard methods. They were then ligated into the pENTR/D-TOPO (Invitrogen) cloning vector and used to transform chemically competent *E. coli* (OneShot TOP10, Invitrogen). Bacterial colonies containing the desired insert were identified by colony PCR screening and amplified by overnight culture in LB media containing 100 µg/ml kanamycin. Recombinant plasmid DNA was isolated from overnight cultures (FastPlasmid Mini kit, Eppendorf, Hauppauge, NY) and used to perform an LR recombination reaction to generate an expression clone in the pDEST17 vector (Invitrogen). This vector is designed to produce recombinant protein in a bacterial expression system with an N-terminal 6 × His tag. Recombinant expression vectors were subsequently transformed into DH5α competent *E. coli* cells and clones containing the correct insert identified as above. Purified DNA from the expression clones was finally transformed into BL21-AI chemically competent *E. coli* (Invitrogen) for recombinant protein expression.

Overexpression of the recombinant ADAMTS10 protein was induced by growth of the BL21-AI cells in the presence of 2% arabinose. Bacterial lysates containing such proteins were
affinity-purified on a nickel resin column (Ni-NTA Superflow, Qiagen, Valencia, CA) and fractions containing recombinant protein were identified on SDS–polyacrylamide gels. Excised bands corresponding to the purified ADAMTS10 protein were then used to immunize a rabbit for the production of polyclonal antisera. Immunoglobulin G (IgG) from the resulting antisera was affinity purified using immobilized protein G (Protein G Dynabeads, Invitrogen) and specific ADAMTS10 antibodies were affinity purified against the recombinant ADAMTS10 immunogen.

Collection and preparation of mouse spermatozoa

All experimental procedures were carried out with the approval of the University of Newcastle’s Animal Care and Ethics Committee (ACEC). Inbred Swiss mice were obtained from a breeding colony held at the institutes’ Central Animal House and maintained according to the recommendations prescribed by the ACEC. Mice were housed under a controlled lighting regime (16L:8D) at 21-22˚C and supplied with food and water ad libitum. Prior to dissection, animals were euthanized via CO₂ inhalation.

Immediately after adult male mice (>8 weeks old) were euthanized, their epididymides and testes were removed and carefully dissected free of fat and overlying connective tissue. The caudal region was isolated, blotted free of blood and immersed under pre-warmed water-saturated mineral oil. Caudal spermatozoa were collected by back-flushing after which the perfusate was deposited into modified Biggers, Whitten, and Whittingham media (BWW; (Biggers et al., 1971)) composed of 91.5 mM NaCl, 4.6 mM KCl, 1.7 mM CaCl₂·2H₂O, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄·7H₂O, 25 mM NaHCO₃, 5.6 mM D-glucose, 0.27 mM sodium pyruvate, 44 mM sodium lactate, 5 U/ml penicillin, 5 µg/ml streptomycin, 20 mM HEPES buffer and 3 mg/ml bovine serum albumin (BSA), then allowed to disperse into the medium for 15 min. Where indicated, negative control (non-capacitated) incubations were conducted using medium prepared without NaHCO₃ while positive control (capacitated) incubations were conducted in media supplemented with 1 mM pentoxifylline (ptx) and 1 mM dibutyryl cyclic adenosine monophosphate (dbcAMP). These treatments have been demonstrated to both suppress and promote sperm capacitation respectively (Nixon et al., 2006). An osmolarity of 300 mOsm/kg was maintained in all media.

Following collection, sperm concentration was determined and the cells diluted as required. Sperm were then assessed for motility and the non-capacitated samples used immediately. Alternatively, populations of capacitated spermatozoa were prepared by incubation for 45 min at 37°C under an atmosphere of 5% CO₂: 95% air. At regular intervals
throughout the incubation, sperm suspensions were gently mixed to prevent settling of the cells and at the end of the incubation, sperm vitality and motility were again assessed. Neither parameter was affected by any of the treatments reported in this study.

To prepare caput and corpus spermatozoa, the appropriate region of the epididymis was dissected and placed in a 500 µl droplet of BWW medium. Multiple incisions were then made in the tissue with a razor blade and spermatozoa gently washed into the medium with mild agitation. The resultant cell suspension was then layered over 27% Percoll and centrifuged (400 × g for 15 min). The pellet, consisting of >95% pure spermatozoa, was washed by gentle centrifugation (400 × g for 2 min) to remove excess Percoll and then resuspended in fresh BWW medium and counted as described above. Similarly, testicular spermatozoa were prepared by decapsulating the isolated testes, making multiple incisions in the tissue with a razor blade and allowing the cells to gently disperse into the medium with mild agitation.

**Immunohistochemical localization of ADAMTS10**

Formalin fixed testis tissue was embedded in paraffin and cut into 5 µm sections. Following de-waxing and rehydration, antigen retrieval was performed by microwaving (500 W) the sections for 20 min in citrate buffer (10 mM trisodium citrate, 4.4 mM HCl, pH 6.0). All subsequent incubations were performed at 37°C in a humid chamber, and all antibody dilutions and washes were conducted in PBS. Sections were blocked at 37°C for 1 h in 10% v/v normal rabbit serum supplemented with 3% w/v BSA in phosphate-buffered saline (PBS). Slides were washed and incubated sequentially in primary antibody (diluted 1:100) and an appropriate Alexa Fluor 488 conjugated secondary antibody (diluted 1:300). After washing, the sections were counterstained with 10 µg/ml propidium iodide, a nuclear dye included to aid morphological assessment. Slides were mounted in antifade reagent (13% Mowiol 4-88, 33% glycerol, 66 mM Tris (pH 8.5), 2.5% 1,4 diazobyclo-[2.2.2]octane), and viewed using a confocal microscope (Carl Zeiss Laser Scanning Microscope 510, Thornwood, NY).

**Immunocytochemical localization of ADAMTS10**

Sperm suspensions were lightly fixed in 1% paraformaldehyde, solubilized in 0.2 M Triton X-100 for 20 min at 4°C, and washed three times with 0.05 M glycine in PBS before being plated onto poly-L-lysine coated glass slides. The cells were blocked with 10% rabbit serum/3% BSA for 1 h at 37°C. Slides were washed with PBS prior to overnight incubation with primary antibody (diluted 1:100) at 4°C. Slides were then subjected to 3 washes in PBS and incubated with an appropriate Alexa Fluor 488 conjugated secondary antibody (diluted 1:300) for 1 h at
37°C. Slides were again washed and mounted in antifade reagent before being viewed by confocal microscopy.

For co-localization studies with cholera toxin B subunit (CTB), live sperm suspensions were mixed with an equal volume of Alexa Fluor 555-labeled CTB (10 µg/ml) and incubated for 15 min at 37°C. The cells were then washed four times in 2 volumes of BWW medium and fixed in 4% paraformaldehyde for 15 min at 37°C. The cells were aliquoted onto poly-L-lysine coated glass slides and allowed to settle before being blocked with PBS supplemented with 3% BSA and 10% goat serum for 1 h at 37°C. They were then sequentially labeled with the appropriate primary and Alexa Fluor 488 conjugated secondary antibodies for 1 h at 37°C in a dark, humidified chamber. The slides were then mounted with anti-fade reagent and observed using a confocal microscope.

In order to assess whether ADAMTS10 localization was influenced by the acrosomal status of spermatozoa, acrosomal exocytosis was induced by incubation of capacitated cells in 2.5 µM calcium ionophore A23187 for 30 min as previously described (Asquith et al., 2005b). Sperm suspensions were then washed, resuspended in hypoosmotic swelling (HOS) medium (Jeyendran et al., 1984) and incubated for an additional 1 h. Following incubation, the cells were sequentially labeled with the appropriate primary and Alexa Fluor-488 conjugated secondary antibodies as indicated above. Spermatozoa were then dual labeled with PNA conjugated to Alexa Fluor 594 and prepared for confocal microscopy as outlined above.

**Electron Microscopy**

Isolated spermatozoa were fixed in 4% paraformaldehyde/0.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.3) followed by dehydration, infiltration and embedding in LR White resin. Sections (70 nm) were cut on an Ultracut S ultramicrotome (Reichert-Jung, Austria) with a diamond knife (Diatome Ltd., Bienne, Switzerland) and placed on nickel grids. All antibody dilutions and washes for immunogold labeling were in Dulbecco’s phosphate buffered saline (DPBS; pH 7.4). Grids were treated with 0.05 M glycine dissolved in DPBS for 40 min followed by washing and blocking in 3 % BSA in DPBS for 1 h at 37°C. Primary antibody diluted to 1:25 was applied and incubated overnight at 4°C. Grids were washed and incubated with 1:20 dilutions of secondary antibody conjugated to 10 nm gold particles for 2 h at 37°C. After washing, sections were post-fixed in 2% glutaraldehyde, dried and stained with 1% uranyl acetate in 40% methanol. Micrographs were taken on a JEOL-100CX transmission electron microscope (JEOL, Tokyo, Japan) operating at 80 kV.
Flow cytometry analysis of ADAMTS10 surface expression

In order to assess the surface expression of ADAMTS10, live spermatozoa were sequentially incubated with anti-ADAMTS10 and Alexa Fluor 488 conjugated secondary antibodies for 15 min at 37°C in a dark humidified chamber, before being washed twice in BWW. Spermatozoa were then counterstained with the vitality stain, propidium iodide (0.5 µM). After washing, the cells were analyzed on a fluorescence-activated cell sorting (FACS) Vantage flow cytometer (Becton Dickinson, San Jose, CA). This system collects fluorescence data in logarithmic mode and light-scatter data in linear mode. Ten thousand cells were counted in each sample at a rate of 50–500 events per sec and data were analyzed using the Cell Quest package.

Analysis of ADAMTS10 expression in sperm membrane rafts

Low-density Brij 35 insoluble membrane fractions (detergent resistant membranes, DRMs) were prepared from mouse spermatozoa as previously described (Nixon et al., 2009b). Briefly, capacitated mouse spermatozoa were washed by gentle centrifugation (400 × g for 1 min) and resuspended in 300 µl of 1% Brij 35 in 25mM HEPES buffer (pH 6.5, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, protease inhibitor cocktail). Following mechanical disruption of the cells by homogenization in a Dounce homogenizer (30 strokes), the cells were incubated for 30 min at 4°C. Cell debris was pelleted by centrifugation at 10,000 × g for 5 min and the supernatant, containing Brij 35-insoluble material, was then mixed with an equal volume of 80% sucrose (w/v) in 25 mM HEPES buffer for a final sucrose concentration of 40%. This suspension was placed in the bottom of an ultracentrifuge tube as the base of a discontinuous sucrose gradient. Additional layers consisting of 30% (980 µl) and 5% (620 µl) sucrose were carefully overlaid and the whole gradient was centrifuged at 100,000 × g for 18 h at 4°C in an SW41 rotor (Beckman Coulter, Inc. Fullerton, CA). Following centrifugation, eleven 200 µl fractions were carefully extracted by pipette from the top of the gradients and assessed for their light scattering properties by measurement of their absorbance at 620 nm and protein concentration using a BCA assay kit (Thermo Fisher Scientific, Rockford, IL). The fractions were then stored at -20°C prior to further analysis.

Dot immunoassays were performed to examine the partitioning behaviour of GM1 gangliosides, and hence DRMs, within the sucrose gradient. Briefly, 50 µl of each of the isolated fractions were diluted 1:1 in PBS, added to wells of a Bio-Dot apparatus (Bio-Rad, Hercules, CA), suctioned onto nitrocellulose membranes and air dried. The membrane was then blocked with 5% milk powder in TBST (10 mM Tris–HCl, pH 7.5, 100 mM NaCl, 0.1% Tween 20) for 1 h at room temperature, followed by incubation with HRP-conjugated cholera toxin in
TBST supplemented with 0.5% milk powder for 1 h. It was then washed five times with TBST and subsequently developed with an enhanced chemiluminescence (ECL) assay (GE Healthcare, Piscataway, NJ, USA) and exposed to Hyperfilm ECL.

The protein composition of low-density Brij 35 insoluble membrane fractions prepared from mouse spermatozoa was assessed by SDS-PAGE and by immunoblotting analysis. For the purpose of SDS-PAGE, equivalent volumes of fractionated protein suspensions were precipitated with methanol / chloroform (2:1 v/v respectively) to collect both the detergent soluble and insoluble proteins. Following centrifugation at 10,000 × g for 1 min, the supernatant was discarded and an additional 300 µl methanol was added. The samples were then re-centrifuged at 10,000 × g for 15 min and the supernatant discarded. The protein pellets obtained from either technique were allowed to air dry, then resolubilized and denatured by boiling in SDS sample buffer (Laemmli, 1970) containing 2% β-mercaptoethanol for 5 min and resolved on 10% SDS polyacrylamide gels according to the methods described by Laemmli (Laemmli, 1970). Resolved proteins were electrotransferred to nitrocellulose membranes (Hybond Super-C, GE Healthcare) under a constant current of 300 mA for 1 h (Towbin et al., 1979) and prepared for immunoblotting with anti-ADAMTS10.

Functional analysis of ADAMTS10 in mouse spermatozoa

At present no functional data is available regarding ADAMTS10, thus precluding the use of a known specific inhibitor for functional studies on this protein. For this reason, two broader specificity inhibitors were employed. The first was tissue inhibitor of metalloprotease 3 (TIMP3), a physiological inhibitor of both matrix metalloproteases (MMPs) and of several ADAMTS proteins (Kashiwagi et al., 2001). In addition, sperm were treated with the broad spectrum metalloprotease inhibitor galardin (HONHCOCH(CH(i-Bu))CO-L-TRP-NHMe isomer 6A or GM6001) (Grobelny et al., 1992). To assess the impact of such inhibitors, as well as anti-ADAMTS10 antibodies, on sperm-zona pellucida binding ability, populations of capacitating mouse spermatozoa were incubated with each reagent for 30 min at 37°C. Following incubation, the spermatozoa were washed and an aliquot of 2 × 10⁴ cells was then deposited into a droplet of BWW containing a minimum of 8 salt stored oocytes (Nixon et al., 2006). The gametes were co-incubated under oil in 5% CO₂ for 30 min at 37°C. Following incubation the oocytes were washed three times by serial aspiration and then incubated for 5 min in a droplet of BWW supplemented with 5 μg/ml of the DNA-specific fluorochrome 4',6-diamidino-2-phenylindole (DAPI). The oocytes were then washed in BWW and mounted on glass slides under coverslips supported on pillars comprising 80% paraffin wax and 20% Vaseline gel. The number of sperm
bound to each zona was subsequently counted using both phase contrast and fluorescence microscopy and expressed as a percentage of the capacitated control sample.

**Blue native polyacrylamide gel electrophoresis**

Following incubation under either capacitating or non-capacitating conditions, suspensions of $1 \times 10^6$ sperm / ml were lightly pelleted (300 x g for 5 min) and resuspended in native protein lysis buffer (1% n-dodecyl β-D-maltoside, 0.5% Coomassie Blue G250 and a cocktail of protease inhibitors [Roche, Mannheim, Germany]) as described (Dun et al., 2011). The samples were gently mixed and then incubated at 4°C on an orbital rotator for 30 min. Following incubation, the lysate was recovered by centrifugation at 14 000 x g for 20 min at 4°C and dialyzed against the Blue native cathode buffer (Invitrogen), to remove excess salt and detergent. Following dialysis, the sample was supplemented with glycerol to a final concentration of 5% (v/v) and resolved on a pre-cast blue native polyacrylamide gel (NativePAGE Novex 4-16%, Bis-Tris; Invitrogen) using a NativePAGE cathode and anode buffer system (Invitrogen). The BN-PAGE electrophoresis apparatus was placed at 4°C and the samples separated at 100 V until the Coomassie dye front reached the bottom of the loading wells. The voltage was then increased to 200 V and the separation continued until the Coomassie dye front reached the bottom of the gel. The gels were then removed from the electrophoresis apparatus and stained with Coomassie G250. Alternatively, the gels were prepared for either immunoblotting with anti-ADMTS10 using standard procedures.

**Co-immunoprecipitation of ADAMTS10 interacting proteins**

Approximately 60 µl (per treatment) of protein G magnetic beads (Millipore) were washed 3 × in PBS. This was followed by conjugation with 5 µg of anti-ADAMTS10 antibody at 4°C overnight with constant mixing. Following conjugation, the antibody-bead complexes were washed 2 × before being covalently cross-linked by incubation in 15 mM DTSSP (Thermo Fisher Scientific) for 2 h at 4°C. The cross-linking reaction was quenched using 1 M Tris and the conjugated beads were washed as above. A control sample of beads was also left non-conjugated and was incubated with PBS only. Both of the bead preparations were then incubated with approximately 100 µg of native sperm lysates (prepared as described above) that had been pre-cleared against non-conjugated beads to limit non-specific interactions. After an overnight incubation at 4°C with constant mixing, the beads were washed 3 × prior to elution of bound proteins by incubation in 0.2 M glycine pH 2.5 for 15 min at room temperature. Precipitated proteins were resolved on 10% polyacrylamide gels and prepared for either silver staining or immunoblotting.
Proteomic analysis of ADAMTS10

To confirm the specificity of the predominant sperm protein recognized by anti-ADAMTS10 antibodies, the protein was excised from a duplicate gel, destained and dehydrated in acetonitrile before being rehydrated in a minimal volume of 20 mM ammonium bicarbonate containing 40 ng/µl trypsin for 16 h at 37°C. The resulting tryptic peptides were purified using a ZipTip and sequenced by matrix-assisted laser desorption time of flight mass spectrometry (MALDI-ToF) using an Ettan MALDI-ToF Pro mass spectrometer (Amersham Biosciences). Briefly, 1 µl fractions of the tryptic peptides were mixed with an equal volume of matrix solution (a saturated solution of α-cyano-4-hydroxycinnamic acid in 0.1% TFA:acetonitrile, 50:50) before being applied in duplicate to the stainless steel sample target plates. The peptide mass spectra were acquired in the reflectron mode and internal mass calibration was performed with two trypsin auto-digestion fragments (842.5 and 2211.1 Da). Peptide mass fingerprints (PMFs) were used as inputs to search the National Center for Biotechnology Information nonredundant (NCBI nr) database using the MASCOT search engine (http://www.matrixscience.com) with a probability-based scoring algorithm. Prior to the database search, known contamination peaks such as keratin and autoproteolysis peaks were removed. Searches were performed without restriction of protein molecular weight (Mr) or pI and with mandatory carbamidomethylation of cysteines and variable oxidation of methionine residues. One trypsin miscleavage was allowed. Peptide mass tolerance and fragment mass tolerance were set to 50 ppm and ± 0.4 Da, respectively. High confidence identifications reported in Fig. S1 were those that possessed statistically significant search scores (greater than 95% confidence interval, equivalent to MASCOT expect value <0.05).

Statistics

All experiments were replicated with material collected from at least 3 different animals and the graphical data presented represent means ± SEM, the standard errors being calculated from the variance between samples. Statistical significance was determined using an analysis of variance (ANOVA).
RESULTS

ADAMTS10 is expressed in the mouse testis and epididymal spermatozoa

To facilitate characterization of ADAMTS10, a truncated recombinant protein incorporating the metalloprotease, disintegrin, cysteine-rich and spacer domains (amino acids 257–886, Fig. S1) was synthesized using an *E. coli* expression system. Unfortunately, despite attempting several variations of the expression protocol, the recombinant ADAMTS10 product proved largely insoluble. Although this precluded its use in functional assays it was able to be used for the production of polyclonal antisera. The antisera were subsequently affinity purified and assessed for their specificity by immunoblotting against cell lysates prepared from mouse lungs (positive control), testes and spermatozoa. As the results presented in Fig. 1A demonstrate, the immune sera labeled a predominant protein of approximately 65 kDa in cell lysates prepared from either the mouse testes or lung. An additional band of approximately 120 kDa, corresponding to the size predicted for the full length, glycosylated protein, was also present in the testes and lung samples, albeit of much lower intensity. In contrast, the predominant band observed in isolated epididymal spermatozoa was approximately 50 kDa. Although this protein was observed in all sperm samples analyzed, the caput and corpus cells displayed an additional band of approximately 120 kDa, whereas an alternative weak band of 65 kDa was detected in corpus and cauda spermatozoa. Given the disparity between the predicted molecular weight of full length and processed forms of ADAMTS10 (approximately 118 kDa and 95 kDa, respectively; Somerville et al., 2004) and that observed experimentally, an additional commercial antibody raised against the catalytic domain was sourced to validate these results. Notwithstanding some minor differences, a similar profile of proteins was labeled with this additional antibody. Notably, a protein of approximately 50 kDa was again the predominant band labeled in cell lysates prepared from epididymal spermatozoa. Although such findings suggest the ADAMTS10 protein is extensively processed, the precise nature of these modification(s) remains to be fully investigated. Nevertheless, we were able to obtain partial amino acid sequence that confirmed the identity of the 50 kDa sperm protein as ADAMTS10 and demonstrate that this processed form retains the catalytic domain (Fig. S1).
Figure 1. Validation of anti-ADAMTS10 antibodies. (A) Recombinant ADAMTS10 protein was used to generate rabbit polyclonal antisera. IgG was isolated by protein G chromatography and
specific ADAMTS10 antibodies were affinity purified against the recombinant ADAMTS10 immunogen. The purified post-immune antibodies were assessed for cross-reactivity in immunoblots of protein lysates prepared from mouse testes and lung, in addition to spermatozoa sampled from the caput, corpus and cauda epididymidis (5 µg per lane). The latter samples were isolated from populations of both non-capacitated (NC) and capacitated (Cap) spermatozoa. After labeling, the blot was stripped and reprobed with anti-tubulin antibodies to confirm equal protein loading across all sperm samples. (B) In order to confirm antibody specificity, a replicate immunoblot was also probed with commercial antibodies directed against ADAMTS10. Each experiment was replicated three times and representative blots are depicted.

**ADAMTS10 is expressed in the peri-acrosomal region of developing spermatids and mature mouse spermatozoa**

Having confirmed the ability of ADAMTS10 antisera to detect the target protein in cell lysates prepared from mature spermatozoa, it was used in conjunction with indirect immunofluorescence to examine the ontogeny of ADAMTS10 expression within the testis and its localization in developing spermatozoa. As illustrated in Fig. 2, ADAMTS10 was not able to be detected in developing germ cells lining the basal margin of the seminiferous tubules. Indeed, the first significant expression of the protein was not observed until the formation of round spermatids. Within these cells, ADAMTS10 staining was primarily localized to a discrete crescent pattern characteristic of that of the developing acrosomal vesicle (Fig. 2A). Intense labeling of this domain remained apparent throughout the later stages of spermatogenesis and was clearly evident in the populations of elongating spermatids lining the luminal border of the seminiferous tubules (Fig. 2B).

To confirm acrosomal localization, enriched populations of round and elongating spermatids were prepared by sediment gradient isolation. These cells were then fixed, permeabilized and labeled with anti-ADAMTS10 before being counterstained with PNA, a marker of the acrosomal membrane (Mortimer et al., 1987). Consistent with the results secured by immunohistochemistry of whole testes sections (Fig. 2), ADAMTS10 expression was initially detected in round spermatids (Fig. 3). Although diffuse labeling of ADAMTS10 was present throughout these cells, an intense pattern of crescent shaped labeling was also apparent. Strong co-localization with PNA confirmed this labeling pattern corresponded to that of the developing acrosomal vesicle. A similar pattern of acrosomal labeling was also observed in elongating spermatids (Fig. 3), raising the possibility that ADAMTS10 is either involved in cytological remodeling of the spermatogenic cells leading to the formation of the acrosomal
vesicle and/or that the protein is transported into this domain during formation of the organelle to fulfill an important function in mature spermatozoa.

Figure 2. Expression of ADAMTS10 protein in mouse testes. (A and B) Paraffin embedded sections of mouse testes were sequentially labeled with anti-ADAMTS10 and Alexa Fluor 488 (green) conjugated secondary antibodies. The sections were then counterstained with propidium iodide (red) to assist with morphological analysis. Control sections were prepared in which the primary antibody was substituted with either (C) buffer only (secondary only) or (D) pre-immune sera. This experiment was replicated three times and representative images are shown. Scale bar = 100 µm.
Figure 3. Co-localization of ADAMTS10 with PNA in isolated populations of developing spermatozoa. Enriched populations of round and elongating spermatids were isolated via sediment gradient filtration and fixed in 4% paraformaldehyde. They were then sequentially labeled with anti-ADAMTS10 and appropriate Alexa Fluor 488-conjugated secondary antibodies (green) followed by Alexa Fluor 594-conjugated PNA (red). Immunofluorescent labeling was detected using confocal microscopy. This experiment was replicated three times and representative images are shown. Scale bar = 10 µm.

To begin to investigate the latter possibility, immunolocalization studies were undertaken to examine the profile of ADAMTS10 expression in maturing spermatozoa recovered from the male reproductive tract. Initial studies conducted on populations of fixed and permeabilized spermatozoa revealed that ADAMTS10 was strongly localized within the peri-acrosomal region of these cells. This pattern of ADAMTS10 localization appeared to be similar in mouse sperm recovered from the testis and the different regions of the epididymis and it did not appear to be noticeably influenced by the capacitation status of these cells (Fig. 4A). An additional focus of protein localization was observed within the sperm tail, however this appeared to change from predominantly principal piece to a more punctate pattern during epididymal maturation. The specificity of these labeling patterns was confirmed by the absence
of staining in the secondary only (prepared by substituting the primary antibody with media alone) and pre-immune controls.

The presence of the ADAMTS10 within the peri-acrosomal region of mature spermatozoa is of potential interest as it intuitively suggests that the protein may participate in the cascade of sperm-oocyte interactions that underpin fertilization. However, since the previous study was conducted on populations of permeabilized spermatozoa, these data provide limited insight into the surface expression characteristics of the protein. A flow cytometry assay was therefore conducted to facilitate the objective measurement of surface protein expression in live cells and the exclusion of dead or moribund cells (identified through incorporation of a cell viability stain). As shown in Fig. 4B, ADAMTS10 was expressed on the surface of less than 5% of live spermatozoa recovered from the testis, caput and corpus epididymidis. The absence of surface labeling in these cells suggests that ADAMTS10 must reside in an intracellular location. This was confirmed by ultrastructural localization of ADAMTS10 which revealed the protein was predominantly expressed the outer acrosomal membrane of these cells (Fig. S2). Interestingly, however, a marked increase in surface expression of ADAMTS10 was detected following sperm entry into the caudal region of the epididymis and a further dramatic increase was again detected following the induction of capacitation in this population of cells. The analysis of these cells by immunofluorescence microscopy confirmed that surface labeling was primarily restricted to the peri-acrosomal region of the sperm head (Fig. 4C).
Figure 4. (A) Populations of spermatozoa were isolated from mouse testis, caput, corpus and cauda epididymidis. The latter samples were either held in a non-capacitated state (NC) or driven to capacitate (Cap). The cells were fixed in 1% paraformaldehyde and permeabilized with 0.2 M Triton X-100 before being sequentially labeled with anti-ADAMTS10 and Alexa Fluor 488 conjugated secondary antibodies (green) and counterstained with DAPI (blue). Control samples were prepared in which the anti-ADAMTS10 antibodies were substituted with either buffer alone (secondary only) or pre-immune sera. This experiment was replicated three times and representative images are shown. Scale bar = 10 µm. (B) To examine the surface expression of ADAMTS10, live populations of spermatozoa were labeled with anti-ADAMTS10 and Alexa
Fluor 488 conjugated secondary antibodies before being counterstained with the vitality stain, propidium iodide. In addition to secondary antibody only controls, a positive control population of spermatozoa were prepared by labeling the cells with anti-flotilin antibodies. Sperm surface labeling was then analyzed using a fluorescence activated cell sorter. Data represent the mean +/- standard error of the mean from three separate experiments. * P<0.05, ** P<0.01. (C) The surface localization of ADAMTS10 in these capacitated spermatozoa was assessed by labeling live populations with anti-ADAMTS10 and Alexa Fluor 488 conjugated secondary antibodies (green) and counterstaining with DAPI (blue).

**ADAMTS10 partitions into membrane rafts and is lost following acrosomal exocytosis**

Our interpretation of the collective data secured in the previous studies is that ADAMTS10 represents a novel sperm protein with a potentially important role in the cellular interactions that underpin fertilization. Since a number of proteins that mediate these interactions have been shown to preferentially partition into membrane rafts, it was of interest to confirm that the ADAMTS10 resides within these microdomains and determine whether it complexes with additional proteins in capacitated mouse spermatozoa. Analysis of raft association was achieved via co-immunolocalization of ADAMTS10 with fluorescently labeled cholera toxin subunit B (CTB), a pentameric label that binds with high affinity to GM1 gangliosides, one of the key components of sperm membrane raft microdomains. As shown in Fig. 5A, ADAMTS10 displayed strong co-localization with CTB within the peri-acrosomal region of the head of the majority of capacitated spermatozoa, thus suggesting it is expressed within membrane raft microdomains in this region of the cell. This interpretation is consistent with the results of immunoblotting experiments in which ADAMTS10 was shown to be abundantly expressed in the light-buoyant density detergent resistant membrane fractions expected of a raft-resident protein (Fig. 5B, fractions 5 and 6).
Figure 5. Co-localization of ADAMTS10 proteins with cholera toxin subunit B (CTB) in mouse spermatozoa. (A) The ability of ADAMTS10 to partition in sperm membrane rafts in situ was examined by co-localization with the raft marker, G_{MI}, in live capacitated spermatozoa. For this purpose membrane rafts were visualized by staining live spermatozoa with Alexa Fluor 555 labeled cholera toxin B subunit (red). The cells were then fixed and labeled with anti-ADAMTS10 and Alexa Fluor 488 conjugated secondary antibodies (green). This experiment was replicated three times with a minimum of 200 spermatozoa being examined in each and representative images are shown. (B) The partitioning behavior of ADAMTS10 into buoyant, low density membrane fractions was examined following fractionation of detergent resistant membranes within a sucrose gradient. Each of the eleven fractions recovered from the sucrose gradient lysate were prepared for dot-blotting with CTB or immunoblotting with anti-ADAMTS10 and HRP conjugated secondary antibodies. Each experiment was replicated three times and representative data are shown. Scale bar = 5 µm.

To achieve the second goal of determining whether ADAMTS10 constitutes part of a multimeric protein complex, native sperm lysates were resolved by BN-PAGE (Fig. 6A) before
being immunoblotted with anti-ADAMTS10 antibodies (Fig. 6B). This analysis revealed cross-reactivity between ADAMTS10 antibodies and at least three high molecular protein bands of approximately 240 kDa, 740, and 800 kDa (Fig. 6B). The apparent molecular weight of these bands is considerably higher than that expected for monomeric ADAMTS10 thus raising the prospect that the protein is assembled into either a homo- or heteromeric complexes on the sperm surface. Since our previous studies have implicated the molecular chaperone, HSPD1, in the assembly of multimeric protein complexes on the surface of mouse spermatozoa (Asquith et al., 2004b), it was of interest that we could demonstrate the presence of at least two putative complexes that harbored both proteins (Fig. 6C, arrowheads). Evidence in support an ADAMTS10 - HSPD1 interaction was provided by the demonstration that both proteins co-localize within the peri-acrosomal region of mouse spermatozoa (Fig. 6D) and HSPD1 was able to be co-precipitated from native sperm lysates using anti-ADAMTS10 antibodies (Fig. 6E). In addition we were able to use this approach to confirm that both anti-ADAMTS10 antibodies recognized the same 50 kDa isoform of the protein (Fig. 6E).

To further characterize the properties of ADAMTS10, its expression was examined following induction of acrosomal exocytosis. The rationale for this study is based on well established evidence that proteins involved in primary sperm-zona pellucida interaction are lost, along with the apical plasma membrane in which they reside, following acrosomal exocytosis. As anticipated, the induction of the acrosome reaction with the calcium ionophore A23187 led to a complete loss of PNA staining within the peri-acrosomal region of approximately 50-60% of the capacitated sperm population (Fig. 7A). Acrosomal loss in each of these cells was also accompanied by a dramatic reduction in ADAMTS10 labeling within the sperm head (Fig. 7B).
Figure 6. Examination of whether ADAMTS10 constitutes part of a multimeric sperm protein complex. Native sperm lysates were resolved by (A) 1D BN-PAGE and (B,C) transferred to
nitrocellulose membranes. The membranes were then probed with (B) anti-ADAMTS10 antibodies or (C) anti-HSPD1 antibodies. This experiment was replicated 3 times and a representative BN-PAGE gel and immunoblots are shown. Arrowheads indicate protein bands that were labeled with both anti-ADAMTS10 and anti-HSPD1 antibodies. (D) The putative interaction between ADAMTS10 and HSPD1 was investigated by co-localization of the proteins in mouse spermatozoa. For this purpose, spermatozoa were stained with anti-ADAMTS10 (red) followed by anti-HSD1 (green) and appropriate Alexa Fluor conjugated secondary antibodies. This experiment was replicated three times with a minimum of 200 spermatozoa being examined in each and representative images are shown. Scale bar = 5 µm. (E) The ADAMTS10 / HSPD1 interaction was further investigated through the use of a co-immunoprecipitation strategy in which native lysates prepared from capacitated spermatozoa were immunoprecipitated with anti-ADAMTS10. Experimental controls included proteins recovered by boiling non-conjugated beads (beads only), proteins recovered from boiling non-conjugated beads following their incubation with native lysate (preclear), whole sperm native lysate (native lysate), anti-ADAMTS10 antibodies only (antibody only), and the first and final bead washes (wash 1 and wash 3, respectively). Proteins that were eluted from ADAMTS10-conjugated beads following their incubation with native sperm lysates were resolved in the final lane (elution). Following separation of the proteins they were transferred to nitrocellulose and immunoblotted with the anti-ADAMTS10 antibodies prepared in our laboratory (anti-ADAMTS10\*) to confirm the efficacy of the immunoprecipitation, before being stripped and reprobed with commercial anti-ADAMTS10 (anti-ADAMTS10\**) to confirm both antibodies recognized the same 50 kDa isoform of the protein. The membrane was then re-stripped and probed with anti-HSPD1. The arrowheads indicate proteins corresponding to ADAMTS10 and HSPD1.
**Figure 7.** Examination of ADAMTS10 behavior in acrosome reacted spermatozoa. Capacitated populations of mouse spermatozoa were induced to acrosome react through incubation with the calcium ionophore, A23187. Following the induction of acrosomal exocytosis, the cells were incubated in HOS media prior to being sequentially labeled with anti-ADAMTS10 and appropriate Alexa Fluor 488-conjugated secondary antibodies (green) followed by Alexa Fluor 594-conjugated PNA (red). Immunofluorescent labeling of viable cells was detected using confocal microscopy. This experiment was replicated three times and representative images are shown. Scale bar = 5 µm.
Inhibitors of metalloprotease function decrease sperm-oocyte interactions

In view of the data presented above, inhibitory studies were undertaken to begin to investigate the function of ADAMTS10 in mouse spermatozoa. In the absence of specific pharmacological inhibitors of ADAMTS10 function, we instead employed the use of two broad specificity metalloprotease inhibitors: tissue inhibitor of metalloprotease 3 (TIMP3), a physiological inhibitor of both matrix metalloproteases (MMPs) and of several ADAMTS proteins (Hashimoto et al., 2001; Kashiwagi et al., 2001); and galardin, a hydroxamic acid known to act as a broad spectrum inhibitor of metalloprotease activity (Grobelny et al., 1992).

At the concentrations employed in the present study (5-100 ng/ml), TIMP3 failed to elicit any marked impact on zona pellucida recognition and adhesion (Fig. 8A). The use of higher concentrations of this inhibitor was negated by the severe adverse effects it had on sperm viability. In contrast, galardin affected a dose-dependent decrease in zona binding in the absence of noticeable effects on sperm motility or viability (Fig. 8B). Similarly, substitution of the inhibitors with anti-ADAMTS10 antisera (20 µg/ml) also elicited a marked reduction in sperm adhesion to homologous zonae pellucidae (Fig. 8C). Indeed, the degree of zona interaction was decreased by approximately four fold to levels that were comparable to those seen in the non-capacitated sperm controls. Importantly, this suppression was not related to a reduction in sperm viability or motility following antibody treatment as both parameters remained at levels comparable to those seen in the untreated controls. The specificity of this inhibition was further attested to by the failure of pre-immune sera (20 µg/ml) or anti-CD59 (20 µg/ml) antibodies to elicit any impact on sperm-zona pellucida interaction. In contrast to the results secured for sperm-zona pellucida binding, anti-ADAMTS10 antibodies had no discernible effect on either sperm-oolemma binding or fusion (results not shown).
Figure 8. Effect of metalloprotease inhibitors on sperm-zona pellucida interactions. Cauda epididymal sperm were capacitated in the presence of varying doses metalloprotease inhibitors.
TIMP3 or galardin) or anti-ADAMTS10 antibodies (20 µg / ml). (A-C) They were then assessed for the ability to adhere to homologous zonae pellucidae. (A and B) Control samples contained vehicle (DMSO) at the same dilution used in the highest inhibitor concentration or (C) pre-immune sera (20 µg / ml) and antibodies against an alternative sperm surface protein (CD59; 20 µg / ml). The mean number of sperm bound per oocyte was recorded and expressed as a percentage of the capacitated control, which typically bound at a rate of approximately 30 - 40 spermatozoa / oocyte. Data represent the results of three replicates and are expressed as mean +/- standard error of the mean. * P<0.05, ** P<0.01
DISCUSSION

The focus of the studies described herein has been the characterization of the novel sperm protein ADAMTS10, a recently identified member of the metalloprotease superfamily. Since the first description of the ADAMTS family by Kuno and co-workers (Kuno et al., 1997) a total of nineteen mammalian ADAMTS proteins have now been identified (Apte, 2009) which possess unique and overlapping functional roles in diverse biological processes ranging from cell migration, connective tissue organization, regulation of angiogenesis and blood coagulation, arthritis and the regulation of fertility (Flannery et al., 1999; Georgiadis et al., 1999; Hurskainen et al., 1999; Shindo et al., 2000; Li et al., 2001b; Zheng et al., 2001; Cal et al., 2002; Russell et al., 2003; Richards, 2005; Stanton et al., 2005). At present there is very limited information regarding the expression of ADAMTS proteins in mammalian spermatozoa. Indeed, only a single study has implicated ADAMTS in sperm function with a report that ADAMTS2 knockout mice are characterized by a male infertility phenotype attributed to defects in spermatogenesis (Li et al., 2001a). In contrast, the endogenous inhibitors of this metalloprotease family, have been identified in the male reproductive tract (Kirchhoff et al., 1991; Robinson et al., 2001; Metayer et al., 2002) and on spermatozoa (Buchman-Shaked et al., 2002), thus strengthening the case for metalloprotease regulation of male reproductive function.

As with other ADAMTS family members, ADAMTS10 is synthesized as a zymogen, possessing an N-terminal signal sequence (Met₁-Ala²⁵) followed by a short prodomain that functions to preserve enzymatic latency and aids in correct protein folding and secretion (Cao, et al., 2000). Experimental evidence secured by Somerville et al., (2004) has demonstrated that furin, a proprotein convertase that is expressed in the testes and epididymidis of various mammalian species (Torii, et al., 1993; Thimon, et al., 2006), is the enzyme most likely responsible for zymogen processing. The amino terminus of the mature protease is predicted to commence at Ser²³⁴, immediately upstream of the catalytic domain. This metalloprotease domain is highly conserved, possessing a characteristic zinc binding signature commonly found in reprolysin-type metalloproteases, the disintegrin-like domain and the first of five thrombospondin type I (TSR) repeat motifs. The presence of the HEXXH consensus sequence is suggestive of catalytic activity (Tang, 2001) and indeed, the metalloprotease activity of ADAMTS10, and several other ADAMTS proteins, is now well established in vitro (Abbaszade, et al., 1999; Kuno, et al., 1999; Tortorella, et al., 1999; Somerville, et al., 2004). On the contrary, the ADAMTS disintegrin-like domains have not yet been shown to possess disintegrin activity (Apte, 2004) and the consensus X(D/E)ECD site responsible for integrin binding is not well conserved in the ADAMTS family. Importantly, the work of Somerville and colleagues (Somerville, et al., 2004) has demonstrated that ADAMTS10 is secreted to the cell surface and is a functional metalloprotease capable of cleaving α₂-macroglubulin.
One curiosity arising from our characterization of ADAMTS10 was that the molecular weight of the predominant protein (50 kDa) recognized in epididymal spermatozoa by anti-ADAMTS10 antibodies differs substantially from that predicted on the basis of the primary sequence for either the full length zymogen or that of the processed mature form of the protein (118 and 95 kDa, respectively). Although post-translational modifications, such as the documented N-glycosylation of ADAMTS10, are known to influence protein resolution in SDS-PAGE gels it is considered unlikely that they alone could account for such large discrepancies in electrophoretic mobility. While exploration of the origin of this alternative sized protein proved beyond the scope of the present work, it is considered likely that it originates from additional cleavage of the parent protein. The latter explanation is consistent with experimental evidence that proteolytic cleavage does occur in the ancillary C-terminal domains of other ADAMTS proteins (Vazquez, et al., 1999; Rodriguez-Manzaneque, et al., 2000; Cal, et al., 2001; Gao, et al., 2002; Somerville, et al., 2003). Such, cleavage events generally occur within the spacer region (Vazquez, et al., 1999; Rodriguez-Manzaneque, et al., 2000; Flannery, et al., 2002; Gao, et al., 2002; Gao, et al., 2004) and have been shown to elicit a profound impact on both substrate specificity and localization of the enzymes. In the best characterized example, ADAMTS4 has been shown to undergo autocatalytic C-terminal processing from a 75 kDa full-length active form to produce isoforms of 60 and 50 kDa (Flannery, et al., 2002; Gao, et al., 2004). Such processing results in the release of the enzyme from the ECM and dramatic alteration of its bioactivity (Gao, et al., 2002) with, for instance, the aggreganase activity profile being reduced to 1% of normal (Kashiwagi, et al., 2004). In the case of ADAMTS10, it has recently been reported that the protein is highly susceptible to C-terminal processing events, analogous to those reported for ADAMTS4, if the protein is expressed in cells cultured in the absence of serum, and hence the protective activity of the proteolytic inhibitors contained therein (Somerville, et al., 2004).

Although further studies are required to investigate the nature of ADAMTS10 processing, it was of considerable interest that post-testicular sperm maturation was identified as the likely staging site for proteolytic cleavage and activation of the ADAMTS10 zymogen. This situation is analogous with that reported for several members of the related ADAM protein family. Indeed many of the ADAM proteins that have proven to be instrumental in male reproductive function are regulated by proteolytic processing during epididymal passage (Schlondorff & Blobel, 1999). The significance of such processing is emphasized by reports that they occur in concert with the acquisition of sperm fertilizing ability in the epididymis (Aitken, et al., 2007). It was also of interest that the surface labeling of ADAMTS10 increased dramatically following sperm capacitation and that the protein co-localized with membrane raft markers in these cells. These findings share striking similarities to our previous data on HSPD1.
(Asquith et al., 2004) and that secured by independent laboratories regarding ZP3R (Kim, *et al.*, 2001; Kim & Gerton, 2003; Buffone, *et al.*, 2008) and a number of additional proteins (Tulsiani & Abou-Haila, 2004) that are progressively released to the sperm surface during capacitation. At present the mechanisms underpinning the maturation-associated changes in the localization of ADAMTS10 remain to be fully explored but it may be mediated through the formation of small fusion pores between the outer acrosomal membrane and the overlying plasma membrane (Dun, *et al.*, 2010). Irrespective of the mechanisms, the localization of ADAMTS10 in capacitated spermatozoa raises the prospect that it may participate in sperm-oocyte interactions. However, the dearth of functional data regarding ADAMTS10 prohibited the use of specific inhibitors to examine this role. Instead, two broad spectrum metalloprotease inhibitors were employed, tissue inhibitor of metalloprotease 3 (TIMP3) and galardin. TIMPs are small, disulphide-bonded proteins that are broadly effective competitive inhibitors of the matrix metalloproteases, yet display much greater selectivity towards both the ADAMs and ADAMTSs (Baker, *et al.*, 2002). TIMP3 is the only known endogenous ADAMTS inhibitor, and has been shown to potently inhibit mouse gamete fusion (Correa, *et al.*, 2000). However, the current study revealed that TIMP3 does not influence the initial binding event between capacitated spermatozoa and the zona pellucida. In contrast, treatment of spermatozoa with galardin, an alternative broad spectrum metalloprotease inhibitor (Grobelny, *et al.*, 1992), initiated a dose-dependent decrease in their affinity for the zona pellucida in the absence of an effect on sperm motility or viability. While these data do not directly implicate ADAMTS10 in the initial stages of gamete interaction, they do provide the first evidence for the involvement of metalloproteases in this process and support the contention that this enzyme activity is an important determinant of sperm-zona interaction. This claim is further substantiated by the use anti-ADAMTS10 antisera, which also proved effective in reducing sperm-ZP interaction. However, we acknowledge that caution must be exercised in interpreting experiments using antibody based inhibition of zona interaction due to the possibility of non-specific steric hindrance.

It therefore remains to be equivocally established whether ADAMTS10 directly binds ligands within the zona pellucida. An alternative tenable hypothesis that is in keeping with the localization of this protein, its putative interaction with the molecular chaperone HSPD1, and the fact that it resides in a multimeric protein complex, is that the proteolytic 'sheddase' activity of ADAMTS10 is required to facilitate the unmasking, maturation and/or activation of alternative proteins involved sperm-oocyte interactions. In this context, the identification of ADAMTS10 as a putative binding partner of a cell surface chaperone draws interesting parallels with recent reports that matrix metalloproteases, such as MMP2 and MMP9, also form dynamic associations with molecular chaperones on the surface of certain tumor cells (Eustace, *et al.*, 2004; Stellas, *et al.*, 2010). In this capacity, the molecular chaperones assist in the activation of
the matrix metalloproteinase thereby promoting increased tumor invasiveness (Eustace & Jay, 2004; Eustace, et al., 2004). Further, antibodies that disrupt these interactions are demonstrably capable of preventing metalloprotease activation and significantly inhibiting the metastatic potential of carcinoma cell lines (Stellas, et al., 2010). In the case of spermatozoa it has been shown that targeted deletion of molecular chaperones, such as calmegin and calsperin, results in a male infertility phenotype associated with impaired sperm transport in the female reproductive tract in vivo and the loss of sperm-zona binding ability (Ikawa, et al., 1997; Ikawa, et al., 2010). These defects in sperm function appear to be due to misexpression, misprocessing and/or misfolding of fertilization-dependent proteins. Indeed, these chaperones appear to be required for ADAM1A/ADAM2 dimerization and/or subsequent maturation of ADAM3 (Ikawa, et al., 1997; Ikawa, et al., 2001; Ikawa, et al., 2010; Ikawa, et al., 2010). It is therefore perhaps not surprising that these infertility phenotypes are mirrored by those reported in reciprocal knockouts of ADAM2 and ADAM3 proteins and that spermatozoa in the latter models also experience the loss of multiple gene products (Nishimura, et al., 2001; Nishimura, et al., 2004). While comparable knockout studies of ADAMTS10 are required to determine if this protein similarly affects the expression profile of other proteins and contributes to defective sperm function, these collective findings raise the possibility that extracellular interaction between chaperones and metalloproteases may be a widespread phenomenon that regulates a range of biological and pathological processes.

Overall, these studies support the notion that sperm-oocyte interactions involve considerable functional redundancy and identify ADAMTS10 as a novel candidate in the mediation of these fundamentally important events. Future investigation of ADAMTS10, focusing on the elucidation of its substrates and hence the proteolytic pathways in which it participates are required to determine its precise role in the regulation of sperm function.

ACKNOWLEDGMENTS

The authors acknowledge the expert assistance of Amanda Bielanowicz in preparation of the electron microscopy images presented herein. We also thank Prof Suneel S. Apte (Lerner Research Institute, Cleveland) for providing valuable feedback and reagents during the early phase of these studies.
Figure S1. Structure of mouse ADAMTS10. (A) Mouse ADAMTS10 is a modular protein of 1104 amino acids comprising a number of distinct functional domains (see key). (B) In order to confirm the specificity of the predominant 50 kDa sperm protein recognized by anti-ADAMTS10 antibodies, the protein was excised and sequenced by matrix-assisted laser desorption time of flight mass spectrometry (MALDI-ToF) using an Ettan MALDI-ToF Pro mass spectrometer (Amersham Biosciences). This strategy returned three high confidence peptide matches corresponding to ADAMTS10 (red, underlined text).

Figure S2. Immunogold labeling of ADAMTS10 in mouse spermatozoa. Mouse spermatozoa were fixed in 4% paraformaldehyde / 0.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.3) followed by dehydration, infiltration and embedding in LR White resin. Sections were blocked in 3 % BSA in Dulbecco’s phosphate buffered saline (DPBS) for 1 h at 37°C before being sequentially labeled with anti-ADAMTS10 (overnight at 4°C) and secondary antibody conjugated to 10 nm gold particles for 2 h at 37°C. After washing, sections were post-fixed in 2% glutaraldehyde, dried and stained with 1% uranyl acetate in 40% methanol. In a number of instances this preparation led to the loss of the majority of the sperm plasma membrane and thus the outermost membrane structure corresponded to the outer acrosomal membrane. In the case of caput sperm cells this outer acrosomal membrane was heavily labeled for ADAMTS10. In
contrast, when the plasma membrane was retained, immunogold labeling specific for ADAMTS10 was able to be detected on the outer leaflet of this structure in capacitated cauda sperm cells. The micrographs, depicting the sperm peri-acrosomal region, were taken on a JEOL-100CX transmission electron microscope (JEOL, Tokyo, Japan) operating at 80 kV. Scale bar = 500 nm, Acr = acrosome, N = nucleus, OAM = outer acrosomal membrane, PM = plasma membrane.
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The role of molecular chaperones in spermatogenesis and the post-testicular maturation of mammalian spermatozoa

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Accepted for publication by Human Reproduction Update
ABSTRACT

BACKGROUND: Spermatogenesis culminates in production of one of the most highly differentiated cells in biology, the spermatozoon. The gametes that emerge from the testes are, however, functionally immature and only acquire full functionality once they have engaged a process of post-testicular maturation in the epididymis and female reproductive tract. Remarkably, this acquisition of sperm function occurs while these cells are transcriptionally and translationally silent and is therefore highly dependent on post translational modifications to their existing protein complement. In this review we consider the emerging roles of several prominent molecular chaperone families in orchestrating both the morphological differentiation of male germ cells during spermatogenesis and their functional transformation following sperm maturation. METHODS: Journal databases were searched using key words including: chaperone, heat shock protein, testis, spermatogenesis, spermatozoa, epididymal maturation, capacitation and fertilization. RESULTS: In the past two decades, molecular chaperones have been acknowledged to play key roles in controlling both the morphological transformation of germ cells during spermatogenesis and the post-testicular maturation of these cells as they transit the male and female reproductive tracts. Furthermore, there is mounting evidence that aberrant chaperone expression may be a major contributing factor to the defective sperm function seen in many cases of male infertility. CONCLUSION: Molecular chaperones are critically involved in all phases of sperm development. Targeted disruption of these proteins has the ability to arrest spermatogenesis, compromise sperm maturation and inhibit fertilization. These proteins therefore hold considerable promise as targets for novel contraceptive strategies and as diagnostic biomarkers for male infertility.

KEY WORDS: Spermatozoa, molecular chaperone, sperm maturation, fertilization
INTRODUCTION

Spermatogenesis involves the passage of diploid germ cells through the reductive divisions of meiosis in order to generate round haploid spermatids that metamorphose into one of the most specialized cells in biology, the spermatozoon, in a process known as spermiogenesis. Remarkably, the morphological transformations associated with spermiogenesis occur in the complete absence of gene transcription, beautifully illustrating how regulated protein translation can control the phenotypic fate of cells during development. The end result of this process is a unique, highly polarized cell that is designed to deliver the paternal genome, centriole, and possibly, key mRNA species to the oocyte at the time of fertilization (Hermo et al. 2010).

Notwithstanding the morphological specialization generated during spermatogenesis, spermatozoa are released from the testes in a functionally immature state. The functional transformation of these cells occurs as they transit the epididymis and ascend the female reproductive tract, taking place in the virtual absence of gene transcription and de novo protein synthesis (Kierszenbaum and Tres 1975; Meistrich et al. 1978; Balhorn et al. 1984; Heidaran et al. 1988). Sperm maturation therefore depends upon the loss, modification, and/or remodelling of existing sperm proteins in response to cues delivered by the male and female reproductive tracts. While many aspects of spermatogenesis and post-testicular sperm maturation remain to be fully elucidated, it is becoming increasingly apparent that these elaborate processes are carefully regulated by a myriad of gene products that are expressed in a phase-specific manner (Hermo et al. 2010). Through the application of advanced biochemical, molecular and proteomic technologies, many of these products have been identified. Interestingly, among those that have proved to be play indispensable roles during all stages of sperm development are several classes of molecular chaperones.

Molecular chaperones are a large family of structurally diverse proteins that are expressed in virtually all cell types (Ellis 1987; Ellis 1996). More than 20 chaperone families, differing primarily with respect to their molecular weight and structural characteristics, have now been described. Owing to the fact that they were originally identified on the basis of their ability to confer cellular resistance to environmental stressors, the majority of these chaperone families are referred to as cell stress response- or, more commonly, heat shock- proteins (HSPs) (Ritossa 1996). In mammals, the HSPs are commonly divided into the HSP100 (HSPH), HSP90 (HSPC), HSP70 (HSPA), HSP60 (HSPD), HSP40 and HSP27 (HSPB) families (Buchner 1996; Bukau and Horwich 1998; Narberhaus 2002). The human genome contains several members from each gene family, a
redundancy that may relate to differences in intra-organelle compartmentalisation in addition to tissue or development-specific expression patterns (Vos et al. 2008). However, the overlapping expression of many different molecular chaperone families or family members indicates that their specialized functions are of critical importance (Vos et al. 2008). Indeed, there is compelling evidence that in addition to their archetypical protective roles, molecular chaperones also participate in a number of normal cellular functions, including metabolism, growth, differentiation, and apoptosis (Bukau et al. 2006) (Fig. 1). Molecular chaperones fulfil these functions by virtue of their ability to selectively recognize and interact with hydrophobic domains that are transiently exposed in their client proteins. Such interactions prevent inappropriate association or aggregation and direct the proteins into productive folding, transport or degradation pathways. Although molecular chaperones were initially thought to be restricted to intracellular organelles, a growing body of evidence suggests that they do exist in other locations, including the cell surface, where it appears likely they fulfil a number of novel secondary roles (Gupta et al. 2008; Triantafilou et al. 2008; Naaby-Hansen and Herr 2010; Sims et al. 2011).

In the current review we consider the established and rapidly emerging roles of several of the more prominent molecular chaperone families in the formation of a functional spermatozoon (Tab. S1). Specifically, we have sought to highlight their roles in promoting the morphological differentiation of the male gamete during spermatogenesis and their subsequent post-testicular functional transformation in the epididymis and female reproductive tract. We present compelling evidence that molecular chaperones are critically important in cellular remodelling events and that aberrant expression is associated with arrested spermatogenesis and pronounced defects in sperm function. Further analysis of molecular chaperones is therefore warranted as a means of providing important insights into some of the most challenging questions concerning the molecular mechanisms regulating sperm function.
Figure 1. Regulation of nascent polypeptide homeostasis by molecular chaperones. Following translational, nascent polypeptides are commonly directed to multi-protein complexes (MPC) comprising molecular chaperones and additional regulatory co-chaperones. The particular composition of such MPC’s varies in accordance with the client polypeptide and cell type. Interactions between molecular chaperones and their client proteins can lead to (A) productive folding into functional proteins, (B) assembly into multisubunit complexes, and (C) intracellular trafficking, in which proteins are translocated across membranous structures to their site of action. (D) If protein folding cannot be achieved, chaperones can target their client polypeptides for degradation through the ubiquitin-proteasome pathway and thereby prevent the accumulation of damaged proteins and/or the formation of protein aggregates.

MATERIALS AND METHODS

Journal databases including PubMed, Science Direct, Ovid, Wiley Online Library, Oxford and Google Scholar were searched using key words including: chaperone, heat shock protein, testis, spermatogenesis, spermatozoa, epididymal maturation, capacitation and fertilization. The most commonly used search included chaperone AND varying combinations of the above words. Journal articles were included based on their quality and relevance. Our journal database searches were not restricted by species, however we focused our review on the human and mouse as these species were found to represent the two most widely studied models of chaperone function.

RESULTS: Families of molecular chaperones implicated in spermatogenesis and/or the post-testicular maturation of mammalian spermatozoa

Chaperonin Containing T-complex/TCP1-Ring Complex (CCT/TRiC)

One of the most highly expressed molecular chaperone complexes in haploid germ cells is the CCT/TRiC complex (Silver 1985). CCT/TRiC is a large, double ring structure possessing a central cavity that is responsible for binding unfolded or denatured polypeptides. Each ring is composed of eight (CCT1 – CCT8) unique subunits of between 52 and 65 kDa (Lewis et al. 1992; Hartl 1996; Liou and Willison 1997; Kubota et al. 1999), that each appear to be
essential for its chaperoning activity (Silver et al. 1979; Miller et al. 2006). Substrate recognition takes place through diverse mechanisms involving hydrophobic and electrostatic interactions. Similar to other chaperone systems, CCT/TRiC also binds ATP and hydrolyses it during protein folding cycles, with flexible protrusions located in the apical domain in each CCT subunit acting as a lid that is responsible for closing the central cavity (Gutsche et al. 1999; Leroux and Hartl 2000; Meyer et al. 2003)(Fig. 2Aii).
Figure 2. Structural domains of some of the key molecular chaperone families involved in the production and functional maturation of spermatozoa. (A) CCT and HSPD1; N-terminal domain (NTD) contains the apical domain (yellow) necessary for peptide binding and recognition. The C-terminal region (CTD) contains the Equatorial domain (pink) which facilitates the binding of each subunit of the complex and contains most ATP contact sites. Linking the CTD to the NTD is the intermediate domain (blue) that regulates the conformational change of the chaperone following ATP hydrolysis. 

(i) The eight subunits of the CCT/TRiC are arranged in dual octameric ring structures. 

(ii) Individual 60 kDa subunits each possess three domains, with the apical domain comprising the hydrophobic residues necessary for substrate binding. Each selective substrate interactions is dependent on structural changes of each subunit which is induced by ATP hydrolysis. 

(B) HSP70 consists of an N-terminal nucleotide binding domain (yellow) that accounts for the ATPase activity of the chaperone. The CTD contains a lid to enclose the central cavity (pink) of the substrate binding domain (blue). This region is linked to the NTD by a highly conserved linker segment (black). 

(C) HSP90 consists of a highly conserved NTD which accounts for ATP hydrolysis (yellow). The NTD is attached to a highly charged linker hinge region (CL) (black) and two other distinct functional regions: a peptide binding domain (blue) and a carboxyl-terminal dimerization domain (EEVD motif) CTD (pink).

Despite the fact that the chaperonin activity of the CCT/TRiC complex has been primarily assessed on the basis of its involvement in the assembly of actin and tubulin filaments (Gao et al. 1992; Lewis et al. 1992; Marco et al. 1994; Siegers et al. 2003), it now appears that it may be far more promiscuous than previously appreciated. Indeed, the presence of eight different subunits enables the complex to potentially interact with a large number of structural features and motifs and thus accommodate a broad variety of substrates (Dunn et al. 2001; Gomez-Puertas et al. 2004). Consistent with this proposal, estimates now suggest that the CCT/TRiC complex may act as the folding machinery for as many as 5–10% of newly synthesized cytosolic proteins (Kubota et al. 1994; Thulasiraman et al. 1999; Yam et al. 2008). The fine-tuning of protein processing by the CCT/TRiC complex involves many co-chaperones that act upstream to facilitate substrate transfer to the central cavity of the complex and may also be responsible for the protection of the newly synthesized polypeptide chains to minimize their chances of aggregation and mis-folding. It has been shown that the activity of the complex is also regulated by the phosphorylation of certain CCT subunits (Yam et al. 2008).

The original TCP-1 polypeptide was identified as a highly expressed mouse testicular protein (Silver et al. 1979; Silver 1981) encoded by a gene located within the t-
complex on chromosome 17 (Silver et al. 1980). The t-complex is of interest as it is known to harbour genes that influence mouse development and male fertility (Silver et al. 1979). For instance, it has been variously reported that t-specified differences exist in sperm antigenic properties (Bennett et al. 1975; Silver 1985) metabolic levels (Artzt et al. 1979; Cheng and Bennett 1980) and sperm-zona pellucida (ZP) receptor activity (Ginsberg and Hillman 1975). Within the testes, this complex is believed to be essential for the cytodifferentiation of spermatids (Soues et al. 2003). In addition to its putative role in spermatogenesis, several components of the CCT/TRiC complex have also been identified as secretory products within the epididymal fluid of the bull (Frenette et al. 2006; Belleannee et al. 2011). Indeed, proteomic analysis of bovine epididymal fluid has revealed the presence of three of the 8 CCT/TRiC subunits (CCT2, CCT7 and CCT8) which, together with a subset of additional chaperones, account for as much as 6% of the total epididymal protein content (Belleannee et al. 2011). At present it remains to be determined whether the additional CCT subunits are also present in epididymal fluid, and therefore whether they exist in a functional complex within this extracellular environment. Nevertheless, the extracellular location of these chaperonin subunits is very intriguing, particularly as some have been shown to reside within prostasome-like (Ronquist and Brody 1985; Fornes et al. 1995) structures known as epididymosomes (Yanagimachi et al. 1985; Fornes et al. 1991; Frenette and Sullivan 2001). These membranous vesicles have been shown to interact with the sperm plasma membrane in vivo (Yanagimachi et al., 1985) and promote the transfer of cargo proteins, thereby contributing to the functional maturation of these cells (Legare et al. 1999; Saez et al. 2003). On the basis of these collective findings, it is conceivable that the molecular chaperones contained within epididymosomes could prevent the mis-folding and aggregation of their cargo proteins in this environment of intense macromolecular crowding (Minton 2005), and/or that they act in concert with lipid transport proteins to facilitate the transfer of these proteins to the sperm surface. A caveat to this model is that CCTs did not feature among the proteins identified in human epididymosomes (Thimon et al. 2008), thus raising the possibility of species specific differences in the function of this chaperone complex.

Our own work has demonstrated that mature human and mouse spermatozoa harbour an intact CCT/TRiC complex that is predominantly expressed within the peri-acrosomal region of the sperm head (Dun et al. 2011; Redgrove et al. 2011). In the mouse this complex is present in testicular sperm but undergoes marked changes in subcellular localization during capacitation, including a striking increase in the surface expression of at least two CCT subunits (Dun et al. 2011). Furthermore, in the spermatozoa of both humans and mice it has been demonstrated that CCT/TRiC participates indirectly in sperm-ZP adhesion. This
interaction appears to be attributed to the ability of CCT/TRiC to form a stable complex with ZP receptors, including ZPBP2 (zona pellucida binding protein 2).

At present the mechanisms underpinning the capacitation-associated translocation of the CCT subunits to the sperm surface remains to be fully explored. Similarly, it is not certain how they interact with the putative ZP adhesion molecules. Nevertheless, it is of considerable interest that the activity of CCT/TRiC is able to be positively modulated by interactions between the complex and caveolin-1, a constituent of specialized membrane microdomains known as membrane rafts (Doucey et al. 2006). This is in keeping with the demonstration that membrane rafts commonly regulate the functions of resident chaperones through the spatial constraint of their substrates (Elhyany et al. 2004). Additionally, it has been demonstrated that chaperones play important roles in maintaining the stability of membrane raft-associated protein complexes (Chen et al. 2005). Such findings take on added significance in light of the key role that membrane rafts play in the capacitation-associated remodelling of the sperm surface and in ZP adhesion (Nixon et al. 2009; Asano et al. 2010).

**Heat Shock Protein 60**

HSPD1 (formerly HSP60) is normally found in the mitochondria where it is responsible for the transportation and refolding of proteins (Cheng et al. 1989; Reading et al. 1989). The protein typically oligomerises into a complex structure arranged as two stacked heptameric rings (Cheng et al. 1990) with a large central cavity in which unfolded client proteins bind via hydrophobic interactions (Fenton et al. 1994) (Fig. 2Ai). HSPD1 has three domains: an apical domain, an equatorial domain, and an intermediate domain (Ranford et al. 2000). The equatorial domain accommodates both the ATPase activity and the binding site for the additional ring (Fig. 2A). The intermediate domain undergoes a conformational change when ATP is bound that allows interactions to occur with client proteins (Ranford et al. 2000). It is well documented that HSPD1 works in conjunction with chaperonin 10 (HSPE1, formerly HSP10), a co-chaperone that causes the central cavity to enlarge and enhances its ability to accommodate client proteins (Ranford et al. 2000).

HSPD1 is expressed during the early stages of human spermatogenesis where it is restricted to the spermatogonial cells that line the basal epithelium (Lachance et al. 2010). Although HSPD1 has been identified in mature human spermatozoa ((Mitchell et al. 2007; Naaby-Hansen and Herr 2010); see below), our knowledge of its function in spermatozoa is largely attributed to work conducted in the mouse. In this species, HSPD1 is expressed in spermatogonia and spermatocytes, but is then apparently lost during the latter stages of spermatogenesis only to be reacquired by spermatozoa as they pass through the caput
epididymidis (Asquith et al. 2005). This differential pattern of HSPD1 expression bears a striking resemblance to that documented for other chaperones, including clusterin, and suggests that these proteins fulfil independent roles during different phases of sperm maturation. In the case of HSPD1, it has been shown that the epididymal secretory form of the protein resides in electron dense, amorphous structures termed dense bodies (Asquith et al. 2005) (Fig. 3). Although they differ substantially from epididymosomes, the appearance of dense bodies within the precise region of the epididymis where spermatozoa acquire the capacity to recognise and bind to the ZP, implicates these structures in the functional remodelling of the sperm surface during epididymal maturation. Indeed, it has been suggested they may mediate the bulk transfer of proteins to the sperm surface during this process (Asquith et al. 2005; Dun et al. 2010). To date only limited proteomic analysis of these structures has been achieved, however, in addition to HSPD1 our data strongly suggests that they also contain HSPE1 and HSP90B1. Evidence from independent groups have also implicated these structures in the transfer of carbohydrate-rich aggregates that promote the formation of sperm rosettes (Monclus et al. 2007) and bactericidal permeability-increasing protein to the acrosomal membrane (Yano et al. 2010).

HSPD1 also appears to fulfil an important function during capacitation. This was first recognised when HSPD1 was identified as a major target for capacitation-associated tyrosine phosphorylation, an event that is critical for acquisition of the spermatozoa’s ability to interact with the oocyte (Ecroyd et al. 2003; Asquith et al. 2004). Interestingly, this modification appears to promote the expression of HSPD1 on the outer leaflet of the plasma membrane of approximately 25% of live capacitated spermatozoa (Asquith et al. 2004). This sub-population of spermatozoa are believed to represent those that are capable of binding to the ZP. This is corroborated by the fact that this expression pattern is found in virtually all spermatozoa bound to the ZP but is lost once the cells have undergone acrosomal exocytosis, as would be expected of cell surface molecules involved in primary ZP interactions (Asquith et al. 2005). Nevertheless, antibodies directed against HSPD1 failed to inhibit zona binding, suggesting that the protein plays an indirect role in this process. Such findings prompted the formulation of a novel hypothesis whereby chaperone activity was implicated in the remodelling of the sperm surface architecture by promoting the assembly and/or external presentation of functional ZP-receptor complexes. Subsequent work to identify the HSPD1 client proteins in capacitated mouse spermatozoa has to date revealed that is interacts with HSPE1, CCT/TRiC, and ADAMTS10 (Walsh et al. 2008; Dun et al. 2011; Dun et al. 2012).
Figure 3. Structures implicated in the transfer of molecular chaperones to spermatozoa during epididymal maturation. Non-membranous structures referred to as dense bodies and membrane bound epididymosomes are among the various mechanisms implicated in the transfer of chaperones to maturing epididymal spermatozoa. (A) Immunofluorescence image of a mouse epididymal section labelled with anti-HSPD1 and a FITC conjugated secondary antibody. Arrowheads indicate the presence of dense bodies that were positively labelled for HSPD1. Scale bar = 25 µm. (B) Electron micrograph of a mouse epididymal section labelled with anti-HSPD1 and a secondary antibody conjugated to 10 nm gold particles. Heavily labelled ‘dense bodies’ (db) are evident throughout the epididymal lumen. Scale bar = 500 nm. (C) Electron micrograph of epididymosomes (es) isolated from mouse epididymal fluid. Scale bar = 500 nm.

Studies on human spermatozoa have also identified HSPD1 on the cell surface (Naaby-Hansen and Herr 2010), although its interaction with alternative proteins and their role in mediation of sperm-oocyte interactions remains to be fully elucidated in our species. Nevertheless, this chaperone is among several that have been identified in the fluids secreted from the oviduct epithelium and shown to interact with the human spermatozoa (Boilard et al. 2004; Lachance et al. 2007). Although a number of different hypotheses for the role of these oviductal secretions exist, one proposal is that they are essential in facilitating acquisition of the spermatozoa’s ability to interact with and fertilize the oocyte (Yang and Yanagimachi 1989; Hunter 1991; Kim et al. 1996; Wang et al. 1998; Slavik and Fulka 1999; Buhi 2002). Conversely, it has been shown that incubation of human spermatozoa with recombinant HSPD1 significantly inhibits the tyrosine phosphorylation of p81 (the major phosphotyrosine-containing protein of human spermatozoa) (Galantino-Homer et al. 1997). It has therefore been suggested that exogenous HSPD1 may counteract the signalling pathway that underpins capacitation-associated tyrosine phosphorylation (Lachance et al. 2007). If similar results were achieved in vivo it would suggest that that protein may act in a manner analogous to that of a decapacitation factor (Nixon et al. 2006). HSPD1 could therefore be one of the oviductal factors that render spermatozoa quiescent while they are stored in the Fallopian tube (Suarez and Pacey 2006).

Heat Shock Protein 70 Family

The 70kDa HSPs (HSP70: HSPA1A, HSPA2/HSP70-2, HSPA4, HSPA5/BiP/GRP78, HSPA6/HSP70B’, HSPA7/HSP70B, HSPA8, HSPA9, HSPA12A, HSPA12B, HSPA13, HSPA14, HSPA1B, HSPA1L, HSPA4L) are a ubiquitously expressed family of folding
catalysts with a modular architecture comprising three major functional domains: a conserved N-terminal ATPase domain, a substrate binding domain, and a C-terminal domain that acts as a lid for the substrate binding domain (Tavaria et al. 1996; Mayer and Bukau 2005) (Fig. 2B). In the ATP bound state, the lid is held in an open position that permits the substrate binding domain to interact with hydrophobic segments within substrate proteins. These transient interactions stimulate ATPase activity and promote the closure of the binding pocket (Mayer and Bukau 2005). This substrate binding and release cycle is augmented by co-chaperones from the family of J-domain proteins (primarily HSP40 in eukaryotes) that target HSP70s to their substrates, and is further fine-tuned by nucleotide exchange factors, which influence the longevity of the HSP70-substrate complex (Youker and Brodsky 2007). HSP70 function is also coupled to the action of other chaperones, such as those of the HSP90, HSP-organizing protein (HOP), and HSP100 (Glover and Lindquist 1998) families. The primary function of HSP70 rests with its ability to transiently bind to partially-synthesized or denatured peptide sequences, thereby preventing their aggregation and allowing them to (re)fold into a functional state. However, by virtue of its ability to stabilize client proteins in a partially-folded state, HSP70 also aids in the transmembrane transport of proteins, and in their assembly into functional complexes (Mayer and Bukau 2005).

**HSPA2**

Eukaryotic organisms express several different HSP70 proteins, all of which share a common domain structure, but possess unique patterns of expression and/or subcellular localization. A novel, testis-specific member of the HSP70 family, known as HSPA2, has been identified in several species (Allen et al. 1988; Maekawa et al. 1989). Targeted mutation of the Hspa2 gene has revealed the chaperone has an essential role in the transition of spermatogenic cells through the late meiotic stages of spermatogenesis (Mori et al. 1997). Specifically, it was shown that homozygous mutant Hspa2 -/- mice support normal spermatogenesis until postnatal day 15 when increasing numbers of pachytene spermatocytes become apoptotic and differentiation beyond this stage begins to falter (Allen et al. 1996; Mori et al. 1997). Spermatogenic cell development appears to arrest in prophase of meiosis I resulting in an absence of spermatids. Detailed characterisation of HSPA2 in these cells revealed two primary roles. Firstly, HSPA2 supports the formation of a heterodimeric complex between CDC2 and cyclin B1 during the transition between G1 to S-phase and then from G2 to M-phases of meiosis (Zhu et al. 1997). Secondly, HSPA2 appears to act as a component of the synaptonemal complexes (Mori et al. 1997) and thereby assists with chromosome cross-over during meiosis. More recent analyses have revealed that, after the
completion of meiosis, HSPA2 acquires a new function as a chaperone of spermatid-specific DNA packaging transition proteins (Govin et al. 2006). These proteins serve as an intermediary, replacing histones before themselves being replaced by protamines during spermiogenesis (Lewis et al. 2003; Govin et al. 2004; Caron et al. 2005; Kimmins and Sassone-Corsi 2005). Owing to its ability to escort transition proteins and mediate their assembly into DNA-packaging structures, HSPA2 acts as a major regulator of genome reorganization in differentiating spermatids.

Interestingly, orthologues of HSPA2 are present in the testes of many animals, suggesting that this chaperone may play a conserved role across phyla (Eddy 1999). Hspa2 mRNA is abundant in human testis but, unlike the mouse, it is not restricted to this tissue with additional transcripts detected in skeletal muscle, the ovary, intestine and brain. Early reports that a HSP70 protein was expressed on the surface of human spermatozoa (Miller et al. 1992) using a monoclonal antibody that recognizes multiple HSP70 family proteins have recently been substantiated by the work of Herr and colleagues (Naaby-Hansen et al. 2010) who demonstrated that HSPA2, in addition to HSPA5 (see below), are among the repertoire of calcium-regulated proteins expressed on the human sperm plasma membrane.

The significance of HSPA2 in mature human spermatozoa has been eluded to by the studies of Huszar et al. who have proposed that the relative levels of HSPA2 expression may be used as an objective biochemical marker of sperm maturity and hence, fertilizing potential (Huszar et al. 2000). Indeed, HSPA2 expression is significantly reduced in infertile patients whose spermatozoa possess increased cytoplasmic retention (Huszar and Vigue 1993) resulting from a developmental defect in the last phase of spermiogenesis (Huszar and Vigue 1990).

In studies designed to examine the stage of the fertilization process compromised by low HSPA2 expression, a substantial defect in ZP adhesion was revealed (Huszar et al. 1994; Huszar et al. 2007). On the basis of such findings it has been proposed that human HSPA2 may play a secondary role in the remodelling the sperm plasma membrane during spermatogenesis (Huszar et al. 2000) to facilitate the formation of the ZP binding sites. Such a notion is in keeping with the biphasic expression of HSPA2, first in spermatocytes related to meiosis, and then at the time of terminal spermiogenesis in elongated spermatids (Govin et al. 2006; Wu et al. 2011). This proposal takes on added significance in light of our own recent work that has identified HSPA2 as a major component of several human sperm protein complexes, a subset of which are expressed on the surface of the cell and harbour known zona adhesion receptors (unpublished observations). Taken together these findings raise the intriguing prospect that, in addition to its well characterised role in
spermatogenesis, HSPA2 may also participate in the assembly of functional ZP receptor complex(es).

**HSPA5**

Another member of the HSP70 family that has been identified in mammalian spermatozoa is that of heat shock 70kDa protein 5 (HSPA5, formerly GRP78/BiP). HSPA5 is commonly localized in the lumen of the endoplasmic reticulum (ER) where it plays a pivotal role in protein transport, folding and assembly (Lee 2005). HSPA5 interacts with several stress sensors (Bertolotti et al. 2000) but disassociates from these sensors as unfolded proteins accumulate in the ER lumen. This dissociation enables HSPA5 to aid in protein folding while simultaneously promoting the activation of the released sensors and the initiation of ER-stress signalling pathways (Lee 2005). It is therefore perhaps not surprising that Hspa5 mRNA expression is rapidly elevated in Sertoli cells following exposure to insults such as nonylphenol (Gong et al. 2009), a widely distributed environment contaminant linked to disruption of testicular development and decrease male fertility. However, in addition to this protective role recent evidence suggests HSPA5 may also play important role(s) in the function of mature human and mouse spermatozoa.

As previously mentioned (see HSPA2 above) early studies reported the presence of multiple forms of HSP70 on the surface of human spermatozoa (Miller et al. 1992). Such results have been substantiated by Naaby-Hansen and Herr (2000) who were able to demonstrate that HSPA5 was among a cohort of proteins that were accessible for surface labelling with both biotin and radiiodine (Naaby-Hansen and Herr 2010). Indeed, this study identified seven members from four different HSP families as putative residents of the human sperm surface. In addition to HSPA5, these included: HYOU1 (hypoxia up-regulated 1), HSP90AA1 (formerly, HSP86/HSPC1), HSPD1, and several isoforms of HSPA2 and HSPA1L (Naaby-Hansen and Herr 2010). Although the HSPA2 and HSPA1L proteins are most likely to be incorporated into the plasma membrane during spermatogenesis, the origin of surface expressed HSPA5, and that of the other chaperones identified in this study, remains to be elucidated. It has been reported that strong HSPA5 expression is present in the cytoplasm of human spermatoocytes and round spermatids (Lachance et al. 2010). However, HSPA5 is also known to be abundantly expressed in the epididymal epithelium, epididymosomes (Lachance et al. 2010), seminal plasma (Pilch and Mann 2006), and oviductal epithelium (Boilard et al. 2004; Lachance et al. 2007; Marin-Briggiler et al. 2010) raising the possibility that additional protein is transferred onto the sperm surface during post-testicular maturation. Such interaction(s) may be mediated by the
chaperone’s ATPase domain, a region that has been shown to possess affinity for sulfogalactosylglycerolipid (Mamelak and Lingwood 1997, 2001; Mamelak et al. 2001), the major glycolipid component of the mammalian sperm plasma membrane.

While the role of human sperm surface HSPA5 remains to be determined, it is noteworthy that it featured among a group of calcium-binding proteins identified in an elegant $^{45}$Ca-overlay assay (Naaby-Hansen et al. 2010). It is therefore possible that it acts in concert with additional Ca$^{2+}$ sensing machinery, such as calreticulin (see below), to modulate intracellular calcium concentrations ([Ca$^{2+}$]). In this context, it is noteworthy that recombinant HSPA5 is able to bind to the sperm acrosomal cap (Marin-Briggiler et al. 2010), and upon doing so is able to augment the cells response to progesterone-stimulus and significantly increase the [Ca$^{2+}$], (Lachance et al. 2010). In an alternative study, exogenous HSPA5 was also found to influence the ability of sperm to engage in oocyte interactions in a calcium-dependent manner (Marin-Briggiler et al. 2010). Indeed, the binding of recombinant HSPA5 initially had a negative impact on ZP interaction. However, this effect was abrogated, and HSPA5 was actually able to enhance sperm-ZP interaction, if the incubation media was modified through the replacement of calcium with strontium. Taken together, these findings lend support to the notion that surface expressed HSPA5 influences [Ca$^{2+}$] and therefore the capacitation and fertilizing competence of human spermatozoa. It is possible however, that the protein may fulfil a different role in the spermatozoa of other species. In the mouse for instance, HSPA5 has also been identified on the extracellular surface of spermatozoa. Yet, in this species the chaperone appears to associate with a number of additional client proteins to form a supramolecular complex (Han et al. 2011). One such protein is ADAM7 (a disintegrin and metalloprotease 7), a protease that is transferred to the sperm surface via epididymosomes as the cells transit through the epididymis (Oh et al. 2009). It has been shown that the incorporation of ADAM7 is indirectly linked to presence of two additional ADAM proteins (ADAM2 and ADAM3) (Kim et al. 2006) that are known to be important for sperm migration in the female reproductive tract and adhesion to the ZP (Muro and Okabe 2011). Two particularly interesting findings to arise from this work are that members of the HSPA5 complex, including ADAM7, reside within detergent-resistant membranes (membrane rafts) and that the assembly of this complex is promoted during sperm capacitation (Han et al. 2011). Such findings share analogy with other putative chaperone complexes (Dun et al. 2011; Dun et al. 2012) and lend support to the concept that the raft environment may stabilize and/or promote the formation of functional chaperone complexes during sperm activation (Dun et al. 2010).
**HSPA8**

Heat shock 70kDa protein 8 (HSPA8, previously known as HSPA10 /HSC70) is a member of the heat-shock cognate (HSC) subgroup of chaperones that, unlike canonical HSPs, is constitutively expressed and performs functions related to normal cellular processes. In addition to traditional roles of protein folding, HPSA8 has been implicated in the catalysis of ATP-dependent uncoating of clathrin-coated vesicles during transport of membrane components through the cell (Gething and Sambrook 1992), promoting lysosomal degradation of intracellular proteins (Chiang et al. 1989; Gething and Sambrook 1992) and may actually have antagonistic effects to HSP70 in terms of the intracellular trafficking of newly synthesized proteins (Goldfarb et al. 2006). The participation of HSPA8 in the dynamics of clathrin-coated vesicles may be important in terms of acrosome biogenesis since this event requires the combined processes of clathrin-coated vesicle trafficking and confluence. In support of this model, HSPA8, and its putative co-chaperone rDJL, have been shown to form a stable complex with clathrin (Yang et al. 2005) and to co-localize within the developing acrosome of differentiating rat germ cells. The importance of HSPA8 in spermatogenesis is also emphasised by its association with the testis-specific serine kinase 6 (TSSK6) (Spiridonov et al. 2005). TSSK6 belongs to a group of proteins that are expressed during the late stages of spermiogenesis and is responsible for the phosphorylation of a myriad of substrates including the histones H1, H2A, H2AX, and H3 (Spiridonov et al. 2005). These phosphorylation events may be important for chromatin compaction and DNA packaging since targeted disruption of the Tssk6 gene produces a sterility phenotype in male mice accompanied by reduced sperm numbers, impaired DNA condensation, and abnormal sperm morphology and motility (Spiridonov et al. 2005). Importantly, HSPA8 does not serve as a substrate for TSSK6 but rather appears to maintain TSSK6 structure and may play a critical role in targeting the kinase to specific subcellular sites.

At present there is limited information regarding the presence or function of HSPA8 in mature human or mouse spermatozoa. However, HSPA8 is present on the surface of the oviductal epithelium and has been implicated in the binding of spermatozoa to these cells (Elliott et al. 2009). Such dynamic interactions are of significant interest as they serve to enhance sperm survival in the female reproductive tract prior to fertilization (Pollard et al. 1991; Suarez et al. 1991; Dobrinski et al. 1997; Fazeli et al. 1999; Fazeli et al. 2003; Brewis et al. 2005; Suarez and Pacey 2006; Holt and Fazeli 2010). In boar spermatozoa this pro-survival property seems to be critically dependent upon HSPA8 since pre-treatment of oviductal epithelium preparations with anti-HSPA8 antibody significantly negated their ability to maintain sperm viability (Elliott et al. 2009). In contrast, the enhancement of sperm survival was able to
be rescued by substituting the oviductal epithelium fraction with exogenous recombinant HSPA8 (Elliott et al. 2009). This pro-survival activity of HSPA8 is conserved across the spermatozoa of several ungulate species (bovine, porcine and ovine) (Elliott et al. 2009; Lloyd et al. 2009). However, the mechanism by which HSPA8 is able to induce such responses has yet to be investigated.

**Calmegin/Calsperin**

Originally identified as calcium-binding, ER-resident chaperones, calmegin (CLGN) and calsperin (CALR3) are the testis-specific homologs of the ubiquitously-expressed lectin chaperones, calnexin (CANX) and calreticulin (CALR), respectively. Despite extensive sequence similarities, the recruitment of unique testicular variants of CANX and CALR may be a reflection of the fact that this organ presents a special case for the control of ER protein folding because of the unique environment in which it functions. Not only do the testes experience temperatures that are typically several degrees below that of the rest of the body, but the developing germ cells also require the assembly of a novel proteome and extensive remodelling of cellular organelles to produce such specialized structures as the acrosomal vesicle and the sperm flagellum (van Lith et al. 2007).

CLGN is expressed exclusively from mid pachytene spermatocytes through to the formation of spermatids (Watanabe et al. 1992). During this key phase of the spermatogenic process, the chaperone is believed to transiently interact with the nascent glycoproteins synthesised within the ER (Ikawa et al. 1997) and destined for the acrosomal matrix and the plasma membrane of mature spermatozoa. Interestingly, coinciding with the loss of the ER during spermatogenesis, Clgn expression is arrested and the protein is not able to be detected in mature spermatozoa (Watanabe et al. 1992; Yoshinaga et al. 1999). In contrast to the situation for HSPD1 described earlier, CLGN is not reacquired during epididymal transit. Nevertheless, despite its absence in mature spermatozoa the chaperone has been identified as being critical for the development of the cells' ability to engage with the oocyte during fertilization. This is evidenced by targeted disruption of the Clgn gene, which leads to the generation of male mice that are virtually sterile due to an inability to navigate beyond the uterotubal junction of the female reproductive tract in vivo and to bind to the ZP in vitro. Interestingly, a similar infertility phenotype has also been documented for Calr3 knockout mice in addition to that of mice lacking ADAM1A (Nishimura et al. 2004), ADAM2 (Cho et al. 1998), ADAM3 (Yamaguchi et al. 2006), and angiotensin-converting enzyme (Hagaman et al. 1998).
Collectively, these findings have led to the suggestion that a number of the proteins required to facilitate zona adhesion must be synthesized within the ER of developing germ cells before being folded and/or assembled through the cooperative action of CLGN and CALR3 (Ikawa et al. 1997; Muro and Okabe 2011). Specifically, calmegin appears to be required for the assembly of the sperm surface s-fertilin complex (heterodimer of ADAM1B and ADAM2) in addition to that of the testis specific t-fertilin complex (heterodimer of ADAM1A and ADAM2). The latter of these complexes appears necessary for the presentation of ADAM3 on the sperm surface. In contrast, CALR3 directly associates with ADAM3 and controls its maturation (Ikawa et al. 2011), suggesting that CLGN and CALR3 have distinct and specific roles in the maturation of ADAM proteins. The fact that ADAM3 is the only protein commonly disrupted or displaced in Clgn and Calr3 knockout sperm (Yamaguchi et al. 2006; Ikawa et al. 2011; Muro and Okabe 2011) suggests that it plays a central role in sperm migration into the oviduct in addition to sperm-ZP adhesion.

Interestingly, the interaction of sperm chaperones and ADAM proteins is not restricted to those mentioned above, nor do they occur exclusively in testicular sperm cells. For instance, it has recently been shown that ADAM7 forms stable complexes with the molecular chaperones, CANX and HSPA5, in sperm membranes (Han et al. 2011). Recent work from our own laboratory has also identified a related member of the ADAM superfamily, ADAMTS10 (ADAM with thrombospondin type 1 motifs number 10), as a putative client protein of HSPD1 (Dun et al. 2012). As indicated above, this molecular chaperone has also been implicated in the capacitation-dependent assembly and/or presentation of multimeric sperm receptor complexes. Taken together, these findings raise the intriguing possibility that interaction between chaperones and ADAM proteins may be a widespread phenomenon that regulates a range of developmental processes associated with sperm maturation.

Calreticulin

Calreticulin (CALR) is a calcium-binding, lectin-like, chaperone that is ubiquitously expressed within the ER of virtually all cells in higher organisms. The protein is highly conserved (Michalak et al. 1992) and divided into three domains: a globular N-terminal domain, a central proline-rich P-domain, and a C-domain that binds Ca\(^{2+}\) with a relatively high capacity but low affinity (Nash et al. 1994; Krause and Michalak 1997). Despite the presence of an N-terminal signal sequence and a C-terminal KDEL ER retention signal, a subpopulation of CALR has been localized to several different intracellular compartments (e.g. nucleus and cytoplasm) in addition to the plasma membrane of many cell types (Krause and Michalak 1997). While it
remains unclear how the protein is differentially localized to these regions it is apparent that it has a myriad of different functions outside the ER environment. For instance, CALR has been implicated in the regulation of intracellular Ca\(^{2+}\) homeostasis (Fliegel et al. 1989; Michalak et al. 1992; Krause and Michalak 1997), modulation of steroid sensitive gene transcription (Burns et al. 1994; Dedhar et al. 1994) and the mediation of integrin-mediated calcium signaling and cell adhesion (Coppolino et al. 1997; Kwon et al. 2000).

In terms of spermatozoa, CALR was first purified as a calcium-binding protein from rat spermatogenic cells (Nakamura et al. 1991) before being localized to the developing acrosomes of these cells during spermiogenesis (Nakamura et al. 1992; Nakamura et al. 1993). Immunohistochemical and ultrastructural studies revealed that CALR was most abundantly expressed in the acrosome of both round spermatids and mature rat spermatozoa, with weaker labelling of other subcellular structures observed in spermatocytes, spermatids, and Sertoli cells. Subsequent studies on mouse (Nakamura et al. 1992), bull (Ho and Suarez 2003) and human spermatozoa (Naaby-Hansen et al. 2010) have revealed that CALR is also expressed in a similar acrosomal location in these cells. However, additional labelling was revealed within the cytoplasmic droplet and midpiece region of human spermatozoa (Naaby-Hansen et al. 2010) and in the principal piece of the bull sperm flagellum (Ho and Suarez 2003).

Although Calr-null mice have been generated by homologous recombination, the embryonic lethality of this mutation (Mesaeli et al. 1999) has prevented a definitive assessment of the role of CALR in sperm function. Nevertheless, it has been demonstrated that mouse spermatozoa are rapidly immobilized, in a dose-dependent manner, when exposed to CALR antisera (Nakamura et al. 1992). Furthermore, CALR has been shown to strongly co-localize with the inositol 1,4,5-trisphosphate receptor (IP\(_3\)R) in the putative Ca\(^{2+}\) storage sites of the acrosome and neck of human and bull spermatozoa (Ho and Suarez 2003; Naaby-Hansen et al. 2010). These latter findings are of significance as IP\(_3\)R has been implicated in the regulation of [Ca\(^{2+}\)], in several model systems (Camacho and Lechleiter 1995; Mohri et al. 1995) and the fact that calcium modulates, directly or via intermediary compounds (Roldan and Fragio 1993; Roldan and Murase 1994; O'Toole et al. 1996), hyperactivated motility and the induction of acrosomal exocytosis (Yanagimachi 1994). Taken together these findings identify CALR as a prime candidate in the regulation of the calcium oscillation patterns observed during hyperactivation and the acrosome reaction.
**Protein Disulphide Isomerases**

The family of protein disulphide isomerases (PDIs: PDIA3, PDIA4, PDIA6, PDILT, PDIR, PDIP, ERP44, ERP29) catalyse the formation, isomerisation and reduction of disulphide bonds. Their localization has mainly been reported in the ER lumen, but increasingly reports are suggesting that some members of the family reside in alternative subcellular locations and possess activities that differ from those displayed by their ER counterparts (Turano et al. 2002; Ellerman et al. 2006; van Lith et al. 2007). The disulphide isomerase activity is achieved by virtue of the fact that PDIs contain four thioredoxin domains (CXXC) (Chivers et al. 1997); two that are redox active and an additional two that are required for either structural or substrate recognition and binding activity (Klappa et al. 1998). A testis specific homologue of PDI (PDILT), is expressed in the seminiferous tubules and the interstitium (van Lith et al. 2007). PDILT has also been localized to round spermatids in mice and is believed to form a multiprotein complex with CLGN in a manner analogous to that of PDI and CANX in somatic cells (van Lith et al. 2007). Functional cooperation between CLGN and PDILT enables recognition of the N-terminal region of nascent polypeptides and simultaneous trapping of free cysteines to be used as substrates to fold protein backbones into their native conformation. It is thought that PDILT may also be important for remodelling events in the ER during meiosis and that it has the potential to assist in the expulsion of excess cytoplasm during spermiogenesis (van Lith et al. 2007).

A number of PDIs have been identified in epididymal fluid and implicated in the folding of epididymal secretory glycoproteins. For instance, a substantial reduction in the abundance of PDIA6, a PDI known to bind in a redox dependent manner to HSPA5 (Mezghrani et al. 2001; Jessop et al. 2009), occurs in human spermatozoa as they transit from the corpus to the cauda epididymidis (Akama et al. 2010). The apparent loss of PDIA6 might reflect the fact that this protein is required during the early phases of epididymal maturation but subsequently shed once the cells have acquired functional competence. Conversely, the expression of PDIA3 remains unchanged in epididymal sperm (Akama et al. 2010), suggesting that its role in disulphide bond formation may be essential for the membrane remodelling events that characterise epididymal maturation and capacitation (Akama et al. 2010).

The events that underpin sperm binding and fusion with the oolemma are among the other well documented functional roles of the PDIs (Ellerman et al. 2006). Typically these events are reliant on extensive conformational changes in the participating fusion proteins. Such changes are, in turn, often mediated by thiol-disulfide exchange(s) that are catalysed by proteins with disulphide isomerase activity (Sanders 2000; Hogg 2002; Matthias et al. 2002).
To date, at least four of the 17 members of the mammalian PDI family (Ellgaard and Ruddock 2005) have been identified in spermatozoa (including: PDI, PDIA3, PDIA4, and PDIA6 (Stein et al. 2006)). Interestingly, each of these PDI proteins have been localized to the equatorial segment of the mature sperm cell (Ellerman et al. 2006), a region that is critical in mediation of sperm-egg fusion (Yanagimachi 1994). Significantly, it has been shown that preincubation of capacitated sperm with anti-PDIA3 antibodies significantly inhibits the formation of the multimolecular complexes that facilitate fusion between the oolemma and the sperm surface (Ellerman et al. 2006).

**Heat shock protein 90**

Unlike most of the other HSPs whose abundance increases upon heat stress, the HSP90 family (HSP90: HSP90AA1/HSP90α, HSP90AB1/HSP90β, HSP90B1/endoplasmic/GP96/GRP94) are already extremely abundant accounting for between 1-2% of the total soluble proteins in non-stressed cells (Lai et al. 1984). HSP90AA1 and HSP90AB1 display modest differences in the C-terminal dimerization domains but both generally form homodimers. Although HSP90 is largely hydrophobic it contains two highly charged domains: one is the hinge-domain located between the N- and C-terminus and the other lies in the C-terminal domain (Fig. 2C). These domains, in concert with exposed hydrophobic surfaces, are involved in defining HSP90 substrate binding characteristics (Binart et al. 1989; Csermely et al. 1998). HSP90 normally associates with co-chaperones such as HSP70 and promotes folding and prevention of protein aggregation (Picard 2002). Like most chaperones, HSP90 utilizes the binding and hydrolysis of ATP via its ATPase domain to drive the opening and closing of its molecular clamp.

Owing to its ubiquitous expression it has proven difficult to definitively assess the function of HSP90 in male gametes using traditional knockout strategies. Indeed, mouse embryos lacking the Hsp90ab1 gene die at implantation (Voss et al. 2000). Nevertheless, the use of gene trap insertions has enabled investigation of the role of Hsp90aa1 in gamete formation (Grad et al. 2010) and revealed that Hsp90aa1 deficient mice are completely sterile. This defect is not attributed to a corresponding loss of Hsp90ab1, the levels of which remain unchanged in Hsp90aa1-/- mice. Similarly, the development of the reproductive system appears to be normal, but spermatogenesis is arrested at the pachytene stage of meiosis 1, a phenotype that resembles that reported for Hspa2 deficient mice. This defect appears to be attributed, at least in part, to the association between HSP90AA1 and CDC2 (Grad et al. 2010). The chaperone also forms important interactions with nuclear
autoantigenic sperm protein, a protein that is essential for binding and transporting the testis-specific linker histone H1t, from the cytoplasm of primary spermatocytes to the nucleus, implicating it as a functional regulator of meiosis 1 (O’Rand et al. 1992). In mature mouse spermatozoa, it has been shown that HSP90AA1 and HSP90B1 are both targets for capacitation-dependent tyrosine phosphorylation (Ecroyd et al. 2003; Asquith et al. 2004; Baker et al. 2006). At present, the significance of the tyrosine phosphorylation of HSP90 family chaperones during capacitation remains to be established. It is possible that it may result in conformational modifications and concomitant changes in substrate specificity, which then facilitates downstream events associated with the fertilization process. In keeping with this notion, HSP90B1, like that of HSPD1, becomes expressed on the surface of approximately 25% of the live capacitated sperm population (Asquith et al. 2004). As mentioned previously, this population are thought to represent the cohort of cells that are capable of interacting with the ZP. This is again corroborated by the fact that HSP90B1 is lost from spermatozoa once they have completed their acrosome reaction (Asquith et al. 2004).
CONCLUSIONS

The preceding data emphasise the notion that a suite of molecular chaperones are intimately involved in modulating the production and functional activity of mammalian spermatozoa. While the pleiotropic function of molecular chaperones in the cellular networks that characterise sperm development remains to be fully elucidated it is apparent that their highly coordinated protein machinery fulfils a range of diverse roles (Fig. 4; Tab. S1). In early germ cells these roles extend to controlling numerous important signalling pathways involved in cell-cycle progression, telomere maintenance, apoptosis, mitotic signal transduction, vesicle-mediated transport and targeted protein degradation (Hartl et al. 2011). Disruption of normal germ cell development can be achieved by the deactivation of a number of chaperones, resulting in azoospermia possibly because of a failure to disassemble the synaptonemal complex that holds homologous chromosomes together during key stages of meiosis. Such targeted gene deletions result in a complete loss of diplotene spermatocytes, stressing their importance in cellular development (Grad et al. 2010). Furthermore, the activation of gene silenced germ cells during the later stages of spermatogenesis adds additional importance to chaperones in the biochemical/physical modifications that occur post meiosis to render the immature cells functionally competent. This is no more evident than when observing the Clgn and Calr3 gene knockout studies performed by Okabe’s group. Although these tests and ER specific chaperones are not involved in cytoskeletal protein assembly, their elimination leads to what appear morphologically functional cells that are nevertheless incapable of passing through the various regions of the female reproductive tract and interacting with the ovulated oocyte. These gene manipulation strategies provide some of the most convincing evidence that chaperones are essential in the activation of morphologically mature sperm, but important questions still remain in respect to their downstream molecular targets.

Interestingly a number of observations of the testis have been made which show that chaperones residing in early germ cells disappear from the germ line only to reappear in the segment of the epididymis where sperm gain the ability to swim and to recognise the ZP (Fig. 4; Tab. S1). In fact the fluids of the epididymis provide an environment in which the functions of molecular chaperones are paramount. The residency of chaperones in epididymosomes and dense bodies are suggestive of their critical load, passing essential cargo from the surrounding support cells of the epididymis and facilitating their transfer to the maturing cell.
Figure 4. Chaperone expression during spermatogenesis and post-testicular sperm maturation. A suite of molecular chaperones have been implicated in the successive maturational phases that culminate in the production of functionally competent mammalian spermatozoa. Many of these molecular chaperones have essential pleiotropic roles in the differentiation and functional maturation of the male gamete, extending from the control of signalling pathways involved in cell-cycle progression and synaptonemal complex assembly (spermatogenesis), to the complex biochemical and biophysical modifications that occur
during post-testicular sperm maturation in the male (epididymal maturation) and female (capacitation) reproductive tracts. Interestingly, a number of the chaperones that reside in early germ cells disappear only to reappear in spermatozoa following their passage through discrete regions of the epididymis. This transfer of chaperones, and their putative client proteins, to the maturing cell may be mediated by epididymosomes and/or dense bodies. Additional chaperones are also secreted by the oviductal epithelium and appear to modulate capacitation and/or elicit a pro-survival effect. Abbreviations: m = mouse, h = human, r = rat, b = bovine, p = porcine, o = ovine, e = equine.

Illuminating data has further revealed the necessary functional alterations that are made to the plasma membrane of spermatozoa in response to the physiological environment of the female reproductive fluids. Not only are a number of key post translational modifications made to chaperones but their surface exposure commensurate with the presentation of a number of potential ZP ligands have enhanced our functional understanding of capacitation. Overlapping expression of many of the key chaperone classes suggests that the functional cooperation of these chaperones leads to the formation of a ‘super-chaperone complex’ on the surface of capacitated mammalian spermatozoa. Whether the assembly of such multimeric zona recognition complexes represents the sole mechanism for sperm–zona recognition or whether there is biological redundancy in this system involving the expression of alternative zona-recognition molecules is still an open question. On the one hand, redundancy might be expected in such an important biological process, on the other, targeted disruption of a single chaperone gene, calmegin, can induce sterility in mice via mechanisms that appear to involve the inhibition of sperm–zona recognition (Yamagata et al. 2002). Further analysis of molecular chaperones is therefore warranted as a means of providing novel targets for contraceptive intervention and as diagnostic biomarkers for male infertility.
### SUPPLEMENTAL DATA

**Supplementary Table 1.** Molecular chaperones implicated in the formation and functional maturation of mammalian spermatozoa.

1Chaperones implicated in each developmental phase are listed and ranked according to the number of primary research articles describing their specific chaperoning activities/functions. The chaperones listed are restricted to those that have been described in relation to human and mouse spermatozoa.

<table>
<thead>
<tr>
<th>Developmental phase</th>
<th>Chaperone</th>
<th>Number of publications</th>
<th>Knockout phenotype or significant role</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Spermatogenesis</td>
<td>HSPA2 (HSP70-2)</td>
<td>14</td>
<td>Mouse: A testis specific chaperone whose deletion leads to infertile phenotype due to arrest of spermatogenesis. Mouse/human: HSPA2 is synthesized during the meiotic phase of spermatogenesis and is abundant in pachytene spermatocytes. <strong>Human</strong>: Appears to be essential for membrane remodelling and therefore a biochemical marker of human sperm function and male fertility.</td>
<td>(Dix et al. 1996; Dix et al. 1997; Mori et al. 1997; Zhu et al. 1997; Eddy 1998, 1999; Mori et al. 1999; Son et al. 1999; Huszar et al. 2000; Feng et al. 2001; Cayli et al. 2003; Govin et al. 2006; Widlak 2006)</td>
</tr>
<tr>
<td></td>
<td>Clusterin (CLU, APOJ, SGP2, SP-40, TRPM2)</td>
<td>13</td>
<td><strong>Mouse &amp; Human</strong>: Secreted by the Sertoli cells and thought to play an important role in the uptake of hydrophobic peptides during spermatogenesis. The Clu gene contains a heat shock element and therefore concentration of CLU increases in the testis on exposure to elevated temperatures. CLU seems to play a role in initially protecting spermatogenic cells from heat damage and then later clearing damaged cells from the tubules.</td>
<td>(Onoda and Djakiew 1990; Morales and Griswold 1991; Roberts et al. 1991; O'Bryan et al. 1994; Wong et al. 1994; Ahuja et al. 1996; Clark et al. 1997; McKinnell and Sharpe 1997; Bailey et al. 2002; Andersen et al. 2003; Plotton et al. 2005; Plotton et al. 2006; Yon et al. 2007)</td>
</tr>
<tr>
<td></td>
<td>HSPD1 (HSP60)</td>
<td>8</td>
<td><strong>Mouse</strong>: Localized to the spermatogonia, primary spermatocytes and Sertoli cells and then lost from more mature spermatogenic cells. <strong>Human</strong>: Localized to spermatogonia, primary spermatocytes and Sertoli cells. A significant reduction of HSPD1 has been recorded in testicular biopsies obtained from adult men with disturbed fertility.</td>
<td>(Meinhardt et al. 1995; Paranko et al. 1996; Werner et al. 1996; Werner et al. 1997; Meinhardt et al. 1998; Asquith et al. 2005; Zhang et al. 2005; Lachance et al. 2010)</td>
</tr>
<tr>
<td></td>
<td>DNAJB3</td>
<td>7</td>
<td><strong>Mouse</strong>: Weakly detected in early</td>
<td>(Berruti et al. 1998;</td>
</tr>
</tbody>
</table>
round spermatids but increases in cytodifferentiating spermatids where it colocalizes with developing acrosome and postnuclear region. Has been implicated in acrosomogenesis and forms stable interactions with both HSPA2 and the deubiquitinating enzyme, USP8, during spermatogenesis. The Dnajb3 transcript and protein are significantly underexpressed in the testis of wobbler mice that possess a male infertility phenotype due to production of abnormal spermatozoa lacking an acrosome and with reduced motility.

Human: A putative Dnajb3 orthologue has been amplified RT-PCR from human sperm cells and anti-DNAJB3 antibodies cross react with a protein in human sperm lysates. The protein may therefore have conserved functions in the human.

HSP90AA1 (HSP86, HSP90, HSP90α) 7 Mouse & Human: Expressed in spermatogonia, spermatocytes, Sertoli cells, Leydig cells and myoid cells in normal testicular tissues, but highly expressed in testes with spermatogenic arrest. Mouse: Gene trap insertions of Hsp90AA1 revealed that, Hsp90AA1 /- mice are completely sterile. This defect is not attributed to a corresponding loss of Hsp90AB1, which remains unchanged. Development of reproductive system appears to be normal, but spermatogenesis is arrested at the pachytene stage of meiosis 1.

HSPA4L (APG1, OSP94) 5 Mouse: Highly expressed in spermatogenic cells from late pachytene spermatocytes. 42% of Hspa4l /- male mice suffer fertility defects associated with reduced sperm number (due to apoptosis of developing germ cells) and motility.

Human: Protein detected in spermatocytes, spermatids and spermatozoa from normal individuals but absent in the testis of individuals without germ cells (Sertoli-cell-only syndrome) or arrested at spermatogonia.

Berruti and Martegani 2001, 2002; Meccariello et al. 2002; Berruti and Aivatiadou 2006; Doiguchi et al. 2007; Kaushal and Bansal 2009)

(Gruppi et al. 1991; Gruppi and Wolgemuth 1993; Biggiogera et al. 1996; Liu et al. 2004; Alekseev et al. 2005; Ferlin et al. 2010; Grad et al. 2010)

(Kaneko et al. 1997; Kaneko et al. 1997; Nonoguchi et al. 2001; Held et al. 2006; Held et al. 2011)
<table>
<thead>
<tr>
<th>Protein</th>
<th>Species</th>
<th>Description</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCT/TRiC</td>
<td>Mouse</td>
<td>Microtubule nucleation during spermatogenesis and an important role in the primary degeneration of spermatogenic epithelium following heat shock.</td>
<td>(Kubota et al. 1994; Hynes et al. 1995; Soues et al. 2003; Zha et al. 2006)</td>
</tr>
<tr>
<td>DNAJB13</td>
<td>Mouse</td>
<td>Highly expressed in the testes where it localizes to annulus during sperm flagellum development and interacts with SEPT4 (an annulus constituent) suggesting that it may be involved in assembling the annulus structure. Ultrasstructural analysis of mature spermatozoa revealed that it is in fact a radial spoke protein of the ‘9+2’ axoneme.</td>
<td>(Liu et al. 2004; Guan and Yuan 2008; Guan et al. 2009; Guan et al. 2010)</td>
</tr>
<tr>
<td>Calmegin</td>
<td>Mouse</td>
<td>Expressed from mid pachytene spermatocytes through to the formation of spermatids. CLGN is believed to transiently interact with nascent glycoproteins synthesised within the ER and destined for the acrosomal matrix and the plasma membrane of mature spermatozoa.</td>
<td>(Watanabe et al. 1994; Ikawa et al. 1997; Yoshinaga et al. 1999)</td>
</tr>
<tr>
<td>Calreticulin</td>
<td>Mouse</td>
<td>Lectin-like chaperone implicated in the regulation of spermatogenesis. CALR is strongly localized to the acrosomal matrix of developing mouse and rat germ cells.</td>
<td>(Nakamura et al. 1992; Nakamura et al. 1993; Watanabe et al. 1994)</td>
</tr>
<tr>
<td>HSPA8</td>
<td>Mouse</td>
<td>Expressed following meiosis in spermatogenesis and therefore a spermatid specific chaperone. Expression is lost in spermatids at step 15. HSPA8 associates with the testis-specific serine kinase 6 (TSSK6) during spermatogenesis.</td>
<td>(Matsumoto et al. 1993; Tsunekawa et al. 1999; Spiridonov et al. 2005)</td>
</tr>
<tr>
<td>HSPB10</td>
<td>Mouse</td>
<td>Major protein of the mammalian sperm tail outer dense fibres. HSPB10 is essential for rigid junction of sperm head and tail with Hspb10 +/- male mice displaying infertility due to detachment of the sperm head.</td>
<td>(Fontaine et al. 2003; Vos et al. 2008; Yang et al. 2012)</td>
</tr>
<tr>
<td>DNAJA1</td>
<td>Mouse</td>
<td>Dnaja1 +/- mice suffer from severe defects in spermatogenesis associated with aberrant androgen signaling. Primary defect in Sertoli cell maintenance of spermiogenesis at steps 8 and 9. These results indicate that DNAJA1 and</td>
<td>(Hu et al. 2004; Terada et al. 2005)</td>
</tr>
<tr>
<td>Protein</td>
<td>Species</td>
<td>Expression</td>
<td>Function</td>
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</tr>
<tr>
<td>DNAJA2</td>
<td>Human</td>
<td>Highly expressed in human testes and spermatozoa where it is proposed to work as a co-chaperone of HSP70s in protein folding and mitochondrial protein import.</td>
<td></td>
</tr>
<tr>
<td>HSPA5 (GRP78, BiP)</td>
<td>Mouse &amp; Human</td>
<td>HSPA5 is developmentally regulated and strongly expressed in spermatocytes and round spermatids in the adult testes.</td>
<td>(Huo et al. 2004; Lachance et al. 2010)</td>
</tr>
<tr>
<td>PDILT (PDIA7)</td>
<td>Mouse &amp; Human</td>
<td>PDILT is a divergent testis-specific PDI with a non-classical SXXC motif that engages in disulfide-dependent glycoprotein folding in the ER of spermatogenic cells. Implicated in acrosome formation and expulsion of the cytoplasmic body during spermiogenesis. Expressed in round spermatids and the interstitium of the testis in rodents and primates where it forms a complex with CLNG.</td>
<td>(van Lith et al. 2005; van Lith et al. 2007)</td>
</tr>
<tr>
<td>HSP90B1 (GP96)</td>
<td>Mouse</td>
<td>Required for normal spermatogenesis. Conditional Hsp90b1 knockout targeted to male germline results in infertility due to production of spermatozoa with large globular heads and abnormal intermediate pieces (phenotype similar to human globozoospermia).</td>
<td>(Asquith et al. 2005; Audouard and Christians 2011)</td>
</tr>
<tr>
<td>HSPA4 (APG2)</td>
<td>Mouse</td>
<td>Required for the progression of spermatogenesis. Hspa4 -/- males display impaired fertility with reduced sperm number and motility. The majority of pachytene spermatocytes in the juvenile Hspa4 -/- mice fail to complete the first meiotic prophase and become apoptotic. Human: Increased HSPA4 expression has been reported in the spermatozoa of men with varicocele and in those with oligozoospermia.</td>
<td>(Ferlin et al. 2010; Held et al. 2011)</td>
</tr>
<tr>
<td>Calsperin (CALR3)</td>
<td>Mouse</td>
<td>Related to CALR but lacks broad lectin activity and does not function as a general chaperone for nascent N-glycoproteins. Instead CALR3 directly associates with ADAM3 and controls its maturation during spermatogenesis.</td>
<td>(Ikawa et al. 2011)</td>
</tr>
<tr>
<td>DNAJB1 (HSP40,</td>
<td>Mouse</td>
<td>Constitutively expressed in mouse testes within</td>
<td>Doiguchi et al. 2007</td>
</tr>
</tbody>
</table>
DNAJ1, HDJ1, HSPF1) spermatocytes and round spermatids. Localizes to middle and the end pieces of flagella as well as in acrosomal region of the head of mature spermatozoa.

PDIA3 (ERP57) 1 Human: Localized in spermatogenic cell cytoplasm from spermatocytes to the spermatozoa phases and present at low levels in Sertoli cells. Primarily detected in sperm acrosome and tail. Expression levels are dramatically decreased in IVF patients with low fertilization rates. (Zhang et al. 2007)

HSPE1 (HSP10) 1 Mouse: Co-chaperone for HSPD1. Detected in spermatogenic cells from spermatocytes to elongating spermatid phase and localized to developing acrosomes and flagellum. (Walsh et al. 2008)

2. Epididymal maturation

Clusterin 23 Mouse & human: Most highly expressed in the caput region of the epididymis correlating with cellular remodelling or differentiation that occurs during these periods of development. The epididymal isoform of CLU has a scaffold domain (five disulfide bonds) and four amphipathic helices. It is thought that CLU acts like a detergent, and binds to hydrophobic complexes and denatured proteins, aiding in their uptake or clearance. CLU is proposed to facilitate a lipid exchange for the delivery of GPI-linked proteins to sperm membranes critical for successful fertilization in humans. (Hermo et al. 1991; Mattmuller and Hinton 1991; Sylvester et al. 1991; Cyr and Robaire 1992; Mattmuller and Hinton 1992; Sensibar et al. 1993; Hermo et al. 1994; Law and Griswold 1994; O'Bryan et al. 1994; O'Bryan et al. 1994; Turner et al. 1994; Hermo 1995; Robaire and Viger 1995; Runic et al. 1995; Ahuja et al. 1996; Morales et al. 1996; Hermo et al. 2000; Turner et al. 2000; Andersen et al. 2003; Ecroyd et al. 2005; Dacheux et al. 2006; Griffiths et al. 2009; Belleannene et al. 2011)

HSPA5 3 Mouse: Forms complexes with ADAM7 (a member of the ADAM family that is associated with epididymosomes and integrated into sperm plasma membrane during epididymal maturation) and CANX in mouse spermatozoa. Human: Shown to reside on the surface of human sperm. Present in the neck region of sperm and possibly involved in intracellular (Naaby-Hansen et al. 2010; Naaby-Hansen and Herr 2010; Han et al. 2011)
<table>
<thead>
<tr>
<th>Protein</th>
<th>Gene Symbol</th>
<th>Species</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSPD1</td>
<td>HSPD1</td>
<td>Mouse</td>
<td>After being lost in late stages of spermatogenesis, HSPD1 is apparently reacquired in the proximal corpus epididymis. The transfer of the protein to epididymal spermatozoa may be mediated by dense bodies in the epididymal lumen. &lt;br&gt;<strong>Human:</strong> HSPD1 is implicated in the functional remodelling of the sperm surface during epididymal maturation. (Asquith <em>et al.</em> 2005; Walsh <em>et al.</em> 2008; Lachance <em>et al.</em> 2010)</td>
</tr>
<tr>
<td>HSPE10</td>
<td>HSPE10</td>
<td>Mouse</td>
<td>Expressed in the epididymal epithelium and co-localizes with HSPD1 in dense bodies in the epididymal lumen. (Walsh <em>et al.</em> 2008)</td>
</tr>
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### 3. Capacitation

<table>
<thead>
<tr>
<th>Protein</th>
<th>Gene Symbol</th>
<th>Species</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSPD1</td>
<td>HSPD1</td>
<td>Mouse</td>
<td>Becomes localized to the plasma membrane overlying the acrosome on the sperm head and is a target for capacitation-associated tyrosine phosphorylation during. Forms multiprotein complexes with ZP receptor proteins, CCT/TRiC and HSPE1. &lt;br&gt;<strong>Human:</strong> Retained in ejaculated spermatozoa and variously reported to localize to the mid-piece and the cell surface. An additional population of HSPD1 is secreted by the uterine and oviductal epithelium and binds to spermatozoa. The ability of exogenous HSPD1 to suppress tyrosine phosphorylation of p81 suggests that it may act as a decapacitation factor. (Asquith <em>et al.</em> 2004; Lachance <em>et al.</em> 2007; Mitchell <em>et al.</em> 2007; Walsh <em>et al.</em> 2008; Naaby-Hansen and Herr 2010; Dun <em>et al.</em> 2012)</td>
</tr>
<tr>
<td>HSP90B1</td>
<td>HSP90B1</td>
<td>Mouse</td>
<td>As with HSPD1, HSP90B1 undergoes tyrosine phosphorylation during capacitation and becomes localized to the plasma membrane overlying the acrosome. (Abbas-Terki <em>et al.</em> 2002; Asquith <em>et al.</em> 2004; Asquith <em>et al.</em> 2005; Walsh <em>et al.</em> 2008)</td>
</tr>
<tr>
<td>HSPA5</td>
<td>HSPA5</td>
<td>Mouse</td>
<td>Forms a stable complex with ADAM7 and CANX in mouse spermatozoa. &lt;br&gt;<strong>Human:</strong> Shown to reside on the surface of human sperm and may be involved in the regulation of intracellular Ca\textsuperscript{2+}. HSPA5 is also secreted by the human and bovine oviductal epithelial cells and has been shown to modulate (Naaby-Hansen <em>et al.</em> 2010; Naaby-Hansen and Herr 2010; Han <em>et al.</em> 2011)</td>
</tr>
</tbody>
</table>
### Ca\(^{2+}\) levels in these spermatozoa.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Species</th>
<th>Description</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clusterin (CLU)</td>
<td>Human</td>
<td>Morphologically abnormal human sperm have an extensive surface coating of native CLU that is apparently absent on normal spermatozoa. The latter cells do however, possess a different CLU isoform within the acrosomal cap that is lost following acrosomal exocytosis.</td>
<td>(O’Bryan et al. 1990; O’Bryan et al. 1994; O’Bryan et al. 1994)</td>
</tr>
<tr>
<td>Calreticulin (CALR)</td>
<td>Mouse &amp; human</td>
<td>CALR is strongly localized to the acrosomal matrix of mature spermatozoa and implicated in the regulation of intracellular calcium concentrations.</td>
<td>(Nakamura et al. 1992; Naaby-Hansen et al. 2010)</td>
</tr>
<tr>
<td>CCT/TRiC</td>
<td>Mouse</td>
<td>Capacitation promotes an increase in surface expression of a number of chaperonin subunits in the anterior head overlying the acrosome.</td>
<td>(Dun et al. 2011; Redgrove et al. 2011)</td>
</tr>
<tr>
<td>HSP90AA1</td>
<td>Mouse &amp; human</td>
<td>Target for capacitation-associated tyrosine phosphorylation in human, rat and mouse spermatozoa. Implicated in acquisition of the ability to fertilize an oocyte.</td>
<td>(Ecroyd et al. 2003; Baker et al. 2006)</td>
</tr>
<tr>
<td>HSPA2</td>
<td>Human</td>
<td>HSPA2 is expressed on the surface of ejaculated human spermatozoa.</td>
<td>(Naaby-Hansen et al. 2010; Naaby-Hansen and Herr 2010)</td>
</tr>
<tr>
<td>PDIA3</td>
<td>Human</td>
<td>Undergoes capacitation-associated post-translational modifications. May be required for membrane protein remodelling during capacitation.</td>
<td>(Zhang et al. 2007; Wiwanitkit 2010)</td>
</tr>
<tr>
<td>HSPE1</td>
<td>Mouse</td>
<td>Able to be co-immunoprecipitated with HSPD1 from capacitated mouse spermatozoa and undergoes a similar relocalization to the sperm surface during this maturational phase.</td>
<td>(Walsh et al. 2008)</td>
</tr>
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</table>

### 4. Fertilization

<table>
<thead>
<tr>
<th>Protein</th>
<th>Species</th>
<th>Description</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSPA2</td>
<td>Human</td>
<td>HSPA2 has been identified as a dominant target antigen for human anti-sperm antibodies that effectively block IVF. Levels of human HSPA2 expression have been correlated with sperm maturity and male fertility.</td>
<td>(Mamelak and Lingwood 1997; Bohring et al. 2001; Ergur et al. 2002; Bohring and Krause 2003; Cayli et al. 2003; Huszar et al. 2006; Naaby-Hansen et al. 2010)</td>
</tr>
<tr>
<td>Calmegin (CLGN)</td>
<td>Mouse</td>
<td>CLGN -/- mice are infertile due to failure of sperm to pass uterotubal junction and interact</td>
<td>(Ikawa et al. 1997; Yamaguchi et al. 2006; Yamaguchi et al. 2007)</td>
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</table>
with ZP. Knockout also leads to disappearance of ADAM3 and an absence of ADAM1A/ADAM2 dimerization.  

<table>
<thead>
<tr>
<th>Protein</th>
<th>Species</th>
<th>Mouse: HSPD1</th>
<th>Mouse &amp; human: CCT/TRiC</th>
<th>Mouse: PDIA3 (ERP57)</th>
<th>Human: HSPA4L (APG1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse: HSPD1</td>
<td>4</td>
<td>Indirect role in ZP interaction suggested on the basis of its interaction with multimeric complex(es) comprising ZP receptors and the fact that it is lost from the sperm surface following induction of acrosomal exocytosis.</td>
<td>May participate indirectly in primary sperm-ZP interactions by virtue of its ability to form multiprotein complexes with ZP receptor proteins. These complexes have been shown to bind to homologous ZP in vitro.</td>
<td>Inhibitors of protein disulfideisomerase inhibit sperm-egg fusion in vitro. Antibodies specific to PDIA3 inhibit gamete fusion raising the possibility that thiol-disulfide exchange is required to produce conformational changes in fusion-active proteins.</td>
<td>Detected in human spermatozoa from normal donors but absent in azoospermic individuals.</td>
</tr>
<tr>
<td>Mouse &amp; human: CCT/TRiC</td>
<td>2</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Mouse: PDIA3 (ERP57)</td>
<td>2</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Human: HSPA4L (APG1)</td>
<td>1</td>
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</table>

(Asquith et al. 2004; Asquith et al. 2005; Dun et al. 2011; Dun et al. 2012)  

(Dun et al. 2011; Redgrove et al. 2011)  

(Ellerman et al. 2006; Zhang et al. 2007)  

(Ikawa et al. 2011)  

(Nonoguchi et al. 2001)
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and contacts between germ cells and Sertoli cells and their regulatory interactions,
testicular cholesterol, and genes/proteins associated with more than one germ cell
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acid binding ability of human sperm reflects cellular maturity and fertilizing potential:
selection of sperm for intracytoplasmic sperm injection. Curr Opin Obstet Gynecol; 18
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ventral prostate, seminal vesicle, testis, and epididymis of rats. Biol Reprod; 49 233-42.


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Silver LM. (1981) A structural gene (Tcp-1) within the mouse t complex is separable from effects on tail length and lethality but may be associated with effects on spermatogenesis. Genet Res; 38 115-23.


The Molecular Basis of Sperm-Oocyte Interaction Final Discussion

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FINAL DISCUSSION

The critical interactions that occur between the gametes during mammalian fertilisation represent some of the most important and complex cellular communication events in all of biology. These interactions are responsible for the propagation of each species, and it is therefore remarkable that their mechanisms remain so poorly defined. While each of the sequential interactions that culminate in fertilisation are fundamentally important, the studies described in this thesis have focused on understanding the molecular basis of sperm-zona pellucida (ZP) recognition. The rationale for this approach is based on the fact that this unique, species-specific, process ranks among the most common causes of human male infertility (Liu and Baker, 2000; Liu and Baker, 2003) and also represents an ideal and, as yet uncharacterised, target for fertility regulation (Dun et al., 2010). The study of these interactions therefore promises to inform the development of novel male contraceptive therapies, progress the diagnosis and treatment of human male factor infertility, aid in the conservation of endangered species and enhance the production of agriculturally significant animals. In our studies, we have specifically sought to delineate the mechanisms that underpin the spermatozoa’s ability to engage in ZP adhesion during capacitation, the final phase of their complex maturation. The significant biochemical insights gained from these studies are described in Chapters 3 and 4.

The purpose of the following discussion is to integrate these findings and to demonstrate how this body of work has advanced our understanding of this fascinating process.

Novel Hypothesis to Describe Sperm-Zona Pellucida Interactions

Until recently, studies into sperm-zona pellucida adhesion have promoted a widely accepted paradigm that this key recognition event is mediated by a single sperm receptor that engages with a complementary ligand within the ZP matrix. While such a model holds obvious appeal, it fails to account for the fact that targeting of individual sperm proteins through selective inhibition strategies (e.g. competitive substrates and mono-specific antibodies) and / or genetic deletion has failed to elicit the anticipated block to sperm-ZP interaction (Dun et al., 2010). Similarly, this simple model is not consistent with the demonstration that sperm-ZP interactions are able to be resolved into multiple adhesion events of both low and high affinity. Indeed, biochemical and biophysical studies indicate that multiple sperm receptors are required to achieve high affinity binding to the complex multivalent glycan ligands present within the ZP (Thaler and Cardullo, 1996b; Thaler and Cardullo, 2002b). These collective data support an alternative novel hypothesis that sperm-ZP interactions require the concerted action of several sperm proteins. However, they also raise the inevitable question as to how the action of these putative sperm receptors is coordinated to optimize the chance of fertilisation. One possibility is that the various ZP receptors are assembled into functional multimeric complex(es). Not only
could such complexes facilitate sequential or hierarchical interactions with ZP ligands but they could also link these events to the activation of downstream signalling cascades that promote the lysis of the acrosomal vesicle and sperm penetration of the zona matrix. However, a limitation of this model is that, prior to the studies described in this thesis, the identification of multimeric complexes on the sperm surface had not been achieved. Through the application of novel technologies I have not only been able advance the notion that discrete zona recognition proteins are assembled into functional, multimeric receptor complex(es) but also begun the analysis into the mechanisms by which such complex assembly is achieved. As indicated below, these studies have served to highlight the fundamental role of molecular chaperones in promoting complex assembly and/or presentation during sperm capacitation (Ecroyd et al., 2003b; Asquith et al., 2004a; Nixon et al., 2005c; Dun et al., 2010; Dun et al., 2011; Redgrove et al., 2011; Dun et al., 2012). The following section summaries the major findings of these studies and highlights a number of the pertinent questions that remain in our current understanding of the mammalian fertilisation process.

**Multimeric Zona Receptor Complexes are Expressed on the Surface of Capacitated Mouse and Human Spermatozoa**

The bio-physical/chemical alterations that ensue following sperm ejaculation into the female reproductive tract were first identified simultaneously by Austin and Chang (Austin, 1951b; Chang, 1951b) more than 60 years ago. Notwithstanding significant recent advances, our understanding of this capacitation process remains incomplete. It is however well known that sperm activation is a direct response to the specific physiological environment encountered within the female reproductive tract and that this event is essential for the acquisition of the ability to recognise and bind to the ZP. Since the balance of evidence indicates that sperm are both transcriptionally and translationally quiescent cells, these changes in sperm function must involve critical modifications in the lipid and protein architecture of the cell. The studies presented in Chapter 3 describe the application of blue native polyacrylamide gel electrophoresis (BN-PAGE) as a novel means of providing direct evidence for the presence and functional significance of multimeric sperm surface complexes. As previously indicated, this methodology was originally developed for the analysis of the large multienzyme complexes comprising the mitochondrial electron transport chain (Schagger and von Jagow, 1991; Schagger et al., 1994), and provides a platform for the electrophoretic resolution and separation of biologically active, native protein complexes. The adaptation of this technology for use in spermatozoa proved to be invaluable for the substantiation of membrane associated protein complexes (Dun et al., 2011; Redgrove et al., 2011). In addition, by coupling the technique with Far-Western blotting, we were able to explore the affinity of these complexes for homologous
zonae pellucidae. In the mouse, this approach led to the identification of two principle complexes of interest (Dun et al., 2011; Dun et al., 2012), the characterisation of which is described in Chapters 5 and 6. Remarkably, in addition to putative zona receptors, both of these complexes were found to contain proteins belonging to the molecular chaperone family. As described in Chapter 7, these findings add to a growing body of literature that suggests chaperones fulfil essential, albeit indirect, roles in the mediation of sperm-oocyte interactions.

In an attempt to explore the relevance of these mechanisms of sperm-oocyte interaction across species, I have also participated in translational studies using human spermatozoa as a model (Redgrove et al., 2011). Remarkably, these studies revealed the conservation of multimeric zona pellucida receptor complexes between human and mouse spermatozoa. Indeed, the successful adaption of BN-PAGE for the analysis of human spermatozoa demonstrated that a number of high molecular weight protein complexes also reside on their surface (Redgrove et al., 2011). Such a finding was not unexpected, given that the recent advances in functional proteomics have provided compelling evidence that a majority of proteins fulfil their functional role in multi-protein complexes (MPCs) (Sali et al., 2003). The use of Far-Western blotting in human spermatozoa revealed at least five predominant protein complexes that possessed affinity for the homologous human zona pellucida proteins. This contrasts the situation in mouse spermatozoa, which only appear to contain 2 predominant zona binding complexes. While the reason for this level of heterogeneity remains to be fully examined it may reflect the fact that, in addition to ZP3, human ZP comprises an extra protein, ZP4, that also participates in primary sperm adhesion (Gupta et al., 2007b; Chakravarty et al., 2008; Chiu et al., 2008; Gupta et al., 2009). It is therefore possible that a subset of these complexes possess affinity for human ZP3 while the remainder mediate adhesion to ZP4. Although the validity of this model is currently being investigated through the use of Far-Western blotting using recombinant human ZP3 and ZP4, such differences nevertheless serve to highlight the anticipated species specificity of gamete interactions.

At the time of thesis preparation two of the five human sperm complexes that show affinity for homologous human ZP proteins had been subjected to comprehensive proteomic analysis. Remarkably this strategy led to the identification of all members of the multimeric zona receptor complex CCT/TRiC, along with a client protein, zona pellucida binding protein 2 (ZPBP2) which is believed to account, in part, for the zona affinity of this complex. The unexpected conservation of this complex in both mouse and human spermatozoa implies that it may be involved in mediation of non-species specific initial interactions between the sperm and ZP. These interactions, which are relatively weak and forgiving of species barriers, precede the high affinity species-specific interactions that are of primary interest for our work. If this is true,
it may account for why mouse sperm can bind to human ZP (Bedford, 1977), but it fails to account for the fact that the reciprocal interaction does not readily occur. The zona pellucida of both species is known to be abundant in carbohydrates with recent reports from Pang et al. (2011) suggesting that binding of sperm to the ZP is attributable to an abundance of a sequence of sugar molecules called sialyl-Lewis$^\text{X}$ (SLeX) at the ends of oligosaccharides that furnish ZP glycoproteins (Pang et al., 2011). These terminal sequences are known to function in the adhesion of other cells, including blood and tumour cells (Feizi, 1985) and present as both N- and O-linked oligosaccharides (Wassarman, 2011). It is well characterised that molecular chaperone complexes can bind to glycoproteins exclusively through a lectin site specific for monoglucosylated oligosaccharides (Saito et al., 1999), which interestingly are moieties that all N-linked oligosaccharides share (Dell and Morris, 2001). The observed transient affinity of mouse sperm with the zona pellucida of the human oocyte might simply be a chaperone-carbohydrate recognition event as describe above however no literature is available to suggest that chaperone recognise SLeX moieties. It should be noted however that the inhibition of homologous sperm-ZP interactions was not successful when utilizing antibodies for the CCT/TRiC complex (Dun et al., 2011; Redgrove et al., 2011), suggesting its indirect role in zona adhesion, however studies to inhibit mouse sperm from binding to human ZP inhibiting using antibodies for the CCT/TRiC complex has not yet been performed. These experiments may provide insight into whether this complex is responsible for the cross species binding of mouse sperm to human oocytes.

In contrast to our findings in mouse spermatozoa (Dun et al., 2011), we currently have no biochemical evidence to suggest that the surface expression of ZPBP2 is altered during the capacitation of human spermatozoa (Redgrove et al., 2011). It is therefore possible that the mechanisms underpinning the assembly, surface presentation and / or functional activation of this particular receptor may differ between the two species. Consistent with this notion, several studies in mouse spermatozoa have shown that these cells are subject to considerable remodelling of the apical plasma membrane (APM) during the latter stages of capacitation. Our own work has shown that this promotes the novel surface exposure of a relatively large cohort of proteins including molecular chaperones and their putative client proteins (Asquith et al., 2004a). By comparison, similar studies of human sperm capacitation have failed to identify these pronounced modifications (Mitchell et al., 2007b). Collectively, these data suggest that unique or alternative proteins / complexes are important for the various cellular interactions that are observed between gametes, but also serve to highlight the need for further research into the molecular composition of these complexes and their varying affinities for the protective matrix that comprise the mammalian zona pellucida.
The second of the human sperm protein complexes that was identified in our study was shown to comprise the various components of the 20S proteasome (Redgrove et al., 2011). While, this multi-subunit protease is traditionally associated with targeted protein degradation, a number of studies have provided evidence for the involvement of the sperm-borne proteasome complex in multiple steps of animal and human fertilization (Zimmerman and Sutovsky, 2009). Among the major findings from these previous studies are that proteasomes are expressed in the mammalian sperm acrosome and on the acrosomal surface and are capable of binding to ubiquitinated proteins that are present on the mammalian ZP. Furthermore, inhibition of ubiquitination and proteasomal proteolysis is capable of blocking fertilisation by preventing sperm-ZP penetration. Our own studies demonstrated that pre-incubation of human spermatozoa with antibodies against 20S proteasome subunits (anti-α3 and anti-β1) were able to significantly inhibit their binding to the ZP of intact human oocytes. It was also shown that strong labelling of the proteasome complex could be detected on ZP following incubation of oocytes with native sperm lysates. These interactions could however be virtually eliminated if the oocytes were incubated with anti-ubiquitin antibodies, to mask the ligands for proteasome adhesion, prior to the addition of the native sperm lysates (Sutovsky et al., 2004; Redgrove et al., 2011). While such studies extend the potential role of the proteasome complex to that of zona binding, a limitation imposed by the conservation of this complex across multiple species, is that it cannot account for the species specificity of this interaction. Ongoing work is therefore focusing on the characterisation of the additional three complexes from human sperm that possess ZP affinity. Such findings also prompted us to conduct a more detailed analysis of the two mouse sperm complexes that possessed affinity for the ZP (Chapter 6).

Biochemical Insight into the Observed Zona Pellucida Affinity of Sperm Multiprotein Complexes

Characterisation of the multimeric zona receptor complexes unearthed during these studies and the molecular mechanisms that facilitate their interaction with ZP ligands currently remain in their infancy with many questions remaining to be resolved. For instance, in contrast to the surface localisation described in our studies, reports from Lin et al., (2007) suggest that ZPBP2 (a key component of the CCT/TRiC complex) is a readily soluble acrosomal protein localized along the rostral ridge. Although the reason for this dichotomy remains to be fully explored, it is possible that different pools of protein exists within sperm and / or that its location may be influenced by the capacitation status of the cells. This indeed may account for the fact that non-capacitated sperm cannot bind to the ZP. As previously mentioned, the latter explanation is consistent with our studies supporting a capacitation driven remodelling of the mouse sperm APM. It is also in keeping with the demonstration that the CCT/TRiC complex also harbours a
least one additional zona receptor, ZP3R/Sp56, that has been variously described as a resident of both the mouse sperm surface (Bleil and Wassarman, 1990a; Suzuki-Toyota et al., 1995; Wassarman, 2009) and acrosomal matrix (Kim and Gerton, 2003b; Buffone et al., 2009). These discrepancies in localisation were recently attributed to a translocation of the protein that occurs during sperm capacitation. Indeed, work by Gerton and colleagues, has shown that ZP3R is among several proteins that are progressively released to the sperm surface during the initial stages of capacitation. Although the exact mechanism underpinning this movement remains to be determined, the authors propose that intact ZP3R remains associated with the acrosomal matrix and is exposed to the external milieu through limited fusion of the outer acrosomal and plasma membranes in preparation for the more complete fusion of these membranes during the course of ZP-stimulated acrosomal exocytosis (Buffone et al., 2008b). Such results encourage a reappraisal of the acrosome reaction as ‘an all or none’ event, suggesting instead that it progresses through a series of intermediate stages that see a step wise release of both intracellular molecules and the receptors necessary for the maintenance of attachment with the oocytes extracellular matrix.

Although a relatively novel concept for the field of sperm biology, the recruitment of proteins onto the plasma membrane is widely acknowledged as a critical step in the activation of a number of cell types. Among the numerous examples, both receptor tyrosine kinases and G-protein coupled receptors recruit, upon activation, effector proteins such as SH2-containing adaptor proteins (Pawson and Nash, 2000) in addition to various enzymes to the plasma membrane (Shenoy and Lefkowitz, 2003). Another class of effector proteins, such as those of the protein kinase C family (Mellor and Parker, 1998), are activated upon translocation to the plasma-membrane microenvironment, independently of receptor binding. The importance of this form of information transfer is highlighted by the fact that, impaired or excessive translocations of specific proteins are known to play important roles in the pathogenesis of diseases (Pinton et al., 2002; Gallo et al., 2005). Detailed knowledge of the translocation of specific proteins, not only in physiological, but also in pathological conditions, may therefore highlight key aspects of both somatic and sperm cell activation. Unfortunately, we do not currently have a large armoury of tools with which to investigate protein translocation in spermatozoa, and while the classical approaches we have employed (immunocytochemistry, immunogold labelling, and Western blotting) are adequate to reveal the existence of the process, these techniques provide limited information on the mechanism of translocation. In cells where this phenomenon has been more widely studied, there is growing recognition of the importance of lipid rafts in creating a platform for the recruitment and/or assembly of receptor complexes (Weintraub et al., 2000; Dykstra et al., 2001). This finding is of considerable interest in light of...
the central role that is emerging for lipid rafts in the capacitation-associated remodelling of the sperm APM (see Chapter 3).

Notwithstanding the promising results of this thesis, targeted ablation of the genes for both ZPBP2 and ZP3R have failed to block fertilisation (Lin et al., 2007; Muro et al., 2011). Indeed, while sperm from ZPBP2 knockout mice display subfertility associated with an impaired ability to penetrate the ZP (Lin et al., 2007), those from the ZP3R null males do not exhibit any marked changes in their ability to engage in ZP binding, undergo acrosomal exocytosis, or fertilise oocytes compared to wild-type sperm (Muro et al., 2011). The latter findings led the authors to propose that ZP3R is either not involved in sperm-ZP binding or, in keeping with our model, that gamete interactions might be functionally redundant, involving multiple proteins.

Among the additional proteins that could participate in this interaction, those that we have identified in complex 2 (HSPD1 and ADAMTS10, Chapter 6) are compelling candidates. Previous work from our laboratory has demonstrated that HSPD1 is a major target for capacitation-associated tyrosine phosphorylation promoting its expression on the outer leaflet of the plasma membrane. This surface expression is thought to represent the sub population of capacitated sperm capable of binding to the zona pellucida (see Chapter 7) (Asquith et al., 2004a; Asquith et al., 2005b). Such studies served to raise the awareness of extracellular chaperones and their importance in the mediation of cellular interactions (see Chapter 7). Indeed, historically chaperones were thought to be limited to roles within intracellular domains (Burel et al., 1992; Welch, 1993). However a growing body of literature has described chaperones as having pericellular and extracellular residency in somatic cells (Calderwood et al., 2007a; Calderwood et al., 2007c; Pockley et al., 2008; Merendino et al., 2010). The role for these cell surface chaperones appears as diverse as the clients proteins with which they interact, but nevertheless, they have been shown to be important for the recovery of cells under stress, the regulation of aspects of the immune response and tumour growth and metastasis (Calderwood et al., 2007b; Pockley et al., 2008). It is widely understood that somatic cells interact with each other to synchronize their behaviour and metabolic activity, and under conditions of environmental change these cells communicate in order to adjust to the prevailing circumstances. It is now believed that some of the key stress signals released by cells under these conditions are molecular chaperones (De Maio, 2011). In an analogous manner, ejaculated sperm respond to the stressful environment of the female reproductive tract by initiating capacitation-associated signalling cascades and, as discussed in Chapter 7, these promote the translocation of a host of molecular chaperones to the surface of the peri-acrosomal region of the cell (Asquith et al., 2004a; Dun et al., 2011). As interesting as these findings are, it is even
more interesting to speculate on how the relocation of the chaperones occurs as the majority lack the consensus signal required for secretion via the classical endoplasmic reticulum (ER)-Golgi pathway (De Maio, 2011). It has now emerged that the translocation of intracellular chaperones to the external membrane may be mediated by their association with export vesicles or liposomes in somatic cells (Vega et al., 2008). Although there is no evidence that sperm chaperones are contained within these lipid structures, it is well established that sperm indeed contain membrane vesicles that participate in remodelling of the APM during capacitation (Langlais and Roberts, 1985). Commensurate is the aggregation of a number of chaperones within membrane rafts and their known association with Gm1 and cholesterol, two key lipids within membrane microdomains, which are fractions of the membrane that have the ability bind to the zonae pellucidae (see Chapter 7) (Bou Khalil et al., 2006b; Nixon et al., 2009b). Further investigation of the residency of chaperones within these lipid transport vesicles and their tight association with varying lipid classes will establish the mechanisms of protein and lipid activation during capacitation.

Interestingly, our collective findings indicate that the surface expression of HSPD1 does not equate to a direct role in mediation of zonae interactions. Instead, on the basis of our early studies we hypothesised that the protein was most likely to fulfil an indirect in ZP interaction by virtue of its ability to promote the assembly and / or presentation of a ZP receptor complex (Asquith et al., 2004a). However, until the studies conducted herein we lacked the evidence to support this model. The identification of ADAMTS10 as a HSPD1 client protein and as a putative ZP receptor (Chapter 6) were therefore extremely exciting discoveries. These findings also share interesting analogies with other cells types in which alternative members of the matrix metalloprotease superfamily form dynamic, surface-orientated associations with molecular chaperones (Eustace et al., 2004; Stellas et al., 2010). However, it remains to be equivocally established whether ADAMTS10 directly binds ligands within the ZP or whether it is the proteins proteolytic 'sheddase' activity (see Chapter 6) that is required to facilitate the unmasking, maturation and / or activation of alternative proteins that are localised within the sperm APM. An additional question remaining from these studies, is that the observed molecular weight of the predominant protein (50 kDa) recognized in epididymal spermatozoa by two unique anti-ADAMTS10 antibodies differs substantially from that predicted on the basis of the primary sequence for either the full length zymogen or that of the processed mature form of the protein (118 and 95 kDa, respectively). At present however, nothing is known regarding the additional processing of the protein that takes place to produce the 50 kDa isoform that is present in epididymal sperm, nor the impact of such processing on the protease activity of the protein. Resolution of these issues is necessary to shed further light on the role of this protein in oocyte interactions.
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The Molecular Basis of Sperm-Oocyte Interaction Thesis Perspectives

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THESIS PERSPECTIVES

These collective findings presented in this thesis have provided an important, novel insight into the molecular mechanisms that underpin sperm-oocyte interactions. Specifically, our studies have generated among the first compelling evidence in support of the role of multimeric sperm receptor complexes in the mediation of sperm-ZP adhesion.

Figure 1. Model to illustrate the major events characterizing sperm capacitation highlighting the discoveries unearthed during these investigations. (A) During their ascent of the female reproductive tract, spermatozoa release decapacitation factors and initiate the process of capacitation. (B) The ensuing sterol efflux renders the sperm permeable to Ca²⁺ and HCO₃⁻ ions thereby increasing intracellular pH and leading to hyperpolarisation of the plasma membrane.
Soluble adenylyl cyclase activity is stimulated, elevating the levels of intracellular cAMP and stimulating signalling pathways that culminate in the tyrosine phosphorylation (YP) of a myriad of target proteins. These biochemical modifications coincide with the aggregation of membrane microdomains in the APM and facilitates the surface expression of chaperones and their client proteins, including a subset of ZP receptors. These collective events render the sperm competent to bind to the ZP. (C) Sperm tethered to the carbohydrates that furnish the ZP initiate the exocytotic events of the acrosome reactions enabling the sperm-ZP secondary binding proteins to be exposed. Following acrosomal loss, the plasma membranes of both gametes fuse and the sperm passes on the paternal genome. Abbreviations: APM, apical plasma membrane; OAM, outer acrosomal membrane.

The major conceptual advances provided by this work (summarised in Fig. 1) is likely to serve a platform for future detailed dissection of sperm-ZP interactions, studies that will ultimately benefit society in terms of defining the causes of male infertility and stimulating the development of novel approaches for contraceptive intervention. Indeed, the inference that sperm contain multimeric zona receptor complexes consisting molecular chaperones delivers an exciting new molecular target for contraceptive development. As we have shown, sperm cells, like of many other cell types (Workman, 2003), contain an abundance of molecular chaperones. These chaperones appear to fulfil an important intermediary role in the assembly, presentation and / or maintenance of the conformational stability of a range of potential ZP receptors. Future development of inhibitors capable of blocking the intrinsic ATPase activity that is essential for chaperone function (Chapter 7) may provide us with novel contraceptive strategies. These developments may fill the current void in the contraceptive armoury of our own species, (McLaughlin and Aitken, 2010) in addition to that of both captive and feral animal species (Hardy and Braid, 2007; Kirkpatrick, 2007; Fayrer-Hosken, 2008). However, in order to meet these objectives, chaperone inhibition must be specific enough to target the essential components of male reproductive system in the target species.
REFERENCES


