OOLEMMA PROTEOMICS:
Identification of Oocyte Cell Surface Protein Complexes
involved in Murine Fertilisation

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I hereby certify that the work embodied in this thesis is the result of original research and has not been submitted for a higher degree to any other University or Institution.

Jonathan W. Paul
For the Honour
Acknowledgements

“Now in the final stages of a seemingly endless PhD project, it has become increasingly clear how indebted I am to those that have helped me along the way. First and foremost I would like to acknowledge and thank my supervisor Dr. Eileen A. McLaughlin, without whom this project and thesis would not have been possible. I cannot thank you enough for your time, or the skills and knowledge you have imparted on me. Your guidance and unwavering support have consistently been above and beyond all hopes and expectations throughout the entire duration of these studies. You once told me that every PhD student is reduced to tears at some point throughout their studies, well thanks to you I was able to beat these odds. To my supervisor Laureate Prof. R. John Aitken, years ago you once told me that it was a privilege to work in the lab, and that we should all be thankful for the opportunity we have been given. As a brash young, Honours student your words were lost on me at the time, but I am pleased to say that I know grasp the magnitude of your words and feel truly privileged to have been given the opportunity to undertake a PhD as part of the Reproductive Sciences Group – Thank you sincerely for giving me that opportunity. Thank you also for your guidance throughout these studies. To this day I remain in awe of your ability to grasp the ‘bigger picture’ amidst the multitude of microcosmic research projects orbiting around you.

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**GENERAL**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>2D</td>
<td>two-dimensional</td>
</tr>
<tr>
<td>amu</td>
<td>atomic mass unit</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>BWW</td>
<td>Biggers, Whitten and Whittingham media</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CEO(s)</td>
<td>cumulus-enclosed oocyte(s)</td>
</tr>
<tr>
<td>CHAPS</td>
<td>3-[(3-Cholamidopropyl) dimethylammonio]-1propanesulfonate</td>
</tr>
<tr>
<td>CO₂</td>
<td>carbon dioxide</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>dH₂O</td>
<td>de-ionised water</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulphoxide</td>
</tr>
<tr>
<td>ECL</td>
<td>Enhanced Chemiluminescence</td>
</tr>
<tr>
<td>FCS</td>
<td>fetal calf serum</td>
</tr>
<tr>
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<td>fluorescein isothiocyanate</td>
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<tr>
<td>IVF</td>
<td><em>in vitro</em> fertilisation</td>
</tr>
<tr>
<td>LC</td>
<td>liquid chromatography</td>
</tr>
<tr>
<td>α-MEM</td>
<td>α-Minimum essential media</td>
</tr>
<tr>
<td>MII</td>
<td>metaphase II</td>
</tr>
<tr>
<td>MilliQ-H₂O</td>
<td>Milli-Q filtered water</td>
</tr>
<tr>
<td>MS</td>
<td>mass spectrometer</td>
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<tr>
<td>MS/MS</td>
<td>tandem mass spectrometry</td>
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<td>polyacrylamide gel electrophoresis</td>
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Abbreviations

PBS phosphate-buffered saline
PI-PLC phosphatidylinositol-specific phospholipase C
PMSG pregnant mare serum gonadotrophin
PVA polyvinyl alcohol
RT room temperature
SDS sodium dodecyl sulphate
TBS tris buffered saline
TBS-T TBS supplemented with 0.1% Tween-20
TEMED N,N,N',N'-tetra-methylethyldiamine
Tris tris[hydroxymethyl]aminomethane
Tween-20 polyoxyethylene sorbitan monolaurate
ZP zona pellucida

UNITS

A amps
°C degrees Celsius
g (weight) grams
g (centrifugation) gravity
h hour(s)
kDa kilodalton
L litre
M molar
min minute(s)
sec second/s
U units (enzymatic activity)
V volts
wk week

PREFIXES

m milli $10^{-3}$
µ micro $10^{-6}$
n nano $10^{-9}$
p pico $10^{-12}$
Membrane fusion events are a fundamental aspect of cellular biology and underpin important processes such as organ formation and fertilisation. Within the latter, proteins that are expressed on the egg surface which are responsible for mediating sperm recognition, binding and fusion to the egg, are yet to be fully determined. Evidence does however suggest that egg surface glycoporphatidylinositol (GPI)-anchored proteins play a role in sperm binding, whilst another class of proteins, known as tetraspanins, appear to be important in downstream events of membrane fusion. Of the tetraspanins, CD9 and CD81 have been identified as fulfilling roles in membrane fusion; identifications are however yet to obtained for the important GPI-anchored protein(s). This research aimed to identify and characterise egg surface proteins implicated in sperm-egg interaction, and embodied attempts to both identify the important GPI-anchored protein(s) as well as expand upon tetraspanin studies through investigations into mice lacking the tetraspanin CD151. Throughout this research, it was hypothesised that membrane fusion events of fertilisation paralleled those of enveloped virus – host cell fusion, for which rearrangement of surface protein thiols is essential.

In vitro binding and fusion experiments were utilised as functional bioassays in the investigation of factors affecting sperm-egg interaction, such as tetraspanin deletion and the xenobiotic modification of cell surface thiols, while mass spectrometry (MS)-based proteomics and bioinformatics-based analyses were employed to compile oocyte protein databases and to identify candidate proteins responsible for mediating sperm-egg interaction, such as GPI-anchored proteins. It was determined that exposing oocytes to compounds with a capacity to alkylate cell surface thiols strongly inhibited sperm-egg binding. Additionally, while CD151 deletion had no effect on sperm-egg binding, the downstream events of membrane fusion were significantly impaired. Ovaries from CD151 null mice also exhibited abnormal phenotypes. In addition, a total of 11 identifications were obtained in the search for the GPI-anchored proteins expressed within eggs, however only 6 of these were deemed to be potential mediators of sperm-egg interaction.
In conclusion, the experiments outlined herein demonstrate a novel inhibitory effect for specific xenobiotics on sperm-egg interaction, and correlate the inhibitory action of these compounds with their capacity to reduce cell surface thiol labelling. A novel role for CD151 in the mediation of sperm-egg fusion was also discovered, while at the same the important GPI-anchored protein(s) implicated in sperm-egg binding may be among 6 identified potential candidates. Together the findings reiterate the consensus that oocytes possess a cell surface protein complex responsible for mediating sperm binding and fusion as separate events, and in light of the demonstrated importance of surface thiols, that events of sperm-egg membrane fusion parallel those of enveloped virus – host cell fusion.
CHAPTER 1:

Introduction and Literature Review
CHAPTER 1 – INTRODUCTION & LITERATURE REVIEW

1.1 Introduction

1.1.1 Perspective on the Study of Reproduction

Sexual reproduction is a process essential to the replication and maintenance of genetic diversity in many plant and animal species. Despite the monumental importance of this event, many of the processes mediating the interaction of male and female gametes remain unknown. This is particularly the case for mammalian fertilisation in that the molecular components that mediate the interaction between sperm and egg are yet to be fully identified.

In light of this, there exists the need to understand this crucial biological event in as much detail as possible. Whilst advances in understanding the biology of sperm-egg interaction are made seemingly every day, there remains much to be discovered with respect to the molecular mechanisms involved in the binding of sperm and egg and how this interaction translates into subsequent membrane fusion. This doctoral dissertation details the planned investigation of the mammalian egg, or oocyte, using the laboratory mouse as the experimental model, in an attempt to gain a better understanding of biological processes and proteins implicated in sperm-egg interaction.

In addition to drawing on the existing pool of literature for this field, evidence continues to emerge highlighting the existence of parallels between sperm-egg fusion and certain types of virus-cell fusion. In light of this correlation and the fact that virus-cell fusion is an extensively investigated field with substantial existing literature, this research looks toward virus-cell fusion as a conceptual model as to how sperm-egg membrane fusion may be mediated, and attempts to draw on the extensive knowledge base in this field to advance our reproductive goals.

1.1.2 Mammalian Fertilisation: Overview and Importance

Following ejaculation, a finite amount of time is required within the female reproductive tract before sperm acquire the capacity to fertilise. First observed in the early 1950s (Austin, 1951; Chang, 1951), this acquisition of fertilising capacity, termed
capacitation, is the culmination of a series of complex molecular changes undergone by sperm within the female reproductive tract. To date, these changes are known to include: (i) the release of sperm surface decapacitation factors, (ii) remodelling of sperm plasma membrane and the distribution of the proteins embedded within it, (iii) hyperactivation of sperm motility, (iv) the tyrosine phosphorylation of multiple sperm proteins, (v) cholesterol efflux from the membrane, which serves to enhance membrane fluidity, (vi) an up-regulation in the generation of reactive oxygen species (ROS), and (vii) the influx of calcium ions ($\text{Ca}^{2+}$) (Cohen-Dayag and Eisenbach, 1994; de Lamirande et al., 1997; Visconti et al., 1998a; Visconti and Kopf, 1998b; Yanagimachi, 1994a). The ultimate endpoint of capacitation is the ability of sperm to undergo a zona pellucida (ZP)-induced acrosome reaction. During sperm-ZP interaction, receptors on the sperm plasma membrane recognise and bind to the ZP protein, ZP3. This binding stimulates fusion of the sperm plasma membrane with the underlying acrosomal membrane resulting acrosomal exocytosis. The hydrolytic activity of the acrosomal enzymes in conjunction with physical shearing forces enables the spermatozoon to penetrate the ZP, and thereby enter the perivitelline space for fusion. For review see (Yanagimachi, 1994a).

Figure 1.1 Capacitation: the acquisition of fertilising potential
Freshly ejaculated sperm must undergo a series of maturational changes within the female reproductive tract to acquire the capacity to fertilise the oocyte. (A) The sperm surface is remodelled including the release of decapacitation factors. (B) Sperm adopt a hyperactivated motility pattern and phosphorylated tyrosine residues appear on the sperm surface (C). These changes occur concurrently within the female reproductive tract and together infer on the sperm the capacity to recognise and bind to the zona pellucida (D).
Aligning virus-cell and sperm-egg fusion, the spermatozoon would represent the virus whilst the oocyte would represent the target cell. Like HIV infection, the final stages of fertilisation can be divided into two distinct events, sperm binding to the oocyte plasma membrane (oolemma) followed by membrane fusion. Recent studies have reported significant advances in attempts to identify proteins implicated in both of these steps and will be addressed.

1.2 Folliculogenesis

1.2.1 Ovarian Development

The ovary has two primary roles within the mammalian body. The first is the production and ovulation of viable eggs for fertilisation, whilst the second is the production of steroid hormones. At the onset of puberty this hormone production functions to promote the development of female characteristics as well as prepare the uterus for fertilisation during the oestrus cycle. In the event that fertilisation should occur, the production of hormones such as progesterone and oestrogen by the ovary then becomes essential to sustaining the initial stages of pregnancy itself (Penzias, 2002).

Although the size and shape of ovaries varies between different mammalian species, the general structure is conserved. All are connected to the fimbriated end of the fallopian tube and all consist of three distinct layers. The outermost layer is the tunica albuginea. This tough fibrous capsule encloses the ovaries and directly overlays the outer cortex. The cortex contains all the follicles within the ovary as well as the remains of ruptured follicles. Strewn throughout the cortex, these follicles are at various stages of development and each is embedded in a vascular fibrous tissue. At the core of the ovary is the inner medulla. The medulla is the site at which blood vessels, the lymphatic system and nerves enter the ovary (see Figure 1.2).
1.2.2 Follicle Development and Ovulation

At the time of birth the mammalian ovary contains a finite number of primordial follicles arrested at the first meiotic division. These primordial follicles are defined by a single oocyte surrounded by a single layer of squamous granulosa cells and represent the pool of female gametes from which mature oocytes will be derived. In a process known as follicle activation, a continuous trickle of these primordial follicles activates and joins the growing follicle pool. The precise mechanism regulating which follicles are activated remains yet to be clearly understood, however at any time the ovary contains a heterogeneous population of follicles at different stages of development. Activation of the primordial oocyte results in the initiation of a pre-programmed course of maturation and development. Upon activation, the previously quiescent granulosa cells that surround and protect the oocyte change from squamous or flattened cells, to cuboidal granulosa cells. At this stage the follicle is now a primary follicle as the granulosa cells begin to divide. As the granulosa cells divide they form concentric layers around the oocyte. For review see (Skinner, 2005).

The granulosa cells in close proximity to, but not in direct contact with the oocyte, are referred to as cumulus cells, whilst those that are in direct contact are referred to as corona radiata. The corona radiata communicate with the oocyte through cytoplasmic
bridges made possible by trans-zonal gap junctions. Together the sub-populations of granulosa cells provide a microenvironment that surrounds and protects the oocyte and supplies the latter with essential growth factors and nutrients essential for survival and growth. Once several layers of cuboidal granulosa cells have accumulated, the follicle is referred to a secondary follicle. At this stage of development, the various sub-populations of granulosa cells that surround the oocyte, collectively called membrana granulosa, begin to secrete follicular fluid. Small pockets of follicular fluid accumulate amongst the membrana granulosa and represent the beginning of a fluid filled cavity called the antrum. At the same time, a thick amorphous layer forms between the oocyte and membrana granulosa and marks the formation of the ZP. As the fluid filled pockets begin to grow, the follicle has reached the secondary-vesicular stage, but as these individual pockets begin to merge formation of the antral cavity is soon complete, giving rise to the antral follicle, also known as a tertiary follicle.

The majority of developing follicles are actually destined to undergo atresia. Increasing levels of luteinizing hormone (LH) and follicle stimulating hormone (FSH) however rescues the dominant follicle, or follicles in many mammalian species, from atresia. LH also stimulates follicles to secrete proteolytic enzymes, which degrade the follicular tissue. Continued degradation results in rupture of the follicle(s) and the cumulus-enclosed oocytes (CEO) are expelled into the fallopian tube (Wassarman, 2002). In addition to stimulating ovulation, LH also induces germinal vesicle breakdown and the resumption of meiosis, however meiosis eventually arrests once again at metaphase II (Korach and Quarmby, 1985).
Figure 1.3 Mammalian folliculogenesis and oogenesis
Activation of primordial follicles (1) stimulates differentiation of squamous granulosa cells (pre-granulosa cells) into cuboidal granulosa cells (2). Granulosa cells proliferate giving rise to several layers of cells (3) and begin to secrete follicular fluid. The oocyte enlarges and the ZP forms (4). Thecal cells now form a basement membrane around the follicle. Follicular fluid secretion results in formation of the antral cavity (5). If rescued from atresia by a surge in LH and FSH levels, the antral follicle(s) progress to ovulation. Redrawn from (Baker, 1982).

1.3 Mammalian Gamete Interaction
Post-coitus, sperm encounter the ovulated oocyte within the fallopian tube, which serves as the site of fertilisation. By the time sperm reach the site of fertilisation they have undergone the prerequisite series of capacitation-associated changes necessary for ultimately achieving fertilisation (Cohen-Dayag and Eisenbach, 1994; de Lamirande et al., 1997; Visconti et al., 1998a; Visconti and Kopf, 1998b; Yanagimachi, 1994a). Upon penetrating the ZP, sperm must then recognise and bind to the oocyte plasma membrane. Together, the molecular machinery present in both cell membranes must
then interact in a precise manner necessary to facilitate the energetically costly event that is the merger of sperm and egg membranes.

1.4 Cell-Cell Recognition and Fusion

1.4.1 Introduction to Cell-Cell Fusion in Biology

Membrane fusion events occur within a wide variety of biological processes, ranging from the mating of single cell yeast though to the formation of complex organs and tissues. Within eukaryotic cells, this fusion process is either endoplasmic or exoplasmic. Endoplasmic refers to fusion events taking place within a cell’s own cytoplasm, such as vesicle transport between organelles or fusion with the inner leaf of the plasma membrane. Exoplasmic events strictly refer to extracellular fusion events, such as membrane fusion between adjacent cells (Rothman and Warren, 1994; White, 1992). Successive exoplasmic fusion events give rise to multinuclear cells and is a process utilised throughout evolution in the formation of complex organs. Bone cells for example, arise from the fusion of mononuclear osteoclast precursors to form differentiated multinucleated bone cells. Cells that remain in this multinucleated state are referred to as syncytia. Examples include skeletal muscle, a syncytia of myoblast cells, while trophoblast fusion gives rise to the syncytiotrophoblast layer of the human placenta (Burger and Verkleij, 1990).

Exoplasmic fusion is also a key process during embryonic development, indeed the very first exoplasmic fusion event occurs when sperm and egg first meet. After penetrating the zona pellucida, the spermatozoon fuses with the egg plasma membrane in order to complete the fertilization process. Unlike syncytia formation, exoplasmic fusion between sperm and egg is a transient event in that the diploid zygote then begins to divide to form individual blastomeres (Shemer and Podbilewicz, 2000).

These fusion events, which have been utilised by nature for fertilisation and the evolution of complex organs, have also been exploited by viruses and other pathogens. Just as a sperm recognises, binds to and then fuses with the membrane of an egg, so too do enveloped viruses bind to and fuse with the membrane of a target cell to infect and ultimately destroy it. The molecular events mediating virus-host cell membrane fusion have been extensively studied and vast repositories of knowledge now exist in this field. By contrast, relatively little is known about the molecular mechanisms mediating cell-
cell membrane fusion, and this is particularly the case where sperm-egg fusion is concerned. The opportunity therefore exists to gain valuable insight into the mechanisms mediating sperm-egg membrane fusion, as well as other somatic cell-cell membrane fusion events, by reviewing existing literature on the topic of virus-host cell membrane fusion.

1.4.2 Virus-Cell Fusion

1.4.2.1 Lipid Bilayer Fusion

In order for fusion between two membranes to occur, the membranes must not only come into close apposition but water must be excluded from the site of initial fusion (Tamm et al., 2003). In biophysical terms, the fusion of lipid bilayers is an energetically costly event (Tamm et al., 2003). However, structural and theoretical studies into membrane fusion have found that the formation of a ‘stalk’ between the two membranes may overcome this problem. An intermediate in the fusion process, the stalk connects only the outer leaflets of the membrane bilayers, effectively creating a diaphragm. It is believed that lateral expansion of this diaphragm then generates tension forces that promote interaction and subsequent fusion of the inner leaflets, effectively achieving pore formation (see Figure 1.4) (Chernomordik et al., 1995; Leikina and Chernomordik, 2000; Yang and Huang, 2002).

**Figure 1.4 Lipid bilayer fusion**

Fusion of opposing lipid bilayer membranes may occur via formation of an intermediate stalk structure. The phospholipid stalk forms when only the outer leaflets of the opposing bilayers have fused (hemifusion) and results in the formation of a diaphragm. Lateral expansion of the resultant diaphragm then generates torsional forces leading to complete bilayer fusion.
During exoplasmic fusion, one may anticipate that fusion-promoting factors in the adjacent membranes act by facilitating the formation of this stalk. While the actual proteins utilised to promote fusion may vary among different cell types, it is anticipated that all serve to endorse the formation of such stalks. Beyond initial stalk formation, membrane fusion is believed to proceed in a similar manner regardless of how the stalk was initially achieved (Chernomordik and Kozlov, 2005).

1.4.2.2 Envelope Virus Fusion
Viruses can be classified into three broad groups: bacteriophage, naked viruses and enveloped viruses. Bacteriophage infect bacteria whilst naked and enveloped viruses infect plant and animal cells. While a naked virus consists merely of a protein coat, or capsid, surrounding the viral genetic material, together called a nucleocapsid, enveloped viruses possess an additional lipid bilayer that encloses the nucleocapsid. Acquired during viral budding from the previous host cell, the viral envelope contains a mosaic of antigens from both the host and the virus and as such is both host cell and viral genome derived. This envelope assists in host cell infection and typically expresses viral fusion glycoproteins in high density.

1.4.2.3 Viral Entry
Enveloped viruses employ one of two routes of entry into host cells. The more complex route entails the binding of a virus to clathrin-coated pits on the surface of a target cell, stimulating internalisation of the virus via endocytosis. The resultant virus-containing vesicle is then fused with a lysosome in an attempt to break down the virus. However, this low pH environment triggers the glycoproteins present in the viral envelope to undergo a conformational change enabling fusion between the viral envelope and the vesicle membrane. This fusion event effectively releases the viral nucleocapsid into the cells’ cytoplasm (Hernandez et al., 1996). The conceptually simpler route occurs at neutral pH and involves the binding of a virus to specific receptors expressed on the surface of a cell. Rather than triggering endocytosis, the viral envelope fuses directly with the cells plasma membrane permitting direct entry of the nucleocapsid into the cells cytoplasm (Hernandez et al., 1996). Whether direct or via means of endocytosis, each fusion pathway is mediated by specific viral genome-derived fusion glycoproteins expressed in the viral envelope.
Figure 1.5 Routes of enveloped virus entry into host cells
Envelope viruses gain entry to host cells by means of low pH mediated membrane fusion or direct entry involving neutral pH membrane fusion. During low pH mediated fusion, the enveloped virus is internalised by endocytosis. The virus-containing vesicle is then fused with a lysosome, upon which the low pH lysosomal contents trigger a series of conformational changes in the viral fusion proteins. Activation of the fusion proteins facilitates membrane fusion between the vesicle and viral envelopes thus enabling release of the viral nucleocapsid into the host cells cytoplasm. During direct entry, viral envelope glycoproteins interact with specific host cell surface receptors. This interaction triggers a series of conformational changes in the viral envelope fusion proteins, which bring about membrane fusion and thus enables release of the nucleocapsid into the host cell cytoplasm.

1.4.2.4 Fusogens
Known as fusogens, viral fusion glycoproteins have been identified for most known animal viruses (Bentz, 1993). Analysis of the viral fusion proteins studied to date reveals a number of attributes common to each of the fusogens. These molecules are composed of either one or two type I integral membrane proteins containing N-linked carbohydrates with the bulk of the proteins mass external to the viral membrane. These proteins are capable of forming higher order oligomers and are expressed on the viral envelope in high density. Most importantly, all contain a ‘fusion peptide’ within a membrane-anchored subunit. In addition, many fusogens are tight complexes of two glycoprotein subunits, which provide the capacity for both binding and fusion activity. Finally many are synthesised as larger precursor proteins that require proteolytic remodelling in order to potentiate their fusogenic nature (Hernandez et al., 1996).
These glycoprotein subunits are referred to as the surface (SU) subunit and the transmembrane (TM) subunit and for almost all virus types, they are synthesised as a single peptide that is proteolytically cleaved to give rise to the monomers (Cohen and Melikyan, 2004). The SU subunit is responsible for binding to the host cell, whilst the TM subunit, which is anchored in the viral envelope by a single membrane-spanning domain (MSD), is responsible for fusion. The fusion promoting peptide is a stretch of approximately 20 non-polar amino acids, and is conserved within, but not between, different virus families. Modelling of these peptides generally reveals an α-helical structure, one face of which is typically very hydrophobic. However fusion peptides may also interact with membranes as a β sheet (Cohen and Melikyan, 2004; Gray et al., 1996; Lear and DeGrado, 1987; Nieva et al., 1994). During fusion, this peptide becomes inserted into the host cell membrane (Harter et al., 1989; Pak et al., 1997; Stegmann et al., 1991), effectively inducing lipid rearrangement. This lipid rearrangement facilitates hemi-fusion and pore formation (Cross et al., 2001; Durell et al., 1997; Qiao et al., 1999) and the fusion peptide has also been demonstrated to participate in pore enlargement (Schoch and Blumenthal, 1993).

1.4.2.5 Viral Fusion Proteins: Low pH Activation

The low pH environment of the endosomes is necessary for activation of the fusion peptides of various virus families including orthomyxo-, flavi-, toga-, bunya-, rhabdo- and arenaviruses. The exact pH necessary for each of these virus families varies, but generally falls within pH 4.8 to 6.5 (Hernandez et al., 1996). Much effort has been directed toward elucidating the crystalline structure of the proteins, in both the inactive neutral pH form as well as the fusogenic low pH conformation. Influenza is one of the most thoroughly characterised low pH-activated enveloped viruses, with atomic resolution structures available for influenza hemagglutinin (HA) in both the pre-fusogenic and fusogenic conformations (Bullough et al., 1994; Wilson et al., 1981). Furthermore, much is now known about the actual steps of the conformational change and how these changes mediate the fusion reaction itself (Gaudin et al., 1995; Hughson, 1995; Stegmann, 1994). In addition, important differences have been discovered in the HA conformational change between different viral strains (Gutman et al., 1993; Pak et al., 1994; Tsurudome et al., 1992). The influenza HA (X:31 strain) is composed of three monomers. Each monomer is comprised of a globular receptor-binding subunit (HA1) and a fibrous subunit containing the fusion peptide (HA2). Together, this trimer
structure projects approximately 130 Å from the target membrane. In the neutral pH structure, the fusion peptides are concealed within the trimer interface by a network of hydrogen bonds. In this conformation, the fusion peptides reside approximately 100 Å from the target membrane and align roughly parallel to the plane of the virus membrane (Wiley and Skehel, 1987).

In the presence of low pH, a series of conformational changes sees HA expose its fusion peptides and bind hydrophobically to the target membrane. In assuming its final low pH conformation, HA then folds back on itself such that its fusion peptides reside near its own transmembrane domain. The target membrane bound to these fusion proteins is thus bent into close proximity with the viral membrane under tension forces necessary to mediate hemifusion, or stalk formation, and the subsequent dilation of a fusion pore. For extensive review see (Hernandez et al., 1996). These events parallel the exposure of oocyte binding sites on the surface of capacitated spermatozoa. It has been demonstrated that a minimum of 3 to 4 clustered HA trimers are required to facilitate the fusion process (Danieli et al., 1996). The timing of initiation of this conformational change is also important. Influenza X:31 can be irreversibly inactivated by pre-treating it with low pH in the absence of a target membrane. In this situation, HA prematurely undergoes its conformational change turning the virus into a fusion-incompetent form (White, 1995). Similarly in spermatozoa, oolemmal binding proteins are only revealed on the cell surface once sperm have acrosome reacted – a prerequisite for successful fertilisation (Yanagimachi, 1994a).

In terms of functional significance, in the presence of low pH, the HA ectodomain converts from a hydrophilic to a hydrophobic entity capable of interacting with the target membrane via exposure of the fusion peptides. In studies utilising synthetic and mutant HA, the ability of the fusion peptides to promote fusion roughly correlate with its capacity to adopt a helical configuration (Gray et al., 1996).

1.4.2.6 Viral Fusion Proteins: Neutral pH Activation
Neutral pH viruses do not require an acidic environment to fuse with a host cell and as such are believed to fuse directly with the target cells plasma membrane. Retro-, herpes-, corona-, paramyxov-, hepadna- and poxviruses are all examples of neutral pH viruses and all require specific host cell factors, or receptors, in the target membrane for
successful fusion. Whilst low pH has been demonstrated to initiate the conformational changes that expose the fusion peptides of low pH viruses, here the conformational change is believed to be triggered by interaction of the viral fusion glycoproteins with a host cell receptor. Upon interaction with the appropriate cell surface receptor(s), transition of the viral fusion protein to a fusogenic state capable of interacting with the host cell membrane occurs, and is envisaged to involve the exposure and repositioning of a previously cryptic fusion peptide.

1.4.2.7 Human Immunodeficiency Virus

HIV is a retrovirus and like all other retroviruses it has one envelope protein responsible for fusion (Hernandez et al., 1996). Components of the envelope protein are synthesised as precursors that require proteolytic cleavage into a transmembrane (TM) subunit and a surface (SU) subunit. These post-translational modifications are essential for retroviral-enveloped proteins to manifest their fusion activity. The retroviral SU subunit has determinants for receptor binding, while the TM subunit anchors the protein in the viral membrane. Located at or near the N-terminus of the TM subunit is a hydrophobic domain that is believed to be the fusion peptide. In terms of HIV, the SU and TM subunits are the proteins gp120 and gp41 respectively. These monomeric proteins, are associated only through non-covalent interactions (Weiss, 1992) and may exist as trimers within the viral envelope (Doms, 2004; Earl et al., 1990; Weiss et al., 1990). The SU subunit, gp120, is responsible for binding to the primary HIV receptor, CD4, expressed on the surface of the target cell (Weiss, 1992), while the TM subunit, gp41, is responsible for fusion. In gp41 mediated fusion, both the amino-terminal fusion peptide and the transmembrane domain play important roles (Freed et al., 1990; Owens et al., 1994).

CD4 is a 59 kDa transmembrane glycoprotein expressed on the surface of T-cells. Spanning the membrane once and consisting of single peptide, under normal conditions this protein plays a role in the initiation of T-cell activation. In terms of HIV infection however, CD4 serves as the receptor for initial contact and binding. Subsequent to binding CD4, gp120 undergoes a series of conformational changes (Clements et al., 1991; Hart et al., 1991; Moore et al., 1990; Sattentau and Moore, 1991; Sattentau et al., 1993) to expose a previously hidden and highly conserved domain within gp41 that binds to a second receptor on the host cell surface (Doms, 2004). During initial
infection, the primary co-receptor bound by gp41 is CCR5. As infection progresses however, mutations in the viral envelope enable the virus to utilise another co-receptor, CXCR4, either instead of, or as well as, CCR5 (Doms, 2004). This is advantageous for the virus because CCR5 is expressed on only approximately 10% of CD4 expressing cells while CXCR4 is expressed on approximately 90% of CD4 positive cells. This adaptive mutation is associated with accelerated HIV disease progression in that it enables infection of a much greater pool of host cells (Doms, 2004). Just as binding to CD4 induces a conformational change in gp120, so too does the binding of gp41 to CCR5 or CXCR4. The conformational change induced in gp41 results in insertion of the fusion peptide into the host cell membrane (Doms, 2004). As in other viral fusion proteins, this fusion peptide is followed by two sequences containing a 4-3 hydrophobic repeat. Based on predictive analysis, this repeat sequence forms a coiled-coil structure (Delwart et al., 1990). Insertion of this domain into the membrane results in the binding of the hydrophobic helical regions, which mechanically draws the viral and host cell membranes into close proximity. The resultant lipid mixing enables stalk formation and subsequent bilayer fusion (see Figure 1.4). Since CXCR4 is expressed on the surface of mature murine oocytes (Holt et al., 2006), it remains a possibility that mammalian sperm-oolemmal interaction may be mediated through similar events to those involved in viral entry into T cells.

1.4.2.8 Disulphide Bonds

Disulphide bonds are a covalent linkage between the thiol groups of two cysteine residues. The formation of such bonds is unique to cysteine in that the other sulphur containing amino acid methionine cannot form disulphide bonds. Within proteins, disulphide bonds perform a series of important roles. Firstly, disulphide bonds fulfil a very well established role in the stabilisation of protein structure. Bonds between two cysteine residues within an amino acid sequence serve to hold the peptide in a folded topology. As a result of such bonds, a protein can maintain a folded topology despite torsional strain favouring the unfolding of the protein to a lower entropy state. Disulphide bonds may also serve as the nucleus of a hydrophobic core for a folded protein, in that hydrophobic residues may condense around the disulphide bond and onto each other. Another important attribute is that the formation of a disulphide bonds lowers the effective concentration of water within the vicinity of the bond. Since water molecules interfere with amide-amide hydrogen bonds within proteins, the presence of a
disulphide bond also serves to maintain these amide bonds, once again promoting stabilisation of protein structure.

Protein disulphides are formed through thiol/disulphide exchange reactions. These reactions are catalysed by enzymes such as protein disulphide isomerase (PDI) and thioredoxin, and usually take place within the oxidising environment of the rough endoplasmic reticulum (RER), as opposed to a cell’s cytoplasm, which typically contains the reducing tripeptide glutathione. Of concern to this research however, are reports that disulphide bonds are prevalent amongst proteins processed within the RER and secreted to the cell surface where they are implicated in mediating virus entry. This is evident in reports that the envelope glycoprotein of HIV for example, contains 9 disulphide bonds (Barbouche et al., 2003; Gallina et al., 2002).

Thiol/disulphide exchange reactions generate disulphide bonds within proteins during protein synthesis in RER. Recently however, it has been shown that thiol/disulphide exchange reactions are also important for driving the conformational changes on the surface of cells during the events of fusogen activation (Barbouche et al., 2003; Fenouillet et al., 2001; Gallina et al., 2002; Ryser et al., 1994). It was postulated that this cell surface thiol/disulphide exchange was mediated by a cell surface isoform of PDI, and strong evidence has recently been obtained to corroborate this (Markovic et al., 2004). Markovic et al., 2004 demonstrated during HIV infection, PDI co-localises with CD4 and CXCR4 in the presence of gp120. The study further demonstrated that the spatial orientation of PDI in relation to gp120, CD4 and CXC4R is critical for PDIs ability to facilitate membrane fusion. In this regard, Markovic and colleagues were able to demonstrate that thiol/disulphide rearrangement in gp120 occurs post-CD4 engagement (Markovic et al., 2004). Binding to CD4 therefore serves as the mechanism for ensuring HIV Env comes into close contact with the cell surface PDI.

In light of the prevalence of disulphide bonds within fusion-mediating membrane proteins and the role of thiol/disulphide exchange, it is feasible that disulphide bonds may play an integral role in regulating the structure of proteins implicated in sperm-egg interaction and that rearrangement of these disulphides may serve to facilitate membrane fusion between the sperm and egg.
1.4.2.9 Cross-Strand Disulphides

It is well established that disulphide bonds play a stabilising role in maintaining protein structure and that rearrangement of these disulphides facilitates membrane merger (Barbouche et al., 2003; Fenouillet et al., 2001; Gallina et al., 2002; Ryser et al., 1994). A variation of the disulphide bond is the cross-strand disulphide (CSD). This class of disulphide is a covalent bond that specifically links adjacent anti-parallel strands of β-sheets and store a great deal of potential energy. In recent times, CSDs have drawn focus from studies investigating exoplasmic fusion events. The rationale is that these bonds provide a possible means by which the previously outlined series of conformational changes essential for successful fusion are made possible.

The first important feature to note concerning CSDs is that the bonds occur in secondary structures already stabilised by non-covalent linkages (Wouters and Curmi, 1995). This raises the obvious question as to why the CSDs are present. An initial clue is that CSDs are relatively rare amongst protein structures and yet over represented in proteins involved in cell entry (Wouters et al., 2004). Sites of hydrogen bonding (H-bonds) occur between adjacent anti-parallel strands within β-sheets. These H-bonded sites alternate with non-H-bonded sites and it is within these non-H-bonded sites that CSDs always occur (Wouters and Curmi, 1995). For H-bonded sites, the backbone carbon atoms on adjacent anti-parallel strands project away from each other and are separated by a distance of 5.5 Å. Within the non-H-bonded sites, these carbons project toward each other and, in the absence of a CSD, are separated by a distance of 4.5 Å. However in the presence of a CSD, created approximately perpendicular to the direction of the anti-parallel sheets, the distance between these carbons is reduced to approximately 4.0 Å. The anti-parallel strands are therefore distorted to accommodate the bond and the result is a localised ‘puckering’ within the β-sheet as the strands tilt toward each other to accommodate the bond (Wouters et al., 2004). In addition to their presence within β-sheets, CSDs are commonly found bridging a β-hairpin. Once again, in this situation a highly strained disulphide linkage forms between two anti-parallel sheets linked to each other by a short turn.

What makes these CSD linkages different to other disulphide bonds is the amount of potential energy they store. This energy is stored in the form of torsional energy within
the highly strained bond itself as well as deformation energy in the overall structure of the sheet (Wouters et al., 2004). A series of studies from the mid 1980s found that one ramification of the high potential energy of CSDs, is a greater susceptibility to cleavage compared to lower energy disulphide bonds (Katz and Kossiakoff, 1986; Weiner et al., 1984; Wells and Powers, 1986; Wetzel, 1987). In terms of viral activity, one can envisage how this increased susceptibility to cleavage would serve the invading virus well. Host cell enzymes present on the cell surface, such as protein disulphide isomerase (PDI) and thioredoxin, are capable of cleaving disulphides. With specific CSDs needing to be cleaved before the essential fusion-promoting conformational changes in the fusogens can become manifest, an increased susceptibility to cleavage of these bonds makes sense. Highlighting this in relation to the previous discussion of HIV infection, there are 9 disulphides with gp120, 3 of which are CSDs. Of these disulphide bonds, at least 2 are known to be cleaved during membrane fusion, with one CSD in CD4 also being cleaved during the fusion process (Barbouche et al., 2003; Gallina et al., 2002; Matthias et al., 2002)

CSDs therefore appear provide a means of maintaining protein structures in a high-energy, pre-activated state with peptides constrained beyond their natural folding. With the reduction of these bonds, comes the release of this strain as the protein spontaneously shifts toward its natural configuration. This shift in protein folding represents the series of conformational change that, in the case of influenza, sees HA insert its fusion peptide into the host membrane and finally draw it back into close proximity to facilitate membrane fusion. This chemistry also accounts for the observation that fusogens can be prematurely activated, such as exposing HA to low pH, rendering them fusion incompetent. This activation is irreversible as the fusogens are unable to spontaneously return to their higher energy folding structure.

Evidence discussed in Section 1.4.1.8 alluded to an important role for disulphides bonds within the molecular machinery that mediates virus-host cell membrane fusion. Expanding upon this, Section 1.4.1.9 has seen evidence discussed pertaining to the existence of a high-energy variant of these bonds, the CSDs, which are found to be over represented in proteins involved in cell entry and may represent a driving force for protein conformational change (Wouters et al., 2004). The application of such important
findings to alternative membrane fusion systems, such as fertilisation, may lead to significant advancement in these lesser-studied fields.

1.5 The Spermatozoon

The principle means by which researchers attempt to identify proteins involved in sperm-egg interaction has been to select candidate proteins of interest then, during *in vitro* sperm-egg interaction studies, test the effectiveness of inhibitors against these proteins to disrupt binding and/or fusion. In the event that these directed inhibition studies allude to a likely role for a particular protein, targeted gene deletion analysis is typically used to confirm the finding. Several examples, where this approach proved fruitful, are outlined below.

1.5.1 The Sperms’ Oolemma Binding Proteins

1.5.1.1 ADAM Proteins

Molecular analysis of the structural domains exhibited by ADAMs has indicated a potentially important role in fertilisation (Primakoff and Myles, 2000). ADAM (an acronym for proteins containing A Disintegrin And Metalloprotease domain) exhibit not only a cysteine-rich domain but a signal sequence, a prodomain, a metalloprotease domain, a disintegrin-like domain and an epidermal growth factor (EGF)-like domain (Iba et al., 2000; Primakoff and Myles, 2000). However, testicular ADAM proteins do undergo some proteolytic processing so that not all these domains are expressed on the surface of spermatozoa. Those that do remain include the disintegrin domain, cysteine-rich domain and EGF-like repeat. Add to this the presence of a transmembrane domain for anchorage, complete with a short cytoplasmic sequence, and ADAM proteins represent strong candidates for proteins with a role in sperm-oocyte recognition.

Within the ADAM family of sperm proteins, primary focus has fallen on fertilin α, fertilin β and cyritestin, also known as ADAMs 1, 2 and 3 respectively. Fertilin β was the first ADAM implicated in sperm-egg recognition. This came about through attempts to identify the antigen to antibody PH-30, which impedes fertilisation in guinea pigs (Primakoff et al., 1987). Identification of fertilin α ensued as fertilin α and fertilin β form a heterodimer (Cho et al., 2000; Primakoff et al., 1987; Waters and White, 1997). Cyritestin was subsequently identified in both mouse and monkey through cloning techniques (Barker et al., 1994; Heinlein, 1996; Wolfsberg et al., 1995). The presence of
these particular ADAMs has since been confirmed in both rodents and primates, while fertilins α and β have also been identified in the rabbit and bull. To date, at least 39 ADAM-family members have been identified across vertebrates as well as invertebrate species such as *Drosophila Melanogaster* and *Caenorhabditis elegans* (Primakoff and Myles, 2000). The fact that approximately half of these are either testis-specific or testes-enriched provides further support for a possible role in sperm-egg fusion.

The presence of a disintegrin-like domain has also generated much interest in these proteins, and rightfully so. Initial studies in the mouse demonstrated that peptide sequences from the disintegrin loops of fertilin β and cyritestin both potently inhibit sperm-egg fusion *in vitro* (McLaughlin et al., 2001). The disintegrin loop peptides from ADAM-1 (fertilin α), ADAM-4 and ADAM-5 however had no effect (Yuan et al., 1997). To further test the importance of these ADAMs, knockout mice with targeted deletions of either fertilin β or cyritestin were generated (Cho et al., 1998; Nishimura et al., 2001; Shamsadin et al., 1999). In both cases males were infertile however both lines manifest defects in sperm function, well upstream of sperm-egg binding and fusion. Thus fertilin β and cyritestin knockout sperm both displayed a reduced capacity to bind to the plasma membrane of egg. Of the few sperm that did bind, a percentage were still able to fuse with and fertilise the egg (Cho et al., 1998; Nishimura et al., 2001). Furthermore, spermatozoa from these males exhibited a reduced capacity to bind to the ZP (Cho et al., 1998; Nishimura et al., 2001; Shamsadin et al., 1999), while fertilin β knockout sperm revealed an additional defect in terms of their capacity to ascend the female reproductive tract (Cho et al., 1998). By contrast, migration of cyritestin knockout sperm from the uterus to the oviduct is normal (Nishimura et al., 2001; Shamsadin et al., 1999). The fact that both mating and sperm motility is normal in fertilin β knockout males suggests a possible role for fertilin β in orchestrating the interaction of spermatozoa with the oviductal epithelia.

These findings also raise questions as to whether these ADAMs are involved in sperm-ZP binding as well as their suspected role in sperm–oocyte fusion. Ideally one would like to be able to attribute such observed defects to the deletion of either fertilin β or cyritestin. Unfortunately, attempts to do so are confounded by findings that fertilin β deletion also results in a lack of expression of fertilin α as well as severely reduced expression of cyritestin. Similarly, cyritestin deletion results in a lack of fertilin α
expression as well as approximately 50% reduction in fertilin β levels (Nishimura et al., 2001). It is also possible that such collateral effects interfere with the expression of other classes of proteins. Sperm deficient in either fertilin β or cyritestin do still manage to fertilise ZP-free eggs, albeit at a reduced rate. These two particular ADAMs are therefore not essential for egg membrane binding and fusion. It is possible that the observed reductions in egg membrane interactions as well as ZP interactions are attributable to alterations in the co-expression of various other proteins. An essential role for ADAM proteins does however remain possible, given that several other ADAMS have now been detected on the surface of sperm (Brachvogel et al., 2002; Choi et al., 2003; Wolfsberg et al., 1995; Zhu et al., 2001).

1.5.1.2 Mouse SLLP1
More recently a new candidate has emerged with a proposed role in sperm-oolemmal binding. Mouse sperm lysozyme-like protein (mSLLP1), a novel c-type lysozyme-like protein, has been located in the equatorial segment of human and mouse spermatozoa following the acrosome reaction and appears to play a role in sperm-egg binding and fertilization (Herrero et al., 2005). Initial experiments with both recombinant mSLLP1 protein treatment of oocytes and anti-mSLLP antibodies revealed a significant inhibition of sperm-oocyte binding. Interestingly, mSLLP was demonstrated to bind in a punctate fashion to the entire perivitelline membrane of zona-free oocytes except the amicrovillar region overlying the meiotic spindle - an area known not to fuse with murine spermatozoa (Herrero et al., 2005). Since mSLLP1, possesses putative N-acetylglucosamine binding residues, the authors speculate that mSLLP1 could bind to hyaluronan and/or related molecules in the perivitelline space and signal to the oolemma that sperm have entered the perivitelline space, possibly triggering the signaling events for sperm phagocytosis. Since mSLLP1 binding sites disappear after fertilization, the authors also postulate a role in the development of the block to polyspermy (Herrero et al., 2005). Production of null mice will confirm the importance of this protein in sperm oocyte recognition.

1.5.2 The Sperms’ Oocyte Fusion Proteins
1.5.2.1 Izumo
Representing a major breakthrough in the field of reproductive biology, Inoue et al., 2005 used the polyclonal antibody OBF13 to successfully identify the sperm protein
The Izumo protein is a type I membrane protein with an extracellular immunoglobulin domain. In addition to a putative glycosylation site, Izumo contains two cysteine residues that might form a disulphide bond (Inoue et al., 2005). In light of the previously outlined importance of thiol/disulphide exchange in virus entry (Section 1.4.1.8), the presence of this possible disulphide site raises the question as to whether this disulphide bond is cleaved during sperm-egg fusion. Cleavage of the bond may indicate that Izumo facilitates fusion through undergoing a series of conformational changes in response to thiol/disulphide exchange, similar to that occurring between viral fusogens and host cell surface proteins.

1.5.2.2 ERp57

A protein that has recently been identified as a mediator of sperm-oocyte fusion is ERp57. During a series of in vitro inhibition studies, Ellerman et al., 2006 investigated the role of PDIs on the sperm surface in mediating sperm-egg interaction. Upon incubating sperm in the presence of PDI inhibitors, the study found that four different PDI inhibitors exhibited a capacity to disrupt sperm-egg interaction by impeding
membrane fusion. Antibody based investigation revealed that the PDI known as ERp57 was expressed on the head of murine sperm, however it was only present on those cells that had undergone the acrosome reaction (Ellerman et al., 2006). The fact that ERp57 can only be detected on acrosome-reacted sperm, as is also the case for Izumo, is consistent with observations that acrosome-intact sperm are not capable of fusing with oocytes.

The establishment of a role for sperm surface ERp57 in sperm-egg membrane fusion is also consistent with the previously outlined viral paradigm, where strong evidence suggests that PDI-mediated thiol/disulphide exchange is essential for membrane fusion not only during HIV infection (Barbouche et al., 2003; Fenouillet et al., 2001; Gallina et al., 2002; Markovic et al., 2004; Ryser et al., 1994), but also during baculovirus (Markovic et al., 1998) and vaccinia virus infection (Locker and Griffiths, 1999).

1.5.2.3 Secretory Protein CRISP-1

As sperm transit the epididymis they are bathed in a dynamic range of endothelial cell-secreted proteins. Incorporation of some of these proteins into the spermatozoon membrane results in extensive remodelling of the sperm surface architecture. A series of epididymal-derived cysteine-rich secretory proteins (CRISP) identified across several mammalian species fulfil exactly this role. CRISP-1, also known as DE, is expressed in the epididymis in an androgen-dependent manner (Cameo and Blaquier, 1976), CRISP-2, also known as Tpx-1, is primarily expressed in the testis (Kasahara et al., 1989) while CRISP-3 exhibits variable tissue distribution (Haendler et al., 1993; Kratzschmar et al., 1996; Mizuki and Kasahara, 1992; Schambony et al., 1998). The overall sequence homologies between CRISP family members ranges from approximately 30 to >80% and all contain a cysteine-rich sequence at the carboxyl terminal.

CRISP-1 was identified as a possible mediator of sperm-egg interaction as a result of a series of observations primarily made in rats. Firstly, pre-incubation of sperm with antibodies against DE inhibits sperm-egg fusion during artificial insemination (Cuasnicu et al., 1984). Similarly, anti-DE inhibits sperm-egg interaction when present in media during IVF (Cuasnicu, 1990). When purified from rat epididymal extracts, CRISP-1 binds to the membrane of both rat and mouse oocytes. Furthermore, this binding results in reduced levels of fertilisation during IVF (Cohen et al., 2000;
Rochwerger et al., 1992). Reduced fertility is also observed in male rats immunised with CRISP-1. Immunisation results in the presence of anti-CRISP-1 antibodies in epididymal and vas deferens fluids. Sperm from these males are able to bind to eggs but lack the capacity for fusion (Ellerman et al., 1998). This finding is in accordance with results published by both Rochwerger et al., 1992 and Ellerman et al., 1998. These studies found that whilst purified CRISP-1 inhibited rat sperm-egg fusion, it had no apparent effect on sperm-egg binding. Although evidence exists supporting a role for sperm-associated CRISP proteins in sperm-egg fusion, the mechanism by which they participate, and the egg binding partners that are involved remain to be elucidated.

1.6 The Oocyte

Throughout investigations into sperm-egg interaction, multiple classes of protein found on the egg surface have been proposed as potential candidates for either sperm binding or fusion partners. Past and present proteins falling into this category include the integrins, in particular α6β1, glycophasphatidylinositol-anchored proteins and tetraspanins, each of which are discussed in brief below;

1.6.1 The Oocytes Sperm Binding Proteins

1.6.1.1 Glycophosphatidylinositol-anchored Proteins

As their name suggests, GPI-anchored proteins are attached to membranes via a GPI lipid anchor instead of a transmembrane domain. This class of extracellular proteins was first implicated in sperm-egg interaction when Coonrod et al., 1999a, utilised phosphatidylinositol-specific phospholipase C (PI-PLC) to specifically cleave GPI-anchored proteins off the surface of sperm and eggs. This study found that treatment of sperm alone had no significant effect on sperm-egg binding or fusion. Treatment of ZP-free oocytes with PI-PLC however, resulted in a dose dependent reduction in sperm-egg binding, which translated into a subsequent reduction in the rates of sperm-egg fusion (Coonrod et al., 1999a). This finding comprised the first evidence supporting a role for GPI-anchored proteins as mediators of sperm-oocyte binding in hamsters. In addition to hamsters, treatment of oocytes with PI-PLC has since been utilised to demonstrate the important of oolemmal GPI-anchored proteins in murine fertilisation as well as the human sperm/hamster oocyte binding and fusion assay (Coonrod et al., 1999b).
To confirm the findings of Coonrod et al., 1999ab, Alfieri et al., 2003 conducted an experiment to ascertain whether GPI-anchored proteins were required for fertilisation in vivo. Through use of the Cre/loxP system, oocyte-specific knockout of a portion of the Pig-A gene, an enzyme involved in GPI anchor biosynthesis, was achieved. In these in vivo studies, knockout females were found to be infertile. In wild type females, examination of oviducts 24 hours post coitum revealed 84% of eggs had been fertilised and progressed to two-cell stage embryos. In knockout females however, only 1% of eggs had been fertilised and progressed to two-cell stage embryos. The latter observation was made despite the presence of multiple spermatozoa in the perivitelline space of many of the eggs (Alfieri et al., 2003). Sperm had therefore managed to penetrate the cumulus oophorus as well as the ZP only to fail in their final attempts to fuse with the oolemma. The fact that multiple spermatozoa had penetrated the ZP suggests that neither sperm-oocyte fusion nor cortical granule extrusion had occurred under these circumstances (Alfieri et al., 2003).

It is clear that GPI-anchored proteins play an important role in fertilisation in both of the utilised experimental models. As such, attention has shifted toward identification of the GPI-anchored proteins liberated by PI-PLC. Coonrod et al., 1999a, 1999b demonstrated that PI-PLC induced infertility was associated with the release of a 70 kDa (pI 5) and 35 to 45 kDa (pI 5.5) protein cluster from the egg surface. The 70 kDa protein is believed to be CD55 (Alfieri et al., 2003). However previous studies on CD55 eliminate it as the egg surface GPI-anchored protein of interest, as CD55 knockout mice exhibit normal fertility (Sun et al., 1999). Advances in this area are thus now dependent on identification of the 35 to 45 kDa protein cluster, or the discovery of previously undetected GPI-anchored proteins on the egg surface.

1.6.2 The Oocytes Sperm Fusion Proteins

1.6.2.1 The Integrins

Initially, integrins were identified as possible mediators of sperm-egg binding and fusion upon the detection of ADAM proteins on the surface of sperm. Representing a ligand-like domain for integrins, the disintegrin domain possessed by ADAMs led to the hypothesis that sperm ADAMs were ligands for integrins expressed on the egg surface. The role of integrins on the egg surface thus received much attention as studies aimed to
dissect two main issues; (i) whether integrins participate in gamete interactions, and (ii) if so, whether sperm ADAMs were indeed the ligands for these integrins.

Integrins are a family of cell adhesion molecules that, in various systems, mediate cell-cell as well as cell-extracellular matrix interactions (Evans, 2002). They exist as heterodimers for which at least 18 α and 8 β subunits have been identified. Different subunit combinations give rise to a total of 24 different integrins. Based on sequence homologies between α subunits as well as general ligand-binding characteristics, these 24 different integrins can be divided into 6 subfamilies. Of these 24 family members, at least 6 different integrins have been reported to interact with at least 8 different ADAMs (Bigler, Takahashi et al. 2000; Bridges, Tani et al. 2002; Cal, Freije et al. 2000; Coonrod, Naaby-Hansen et al. 1999b; Eto, Huet et al. 2002; Nath, Slocombe et al. 1999; Nath, Slocombe et al. 2000; Takahashi, Bigler et al. 2001; Zhang, Kamata et al. 1998; Zhou, Graham et al. 2001).

Initial results obtained from a series of in vitro experiments pointed to a possible role for egg integrins in gamete membrane fusion. The integrin of primary interest, α6β1, was implicated as a receptor for both fertilin β and cyritestin (Almeida et al., 1995; Bigler et al., 2000; Coonrod et al., 1999b; Takahashi et al., 2001). However, studies utilising knockout animals to test these findings have so far been unable to support a role for egg integrins in gamete membrane fusion. Eggs from multiple strains of null mice lacking a variety of subunit knockout combinations exhibit normal fertility (He et al., 2003). Nonetheless, it remains possible that integrins contribute in one way or another to the gamete interaction but may not be essential in the mouse or human (He et al., 2003; Sengoku et al., 2004).

1.6.2.2 The Tetraspanins
Although the existence of tetraspanins was first realized in 1990, the broad functional importance of this protein family has only recently been appreciated. Widely expressed in eukaryotic organisms, at least 32 members have been identified in the mammalian tetraspanin family, at least 35 in Drosophila Melanogaster (Fradkin et al., 2002; Todres et al., 2000), 20 in the genome of Caenorhabditis elegans (Todres et al., 2000) and in 4 fungal species (Gourgues et al., 2002). Tetraspanins are a family of related proteins that, as their name suggests, span the plasma membranes of eukaryotic cells four times. With
both the N and C termini residing in the intracellular environment, the structure results
in two extracellular loops, EC1 and EC2 (Boucheix et al., 1991). While extracellular
loop EC1 is small, EC2 is large and contains both a constant and a variable domain
(Seigneuret et al., 2001). Members of this family typically contain 4 to 6 conserved
extracellular cysteine residues linked into 2 to 3 disulphide bonds. Of these cysteine
residues, 4 are absolutely conserved in all tetraspanins analysed to date. Included in
these conserved cysteines is a cysteine-cysteine-glycine motif that occurs approximately
28 to 47 amino acid residues after the third transmembrane domain (variable region of
EC2), and another cysteine approximately 11 amino acids before the fourth
transmembrane domain (border of the EC2 variable domain). In addition to these
conserved cysteines, tetraspanins also typically contain conserved polar amino acids
within transmembrane domains 1, 3 and 4 (Hemler, 2003).

A crucial aspect of tetraspanin biology is their proposed role in membrane organisation
(Hemler, 2003). As investigations into tetraspanin function continue, accumulating
evidence identifies these proteins as mediators of protein interactions at the primary,
secondary and tertiary level. Primary interactions are direct and detergent stable.
Integrins, immunoglobulin superfamily members and membrane-anchored growth
factors are all classes of protein with which tetraspanins form primary interactions.
Human hepatitis C virus (HCV) is a member of positive strand RNA viruses that form
the genus Hepacivirus of the Flaviviridae family of viruses. The HCV envelope protein,
E2, binds to the major extracellular loop of human CD81, a tetraspanin expressed on
various cell types including hepatocytes and B lymphocytes (Seeger, 2005).

Tetraspanins also form secondary interactions with other tetraspanins. CD81 is known
to interact with CD9 and CD151. Many of the proteins that interact with tetraspanins
such as CD9P-1 and EWI-2 (Charrin et al., 2003; Charrin et al., 2001), also interact with
each other. The result is a large network of proteins linked by either direct or indirect
associations with tetraspanins (Hemler, 2005). The sum of these interactions is referred
to as the tetraspanin web (Boucheix and Rubinstein, 2001; Hemler, 2005). Tetraspanin
webs give rise to tetraspanin-enriched microdomains. The lipid composition within
these microdomains is unique and is distinguishable from classic membrane rafts by
several features (Hemler, 2003). These specific microdomains provide a means by
which tetraspanins regulate critical cellular functions that require cooperation of
multiple proteins (Stein et al., 2004). Emerging data suggests the tetraspanins CD9 and CD81, as well as these lipid microdomains may be implicated in sperm-egg interaction (Hemler, 2005).

CD9 was first implicated in fertilisation when Chen et al., 1999 observed that the antibody JF9, which was directed against CD9, inhibited sperm-egg binding and fusion in a dose dependent manner in vitro (Chen et al., 1999). These results were promising and an unequivocal role was later established when three different laboratories generated mice deficient in CD9 (Kaji et al., 2000; Le Naour et al., 2000; Miyado et al., 2000). Despite normal health and vitality, females in all cases exhibited severely reduced fertility attributable to a lack of fusibility of their CD9-deficient oocytes. Whilst no significant differences were observed for sperm binding capacity between control and CD9-deficient eggs, CD9-deficient eggs rarely fused with spermatozoa during either in vivo or in vitro fertilisation studies. Despite their lack of fusibility, the use of intracytoplasmic sperm injection (ICSI) to bypass this defect in fusion resulted in viable fertilised eggs that apparently developed to term (Miyado et al., 2000).

These observations raise obvious fundamental questions regarding the role of CD9 in gamete fusion. Does CD9 function directly as a binding partner for proteins on the sperm surface? Does this molecule mediate the formation and stabilization of a receptor complex within the egg membrane? Recent studies on tetraspanins in general, have identified the large extracellular loop, EC2, as a region of functional significance (Hemler, 2003). As such, investigations into the role of CD9 during sperm-egg interaction have focused on this domain. A cis function for CD9 was revealed when a bacterially expressed mouse CD9 EC2 construct was pre-incubated with either sperm or eggs during in vitro fertilisation studies. Pre-incubation of sperm with EC2 had no effect on sperm binding or fusion, whereas pre-incubation of eggs with EC2 resulted in an inhibition of fusion (Zhu et al., 2002). This indicates that the EC2 domain of CD9 plays a significant role in the interaction of CD9 with other egg membrane proteins, and that the presence of the EC2 construct impedes sperm-egg fusion by disrupting these interactions.

It does however remain possible that the EC2 domain of egg CD9 interacts directly, in trans, with a ligand on the sperm surface. Studies by Waterhouse et al., 2002 revealed
that the pregnancy specific glycoprotein 17 (PSG17) binds directly to macrophages in a CD9-dependent manner. It was established that residues within the EC2 loop of CD9 played a key role in this interaction (Waterhouse et al., 2002). Interestingly, PSG17 results in substantial competitive inhibition of sperm-egg fusion when eggs are pre-incubated in its presence (Ellerman et al., 2003). Although PSG17 is not expressed on the surface of mammalian sperm, it is an example of a system whereby a sperm antigen could possibly bind directly to CD9. Despite the suggestion of direct interaction of PSG17 with macrophage CD9, it does remain possible that, like the bacterially expressed mouse EC2 construct, PSG17 binding to EC2 simply impedes CD9 interactions with other egg membrane proteins thereby disrupting receptor complex formation.

Through messenger RNA (mRNA) microinjection, it has been possible to identify regions of the EC2 loop essential to CD9 function during sperm-oocyte fusion. Infertility in CD9 deficient eggs can be rescued via injection of polyadenylated mouse CD9 mRNA at the egg germinal vesicle phase. Expression of the exogenous transcript restores fusibility in the otherwise CD9 deficient eggs. Fertility can also be restored, up to approximately 90% of that for wild type eggs, via injection human CD9. Similarly, injection with mouse CD81 restores fertility up to approximately 50%. More recently it has been demonstrated that fertility in CD81 null mice is also compromised due to a deficiency in sperm-oocyte fusion (Rubinstein et al., 2006a). Analysis of hepatitis virus binding to CD81 had previously demonstrated that a single amino acid (F186) substitution in the EC2 domain of CD81 dramatically affects virus binding (Higginbottom et al., 2000). To determine whether the equivalent region of CD9 was also important, two mutant CD9 mRNA constructs were generated and tested for their capacity to restore fusion. The mutant CD9 proteins contained a substitution of either amino acid residue 174 from phenylalanine to alanine (F→A) or substitution of amino acid residues 173-175 from serine, phenylalanine and glutamine to alanine, alanine, alanine (SFQ→AAA) (Zhu et al., 2002). The ability of the CD9 constructs to restore fertility in CD9 deficient eggs was either greatly reduced in the case of the F→A (174) mutant or completely abolished in the SFQ→AAA (173-75) mutant (Zhu et al., 2002). Substantial data exists supporting the correct folding of the SFQ mutant (Zhu et al., 2002) suggested that these results were not attributable to protein misfolding. This same
SFQ mutation in a GST construct of the CD9 EC2 also effectively abolished the binding of the EC2 loop to PSG17-coated beads (Ellerman et al., 2003). These findings clearly identify the SFQ sequence of EC2 as a critical CD9 domain during sperm-egg interaction.

With an unequivocal role for CD9 in sperm-egg interaction established, studies to gain an understanding of the protein interactions mediated by this tetraspanin are ongoing. Generation of double knockout mice indicated that CD9−/−/CD81−/− KO mice are completely infertile and that CD9 and CD81 play complementary roles in sperm-oocyte fusion (Rubinstein et al., 2006a). These authors also demonstrated that one of the two common partners of the CD9 and CD81 tetraspanins, CD9P-1, a member of a novel subfamily of proteins with Ig domains (Charrin et al., 2001) is present on freshly ovulated oocytes and may play a crucial role in sperm-oocyte fusion (Rubinstein et al., 2006a).

1.7 Xenobiotics and Fertility
Since the 18th and 19th centuries, the industrial revolution has resulted in a significant increase in the release of novel contaminants into the atmosphere and waterways (Miller et al., 2004). Refined chemicals are now a part of our everyday lives, be it in the form of prescription medications, industrial byproducts or environmental pollutants, and many can have deleterious effects on the mammalian body including the reproductive system (Miller et al., 2004) There are a variety of factors that govern the severity of effects upon exposure to a particular chemical or agent, these include variables such as the level of exposure and the manner in which the agent was encountered, such as inhalation, ingestion or absorption through skin, whether exposure occurred during a vulnerable developmental period such as embryonic development or pregnancy, and of particular concern is whether the agent has the capacity to mimic endogenous compounds, such as hormones, or alter the action of hormones or their levels within the body (Miller et al., 2004). The potential to do harm possessed by these ‘xenobiotic’ agents has not gone unnoticed and studies have already confirmed their ability to enter the body and reach organs such as the ovaries where they have the potential to induce damaging effects (Hunt et al., 2003).
By definition a xenobiotic is a chemical compound or substance that is foreign to an organism or biological system. Xenobiotics are therefore are not present as natural products or contain structural components that cannot be synthesized biochemically (Rieger et al., 2002). This is of major concern in that the relatively recent of emergence of a plethora of these highly refined compounds means that cells are largely ill equipped to deal with them. Catabolic enzymes and pathways do not exist within cells to enable use of these compounds as carbon sources. Thus exposure to significant levels of xenobiotic agent, whether by means of occupational hazards or bioaccumulation, is of primary concern in relation overall health and is particularly relevant to reproductive fitness.

A primary example is the investigation of the oestrogenic compound bisphenol A (BPA). A study performed by Hunt et al., 2003 reported two major findings with respect to BPA. The first point of concern was that when BPA is present in an animal’s housing or environment, this xenobiotic has the capacity to not only enter the body but also reach the ovaries. BPA is a compound commonly used in the production of epoxy resins and plastics. When female mice were inadvertently exposed to BPA through damaged plastics in housing and water containers, it was found that BPA managed to reach the ovaries of the exposed mice. The second point of concern was that upon reaching the ovaries, BPA induced detectable effects on meiosis. Thorough investigation of this phenomenon revealed that short term and low dose exposure of female mice to BPA increased the incidence of chromosomal abnormalities during meiosis of the oocytes (Hunt et al., 2003). The study therefore demonstrates that xenobiotic compounds present in the housing environment of animals have the capacity to reach reproductive tissues and induce adverse effects. Chromosomal abnormalities, such as those induced by BPA, could prove disastrous for future generations, which may suffer from an increased incidence of genetic disease or even embryonic lethality. Subsequent studies have since reiterated the toxic effects of BPA on the female reproductive system (Kato et al., 2003; Markey et al., 2002; Mlynarcikova et al., 2005).

As well as mimicking biological compounds, many xenobiotic agents possess innate chemical activities such as a capacity for alkylation and redox cycling. Alkylation involves the addition of alkyl groups to chemical compounds through replacement of hydrogen, or where arene rings are concerned the process is often referred to as
arylation. In relation to membrane fusion, chemicals with thiol alkylating properties may be anticipated to block membrane fusion by interfering with thiol/disulphide exchange. Redox cycling on the other hand is an enzyme-dependent process that can occur when a compound is enzymatic reduced to contain one electron more than the parent compound. The unstable reduced compound then donates its additional electron to molecular oxygen ($O_2$) to generate the ROS known as superoxide anion ($O_2^-$). In addition to the generation of $O_2^-$, the process also regenerates the original parent compound. The net result is a cyclic process based around enzyme-catalysed oxidation/reduction (redox) events that lead to the continuous generation of ROS. Agents with a capacity for ROS generation may be anticipated to disrupt membrane fusion by inducing the peroxidation of membrane lipids. Where such peroxidative damage involves a sustained attack on the unsaturated fatty acids of the plasma membrane, the result is a pronounced decrease in membrane fluidity, which has negative ramifications with regard to membrane fusogenicity.

In light of the evidence that xenobiotics are clearly able to induce negative effects on oocytes, it was plausible that exposure of oocytes to a range xenobiotics may lead to the identification of a compound(s) with the capacity to modify egg membrane proteins in such a manner as to inhibit or block sperm-egg binding and/or fusion. If such a compound were found, understanding the biochemistry of the xenobiotic and the modifications it induced might provide a valuable insight into the fundamental mechanisms mediating sperm-egg interaction.

### 1.8 Aims and Hypothesis

The studies outlined in this thesis represent a planned body of research directed toward elucidating the mechanisms that mediate sperm-egg binding and fusion in the mouse oocyte. Prior to commencing this research, it was hypothesised that the oocyte mediated sperm-egg binding and fusion by means of a receptor complex expressed on its cell surface, and that this complex regulated binding and fusion as separate protein-protein interaction events. In addition, similar to virus–cell fusion, it was hypothesised that disulphides along with thiol/disulphide exchange played an important role in this interaction.
To investigate these series hypotheses, research presented herein was conducted to address three major aims:

(i) To characterise the factors that affect the oocyte’s ability to mediate sperm-egg binding and fusion

(ii) To implement a combination of proteomic and bioinformatics techniques to identify candidate proteins expressed by the oocyte that might be responsible for mediating sperm-egg binding and fusion, with emphasis placed on the identification of oolemmal GPI-anchored proteins

(iii) To perform characterisation studies of the identified candidate proteins with the aim of confirming a positive role for these proteins in mediating sperm-egg binding or sperm-egg fusion.

Within this broad series of aims, the research was further sub-divided and carried out to address specific questions. These questions were raised as a result of shortfalls in the existing literature and are outlined in greater detail within each of the relevant chapter aims sections.
CHAPTER 2:

Materials and Methods
CHAPTER 2 – MATERIALS & METHODS

2.1 Reagents and Solutions
Reagents, unless otherwise stated, were obtained from Sigma Chemical Company (Sigma-Aldrich, Castle Hill, NSW 1765, Australia), Crown Scientific (Minto, NSW, 2566, Australia) or Invitrogen Australia Pty Limited (Mount Waverly, VIC, 3149, Australia). Detailed outlines of buffers, solutions, media and antibodies may be found in the appropriate appendices.

Appendix A: Whole Ovary Proteomic Identifications
Appendix B: Isolated Oocyte Proteomic Identifications
Appendix C: Buffers, Solutions and Media
Appendix D: Antibodies, Enzymes and Cellular Probes
Appendix E: Abstracts, Presentations and Publications

2.2 Animals
All animal experiments presented in this thesis were subject to approval by the University of Newcastle Animal Care and Ethics Committee under the approval number 816. Female mice (6-8 weeks old) of either Swiss (CD1) or C57 Black (Wild Type or CD151 Null) lineage were obtained from the University of Newcastle Central Animal House. Male mice (>8 weeks old) of either Swiss (CD1) or C57 Black (Wild Type) Lineage were also obtained from the Central Animal House. All mice were housed in the Medical Sciences Building animal holding facility. Animals were maintained at a constant temperature of 22°C on a 12 h light cycle with food and water available ad libitum. Animals were euthanised by carbon dioxide asphyxiation.

CD151 Null females utilised during these investigations were generated by a collaborative research team by means of Cre-mediated recombination. Mice were subjected to initial characterisation studies as outlined by Wright et al., 2004.
2.3 Superovulation
For collection of germinal vesicle (GV) phase oocytes, follicle development in Swiss female mice was stimulated by intra-peritoneal injection of 10 IU of pregnant mare serum gonadotrophin (PMSG). Ovaries were excised 48 h later. For collection of ovulated oocytes, follicle development in Swiss or C57 Black female mice was stimulated by intra-peritoneal injection of 10 IU of PMSG. Ovulation was induced 48 hours later by a second intra-peritoneal injection of human chorionic gonadotrophin (hCG). Ovaries plus oviducts were excised 12 h post hCG injection.

2.4 Oocyte Isolation, Preparation and Maturation
2.4.1 Isolation of Germinal Vesicle Phase oocytes
Approximately 48 h post PMSG injection, ovaries from Swiss mice were excised and washed through $3 \times 100 \mu L$ α-minimum essential media (α-MEM) droplets on a Petri dish. α-MEM was supplemented with 1 μM Milrinone, to maintain oocytes in meiotic arrest, as well as 5% fetal calf serum (FCS) or 1 mg/mL polyvinyl alcohol (PVA). The ovaries were then repeatedly aspirated using a 30 gauge needle resulting in the release of cumulus-enclosed oocytes (CEOs). Oocytes were then denuded of cumulus cells by mechanical sheering using a pulled Pasteur pipette with a bore diameter of approximately 80 μm in diameter.

2.4.2 In Vitro Oocyte Maturation
To maintain oocytes at the immature GV phase, GV oocytes were suspended at all times in α-MEM supplemented with 1 μM Milrinone. To obtain in vitro matured metaphase II (MII) oocytes, denuded ZP-intact oocytes were washed through $3 \times 100 \mu L$ droplets of α-MEM devoid of Milrinone. Oocytes were then incubated overnight (16 hours) in Milrinone deficient media to allow meiosis to resume then once again arrest, this time at MII phase of meiosis II.

2.4.3 Isolation of Metaphase II Oocytes
Twelve hours post-hCG injection (see superovulation regime above), ovaries and oviducts were excised and placed into α-MEM supplemented with 5% FCS, 3 mg/mL bovine serum albumin (BSA) or 1 mg/ml polyvinyl alcohol (PVA). Using fine tip forceps, oviductal ampullae were ruptured resulting in the extrusion of bundles of
CEOs. Bundles of CEOs were collected and transferred to 1 mg/mL Hyaluronidase solution in α-MEM for 5 min to remove cumulus cells. The denuded oocytes were washed through 3 × 100 µL media droplets to remove unwanted granulosa cells ready for further use or ZP removal.

2.4.4 Zona Pellucida Removal

Tyrodes solution was acidified by adjusting the pH to 2.2 and an 80 µL aliquot placed onto a Petri dish. Groups of 10 – 15 oocytes were injected into the droplet and mixed briefly. Oocytes were carefully observed and immediately removed from the droplet once ZP dissolution was complete (15 – 25 sec total). Oocytes were quickly washed through 3 × 100 µL α-MEM droplets. Once all ZP had been removed, oocytes were incubated for 1 – 3 h at 37°C, 5% CO₂. This period was essential to allow oocytes to recover following harsh acid treatment.

2.5 Sperm-Egg Binding and Fusion Bioassay

2.5.1 Sperm Isolation and Capacitation

Male mice were euthanised and their cauda epididymides removed and placed under medium-saturated mineral oil next to a 500 µL α-MEM droplet. Epididymal tubules were snipped and using fine-tip forceps, strands of concentrated sperm were then transferred into the medium droplet. The sperm concentrates were incubated for 10 min to allow sperm to swim out before the medium droplet was extracted and transferred to a 1.5 mL Eppendorf tube. To allow sperm to undergo capacitation, the tube was incubated at 37°C, in an atmosphere of 5% CO₂/95% air, for 60 min, with resuspension every 15 min.

2.5.2 Gamete Co-Incubation

To enable the detection of successful sperm-egg fusion events, oocytes were incubated for 15 min in 10 µg/mL 4’,6-diamidino-2-phenylindole (DAPI) in α-MEM to preload the eggs with the nucleic acid stain. The DAPI loaded oocytes were washed twice then transferred to 100 µL aliquots of capacitated mouse sperm at 3.0 × 10⁵ sperm/mL, with a maximum of 15 oocytes present in each droplet. Oocytes were gently mixed through the sperm droplet and the gametes co-incubated at 37°C for 60 min, with gentle mixing.
every 15 min. Oocytes were then collected and washed through 3 × 100 µL media droplets to remove any unbound or loosely attached sperm.

2.5.3 **Fluorescence Microscopy**

Using a super PAP pen (Daido Sangyo Co. Ltd. Tokyo, Japan), hydrophobic barriers were drawn onto glass slides (Menzel-Gläser Co. Saarbrückener, Str. D-38116 Braunschweig). Due to the large size of oocytes, grease spots (1:1 paraffin wax: Vaseline) were placed near each corner of the slide. Oocytes were then collected in 5 µL volumes of media and expelled onto the slides within the liquid barrier. Coverslips (25 × 40 mm; Menzel-Gläser Co. Saarbrückener, Str. D-38116 Braunschweig) were applied and, while looking down the dissection microscope, very carefully compressed until contact with the surface of the oocytes was made. Slides were stored in a humidified lightproof container until viewed at 200 × magnification using an Axioplan 100 epifluorescent microscope (Carl Zeiss Pty. Ltd. North Ryde, NSW 2113 Australia).

To assay sperm-egg binding, oocytes were visualised under phase contrast and the number of spermatozoa bound to each egg was counted. To assay successful fusion events, oocytes were visualised using epifluorescence illumination (excitation 365nm, emission >420nm) and the number of fluorescent sperm nuclei was counted for each egg (Wortzman et al., 2006). Fluorescent sperm nuclei constitute successful fusion events, as DAPI present in the eggs cytoplasm is only able to label the sperm genetic material once membrane fusion has occurred. Sperm-egg binding and fusion rates were reported as means ± the standard error of the mean (sem). Data was analysed using the Student T-test (unpaired). The reported ‘P’ value is the probability that a difference between experimental groups happened by chance. Observed experimental differences were deemed significant when P ≤ 0.05.

2.6 **Oocyte Surface Labelling and Modification**

2.6.1 **CD55 Detection**

To demonstrate effective cleavage of GPI-anchored proteins, the release of the known GPI-anchored protein CD55 (decay accelerating factor, DAF) was monitored. For the purpose of this assay, ZP-free oocytes (see Section 2.4) were incubated in the presence of PI-PLC, glycosphatidylinositol-phospholipase D (GPI-PLD) or angiotensin
converting enzyme (ACE) at 5 U/mL in α-MEM for 60 min. Oocytes were then washed through 3 × 100 μL media droplets before being incubated in the presence of 1:50 goat anti-mouse CD55 for 30 min at 37°C. Oocytes were again washed and then incubated in 1:50 donkey anti-goat IgG-biotin for 30 min at 37°C. Oocytes were again washed and transferred to droplets containing 1:100 streptavidin-FITC for 30 min at 37°C. Following a final series of washes, oocytes were then mounted on slides with raised coverslips and viewed at 400 × magnification using an LSM510 laser scanning confocal microscope (Carl Zeiss Pty. Ltd. North Ryde, NSW 2113 Australia). Excitation and emission wavelengths were 488nm and 500-530nm, respectively.

2.6.2 PI-PLC Release of GPI-Anchored Proteins

For the purpose of repeating the observations of Coonrod et al., 1999b, ZP-free oocytes were incubated in the presence of PI-PLC at final concentrations of 0, 0.05, 0.5, 1.0 and 5.0 U/mL for 60 min at 37°C. A control in which oocytes were incubated in 5.0 U/mL heat inactivated (95°C for 5 min) PI-PLC was also included. Oocytes were then washed through 3 × 100 μL media droplets in readiness for the sperm-egg binding and fusion assay.

For the purpose of pooling oolemmal GPI-anchored proteins for subsequent proteomic analysis, oocytes were either mock-treated (medium only) or treated with 1 U/mL PI-PLC in α-MEM/PVA for 60 min at 37°C, with mixing every 15 min. Oocytes were then removed and the supernatant droplets collected. Approximately 35 – 40 oocytes were incubated per 100 μL droplet.

2.6.3 Surface Protein Biotinylation and Detection

During the use of biotin to label oocyte cell surface proteins, oocytes were isolated into the BWW/PVA medium, as opposed to α-MEM, which contains a primary amine and is therefore not suitable for use during the labelling process. Oocytes were incubated in the presence of 1 mg/mL Sulfo-NHS-LC-Biotin (Quantum Scientific Pty Ltd. Paddington QLD, 4064 Australia) for 30 min at 37°C. Following this incubation, oocytes were washed through 3 × 100 μL media droplets to remove any unbound biotin.
To confirm successful labelling of the oocyte surface proteins, 5 – 10 oocytes were incubated in the presence of 1:2500 streptavidin-FITC for 30 min at 37°C. Oocytes were transferred through 3 × 100 µL washes before being mounted on slides with raised coverslips and viewed by epifluorescence microscopy. 5 – 10 oocytes that had not been exposed to biotin were also analysed as a control against non-specific streptavidin-FITC labelling.

For Western blot detection of the biotin-labelled surface proteins, the remainder of the oocytes were then extracted into 0.5% Triton X-100.

2.7 Xenobiotic Modification of Oocytes

2.7.1 Xenobiotic Treatments

To investigate the impact of exposure of oocytes to various xenobiotic agents on sperm-egg interaction, ZP-free oocytes were incubated in the presence of a range of xenobiotic agents. For the purpose of these treatments, it was necessary to use BWW/PVA medium as opposed to α-MEM/PVA or α-MEM/FCS. Depending on the solubility properties of the individual xenobiotics, the agents screened were dissolved either directly into BWW/PVA or dimethyl sulfoxide (DMSO). Agents dissolved in DMSO were prepared as 1000 × stocks then diluted 1:1000 into BWW/PVA to give working solutions of the desired concentration at safe DMSO final concentrations. A list of the xenobiotic agents and the range of concentrations at which they were investigated is given in Table 2.1.

<table>
<thead>
<tr>
<th>Xenobiotic</th>
<th>Properties</th>
<th>Concentrations Utilised (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-Benzoquinone</td>
<td>redox-active/alkylating</td>
<td>0.1 - 10.0</td>
</tr>
<tr>
<td>Hydroquinone</td>
<td>relatively inert</td>
<td>10</td>
</tr>
<tr>
<td>Duroquinone</td>
<td>redox active/non-alkylating</td>
<td>10</td>
</tr>
<tr>
<td>Menadione</td>
<td>Redox-active/alkylating</td>
<td>10</td>
</tr>
<tr>
<td>Bis maleimide</td>
<td>non-redox active/alkylating</td>
<td>0.1 – 10</td>
</tr>
<tr>
<td>Iodoacetamide</td>
<td>alkylation agent</td>
<td>10</td>
</tr>
<tr>
<td>pCMBS</td>
<td>alkylation agent</td>
<td>10</td>
</tr>
<tr>
<td>TCEP</td>
<td>reducing agent</td>
<td>1000</td>
</tr>
</tbody>
</table>

During sperm-egg binding and fusion assays, oocytes were incubated in the presence of the xenobiotic agents for 15 min at 37°C. For the purpose of benzoquinone, bis
maleimide and iodacetamide time dependency studies, oocytes were incubated in the presence of the agents for 2 or 20 min at 37°C. Following the appropriate incubation periods, the washed oocytes were washed through 3 × 100 µL BWW/PVA droplets in readiness for the sperm-egg binding and fusion assay (see Section 2.5.2), or incubated in the presence of AlexaFluor 488 C₅ maleimide for the detection of surface thiols (see Section 2.7.2).

2.7.2 AlexaFluor 488 Detection of Surface Thiols
Following the successful modification of sperm-egg binding and fusion rates with various xenobiotic agents, oocytes were visualised via confocal microscopy to investigate the status of any surface thiol residues that might have been present. Following xenobiotic treatment, oocytes were washed then incubated in BWW/PVA supplemented with 10 µM AlexaFluor 488 C₅ maleimide for 15 min at 37°C. The oocytes were then transferred through 3 × 100 µL washes before being mounted on slides with raised coverslips. Labelling intensity was viewed quantitatively at 250 × magnification using a LSM510 laser-scanning microscope (Carl Zeiss Pty) equipped with Argon laser: excitation wavelength of 488 and emission spectra of 500-530nm (FITC).

2.8 CD151 Detection and Expression
2.8.1 Ovarian Expression of CD151
Ovarian gene expression analysis was performed by Janet Holt utilising CodeLink™ Mouse Whole Genome Array slides (GE Healthcare Bio-Sciences Pty Ltd, Castle Hill NSW 2154 Australia) as outlined in Holt et al., 2006. Briefly, cDNA was generated from approximately 2 µg of total RNA from neonatal or adult mouse ovaries. An in vitro transcription was performed, incorporating biotinylated UTP in the resulting amplified RNA (aRNA). 10 µg of aRNA was hybridized with the Mouse Whole Genome Slide and detection of hybridisation carried out by probing with streptavidin-Cy5. Slides were scanned in an Axon scanner and data analyzed with proprietary CodeLink™ Expression Analysis Software (GE Healthcare Bio-Sciences Pty Ltd) (Holt et al., 2006).
2.8.2 Proteomic Confirmation of CD151 Deletion by Immunoblotting

Superovulated ovaries were excised from two wild type and two CD151 null females and washed in α-MEM. Ovaries were mechanically homogenised in 2D rehydration buffer (see Appendix C), supplemented with Complete Protease Inhibitor Cocktail tablets (Roche Diagnostics Australia Pty Ltd. Castle Hill NSW, 2154 Australia), by 3 × 20 sec burst with a Polytron PT2100 homogeniser (Kinematica AG, Pty Ltd. Luzernerstrasse, Lucerne, CH-6014 Switzerland). The samples were then incubated at 4°C for 60 min on a rotary mixer before being centrifuged at 10,000 × g for 15 min. Supernatants were extracted and proteins levels determined using the 2D Quant Kit (Amersham Biosciences, Piscataway, NJ, 08855-1327 USA) according to manufacturer’s instructions. 2 µg of each protein extract was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrophroblotted onto nitrocellulose membranes by Western transfer at 300 mA for 60 min. Membranes were blocked in 3% BSA (Research Organics, Cleveland, Ohio, 44125) in Tris-buffered saline (TBS) for 60 min. Membranes were then probed with rabbit anti-human/mouse CD151 (1:2500 dilution; obtained from Prof. Leonie Ashman of the Molecular Oncology Group, School of Biomedical Sciences, University of Newcastle, NSW, 2308 Australia) in 1% BSA in TBS supplemented with 0.1% Tween-20 (TBS-T; Ameresco. Solon, Ohio, 44139 USA) overnight at 4°C. Membranes were washed for 4 × 5 min in TBS-T and incubated in HRP-conjugated goat anti-rabbit IgG (1:3000 dilution: MERK PTY. Kilsyth, Victoria 3137 Australia) for 60 min at room temperature (RT). Following 4 × 5 min TBS-T washes, blots were developed using Enhanced Chemiluminescence (ECL) reagent and Hyperfilm (GE Healthcare Bio-Sciences Pty Ltd) in conjunction with standard chemiluminescence techniques.

2.8.3 Immunocytochemistry

Denuded, ZP-free oocytes were obtained from superovulated CD151 null and wild type females as previously outlined. For CD9 labelling, oocytes were incubated for 60 min in 100 µL droplets of α-MEM/PVA supplemented with 1:50 rat anti-mouse CD9 (KMC8, BD PharMingen, San Diego, CA 92121 USA). Oocytes were then washed through 3 × 100 µL droplets before being incubated for 60 min in 1:50 goat anti-rat IgG-FITC. Oocytes were then transferred through 3 × 100 µL washes before being mounted on slides with raised coverslips. Labelling intensity was viewed quantitatively using a
LSM510 laser-scanning microscope (Carl Zeiss Pty) equipped with Argon laser; excitation wavelength of 488 nm and emission spectra of 500-530nm (FITC).

For CD81 detection, oocytes were incubated for 60 min in 1:50 dilution hamster anti-mouse CD81 (EAT-2 clone; Molecular Oncology Group). Washed oocytes were then incubated in biotin-conjugated goat anti-hamster IgG at 1:50 dilution for 30 min, followed by 3 more washes and 30 min incubation in streptavidin-FITC (1:100 dilution; Chemicon Int., Temecula, CA, 92590).

2.8.4 Histology and Immunohistochemistry

Following the superovulation and collection of oocytes as outlined above, excised ovaries were de-capsulated (stripped of surrounding membrane, oviductal tissue and fatty deposits) and washed through 3 × 100 μL droplets of α-MEM/PVA. Ovaries were then fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) and embedded in paraffin. 5 μm thick sections were cut and mounted 3 per slide. For histological visualisation by light microscopy, the sections were de-waxed and stained with haematoxylin and eosin using standard procedures (Hutt et al., 2006).

For immunohistochemistry, sections were de-waxed by 3 × 5 min incubations in xylene and rehydrated. Antigen retrieval was performed by means of boiling for 3 × 3 min in 0.1 M sodium citrate. Sections were outlined with hydrophobic barriers (Super PAP Pen; Daido Sangyo Co. Ltd) and blocked in 3% BSA in Phosphate Buffered Saline (PBS) containing 0.1% Tween-20 (PBS-T) for 60 min at RT. Primary antibodies were then applied and sections and incubated overnight at 4°C (rat anti-mouse CD9 at 1:100; hamster anti-mouse CD81 at 1:250). Following 3 × 5 min washes in PBS-T, sections were incubated for 60 min at RT in their respective appropriate secondary antibodies (alkaline phosphatase-conjugated goat anti-rat IgG at 1:50, or biotin-conjugated anti-hamster IgG at 1:100). For CD9 labelling, sections were then given 3 × 5 min washes in PBS-T and developed using standard procedures for alkaline phosphatase conjugated antibodies and counterstained with methyl green. For CD81 labelling, slides were overlayed with 1:100 streptavidin-HRP for 60 min at RT then washed for 3 × 5 min in PBS-T. Slides were then incubated with DAB (3,3’-diaminobenzidine
tetrahydrochloride) for 10 min at RT to develop the HRP-mediated staining prior to rinsing in PBS, and counterstaining with haematoxylin and mounting.

2.9 Protein Extraction and Purification

2.9.1 Solubilisation of Oocyte and Ovarian Proteins

Throughout these investigations proteins were routinely solubilised from both isolated oocytes and whole ovaries. During experiments utilising biotin-labelled surface proteins, ZP-free oocytes were transferred to Eppendorf tubes containing ice cold 0.5% Triton X-100 in Dulbeccos PBS. Approximately 200 oocytes were solubilised per 50 µL of 0.5% Triton X-100 solution. Oocytes were vortexed and extracted on ice for 60 min, with vortexing every 15 min. Extracts were then stored at -80°C until further use.

To solubilise proteins from whole ovaries, ovaries were excised from mice following superovulation and transferred to α-MEM/PVA. Ovaries were decapsulated and washed through 3 × 200 µL media droplets. Washed ovaries were then transferred to 2D rehydration buffer supplemented with Complete Protease Inhibitor Cocktail tablets (Roche Diagnostics) and mechanically homogenised by 3 × 20 sec burst with a Polytron PT2100 homogeniser (Kinematica AG). Extracts were then incubated at 4°C for 60 min on a rotary mixer. Following extraction, samples were centrifuged at 10,000 × g for 15 min to pellet any insoluble debris. Supernatants were extracted and protein levels determined using the Amersham 2D Quant Kit (Amersham Biosciences) according to manufacturer’s instructions.

For 2D LC-MS/MS analysis (see Section 2.10.6), denuded ZP-intact oocytes were collected in a 6 – 8 µL volume of BWW/PVA and transferred to a low-bind Eppendorf tube. The tube was snap frozen and stored at -80°C. Aliquots of oocytes were added to pool consecutive rounds of oocyte isolations. The pool, which totalled 2346 oocytes, was then thawed on ice before 200 µL of oocyte lysate buffer (see Appendix C) supplemented with Complete Protease Inhibitor Cocktail tablets (Roche Diagnostics) was added. Oocytes were incubated for 60 min at 4°C on a rotary mixer. Complete dissolution of oocytes and the ZP was visually confirmed using a dissection microscope. The oocyte lysate was then stored at -80°C until required for further use.
2.9.2 Affinity Purification of Surface Proteins

To isolate egg surface proteins from whole oocyte extracts, biotin-labelled egg surface proteins (see Section 2.6.3) were purified by magnetic separation using M280 Streptavidin Dynabeads (Invitrogen). To ensure adequate suspension of Dynabeads, the vial of M280 streptavidin Dynabeads was placed on a roller mixer for 5 min at RT. 400 µL of the Dynabead suspension was then extracted and transferred to a 500 µL Eppendorf tube in a magnetic rack. The magnetic slide was inserted and the beads pelleted against the side. The supernatant was then carefully extracted and discarded. The magnetic slide was then removed and the beads washed by resuspension in 500 µL of Dulbeccos PBS. A series of 5 such washes were performed, after which beads were resuspended and divided equally between two 500 µL Eppendorf tubes. Beads were again pelleted and resuspended in either 0.5% Triton X-100 in PBS for the control, or 0.5% Triton X-100 in PBS containing extracted proteins from biotinylated ZP-free oocytes. Tubes were incubated at RT for 60 min on a rotary mixer to allow the biotin-streptavidin interaction to take place. Tubes were magnetically separated and the supernatant of unbound lysate removed and retained. Beads were then washed 4 × by resuspension in 200 µL of Dulbeccos PBS, with each wash volume retained. Following the final wash, beads were suspended in 30 µL of SDS extraction buffer and heated to 95 °C for 10 min, with vortexing every 5 min. To achieve maximum supernatant recovery, tubes were then centrifuged at 10,000 × g to produce a highly compacted pellet. Supernatants containing any proteins released from the beads following SDS boiling were collected and stored at -80°C until further use.

2.9.3 Protein Precipitation

In preparation for proteomic analysis, proteins were routinely recovered from media solutions by means of a chloroform-methanol precipitation protocol adapted from Wessel and Flugge, 1984. Sample volume was made up to 400 µL using dH₂O in 1.5 mL low-bind Eppendorf tubes and mixed. The samples were then diluted with 400 µL of 100% methanol and mixed by vortexing. 200 µL of chloroform was then added to each tube. Tubes were then vortexed before layers were phase separated by 3 min centrifugation at 14,000 × g. The upper aqueous layer was then carefully removed without disturbing the protein layer at the phase interface. 300 µL of 100% methanol was then added and the tubes vortexed. Precipitated protein was pelleted by
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centrifugation at 14,000 × g for 15 min at 4°C and the supernatant removed. Tubes were then pulse spun to bring down residual supernatant and the residual supernatant carefully removed. Protein pellets were air-dried before being resuspended in the desired buffer (Wessel and Flugge, 1984).

2.9.4 Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

One-dimensional SDS-PAGE was routinely used to separate extracted oocyte proteins in accordance with previously outlined methods (Laemmli, 1970). Polyacrylamide gels were cast in a commercial, small format (0.75mm × 6 cm × 10 cm) gel chambers (Bio-Rad Pty Ltd. Hercules CA 94547 USA) whereby a 10% polyacrylamide resolving gel was overlayed with a 4% polyacrylamide stacking gel. The cast gels were then placed in a commercial electrophoresis tank (Bio-Rad), and the tank filled with 1× SDS-PAGE running buffer (see Appendix C). The desired quantity of protein (2 – 4 µg) from each sample was solubilised in reducing SDS-PAGE loading buffer (see Appendix C) and denatured by heating to 95°C for 5 min. Each sample was then pulse spun to spin down any condensed vapour droplets before loading into the wells of the stacking gel.

A Fermentas Pre-stained Protein Ladder® (Quantum Scientific) comprised of proteins of known molecular mass was loaded in appropriate gels lanes to provide a molecular weight reference. Electrophoresis was commenced at a constant 85 V until proteins had migrated into the stacking gel. Upon migrating to the stacking gel/resolving gel interface, voltage was increased to a constant 150 V. Electrophoretic separation preceded until the blue dye front neared the bottom edge of the gel (approximately 60 min). Each gel was then removed from the glass plates and the stacking gel discarded. Depending on experimental requirements, the gels were either silver stained (see Section 2.9.5) or prepared for Western blotting (see Section 2.9.6).

2.9.5 Silver Nitrate Staining

Upon completion of SDS-PAGE, the profile of separated proteins present in each gel was visualised using a silver staining protocol modified from Blum, 1987. Briefly, each gel was pre-fixed in a 50% (v/v) methanol/12% (v/v) acetic acid solution for 30 min, and then washed for 2 × 15 min in a 10% (v/v) ethanol/5% (v/v) acetic acid solution. A 10% (v/v) ethanol solution was then used to wash the gels for a minimum of 4 × 8 min
washes. Gels were routinely left in a 10% ethanol solution overnight in order to ensure effective washing of acetic acid from the gel. The 10% ethanol solution was decanted and the gels suspended in a 0.02% sodium thiosulfate solution for 3 min. The sodium thiosulfate solution was poured off and the gels rinsed for $3 \times 20$ sec in dH$_2$O. Next, the gels were immersed in a 0.1% (w:v) solution of silver nitrate for 6 min, after which the silver nitrate solution was poured off and the gels once again rinsed in dH$_2$O for $3 \times 20$ sec. The silver stain was then developed by immersing the gels in a solution containing 3% sodium carbonate, 0.05% formaldehyde and 0.0003% sodium thiosulfate. Gels were exposed to the developing solution until the desired level of staining was achieved (approximately 5 min). The developer was then decanted and the staining reaction stopped through the addition of a 1% (v/v) acetic acid solution for 5 min (Blum et al., 1987). The gels were then transferred to dH$_2$O before being scanned and dried down.

2.9.6 Western blotting/electroblotting

Upon completion of SDS-PAGE, proteins were routinely transferred to nitrocellulose membranes by Western blotting (Towbin et al., 1979). After removal from the electrophoresis apparatus, gels were placed into Western blot transfer buffer (see Appendix C) and allowed to equilibrate for 10 min. Furthermore, for each transfer being performed, a $6 \times 8$ cm piece of nitrocellulose membrane, 2 gauze sponges and 4 sheets of filter paper (8 cm $\times$ 10 cm) were pre-wet in Western blot transfer buffer. The blotting cassette was then assembled in the following order: 1 gauze sponge, 2 sheets of filter paper, gel, nitrocellulose membrane, 2 sheets of filter paper and finally 1 gauze sponge. Each layer was assembled exercising care to eliminate bubbles between layers. The cassette was closed, loaded into Western blotting tank and a constant current of 300 mA applied for 1 h. The membrane was removed from the cassette and prepared directly for immunoblotting or air dried for later use.

2.10 Protein Identification by High Pressure Liquid Chromatography Electrospray Ionisation-Ion Trap-Mass Spectrometry (HPLC ESI-IT-MS)

2.10.1 Generation of Peptides from Oocyte Proteins

For LC-MS/MS analysis of protein fractions obtained from isolated oocytes (biotin-purified surface protein fraction and the remaining egg lysate fraction), protein concentration was determined using the bicinchoninic acid (BCA) protein assay
(Quantum Scientific Pty Ltd. Paddington QLD, 4064 Australia). Proteins were then chloroform-methanol precipitated (see Section 2.9.3) and resuspended in 250 µL of 25 mM ammonium bicarbonate (NH₄CO₃) containing an appropriate concentration of sequencing grade Trypsin (Promega Corporation. Annandale, NSW, 2038 Australia) to give a trypsin : protein ratio of 1:100. Samples were incubated at 37°C overnight in an orbital mixer at 750 rpm. Further trypsin was added to a final ratio of 1:50 and the samples incubated for a further 2 h. Samples were then transferred to a v-bottomed 96 well plate (Greiner Bio-One Inc. Monroe, North Carolina, 28111, USA) and vacuum centrifuged to dryness using a CHRIST RVC 02-25 evaporative centrifuge coupled to a CHRIST CT02-50 condenser unit (Martin Christ Gefriertrocknungsanlagen GmbH. 37507 Osterode am Harz, Germany). Tryptic peptides were then resuspended 10 µL of 0.1% formic acid and analysed using nano-scale reversed-phase liquid chromatography in conjunction with online electrospray ionisation (see Section 2.10.4).

For LC-MS/MS analysis of the GPI-anchored protein fraction, the protein extract was chloroform-methanol precipitated (see Section 2.9.3) and the dry pellet shipped to the Australian Protein Analysis Facility (APAF), North Ryde, NSW, 2109, Australia.

During APAF processing, the sample was resuspended in 10 µL of 50 mM NH₄CO₃ containing 6 M urea. The proteins were then reduced with 200 mM dithiothreitol (DTT) before being alkylated with 2 mM iodoacetamide. Sample volume was made up to 100 µL in 50 mM NH₄CO₃ and 8 µL of sequencing grade trypsin (Promega) at 15 ng/µL was added. The sample was incubated for 16 h at 37°C, after which sample volume was reduced by vacuum centrifugation to 6 µL.

The digested peptides were separated by nano-LC using a CapLC system (Waters Corporation, Milford, MA, 01757 USA). Samples (6µL) were injected onto a micro C18 pre-column for pre-concentration and desalted with 0.1% formic acid at 30 µL/min. After a 3 min wash the pre-column was switched online with the analytical column containing C18 RP silica (Atlantis, 75 µm × 100 mm, Waters Corporation). Peptides were eluted from the column using an extended linear solvent gradient, with steps, from water : acetonitrile ratios of 95:5 (+ 0.1% formic acid) to 20:80 (+ 0.1% formic acid) at 200 nL/min over a 60 min period. The LC eluent was subjected to positive ion
nanoflow electrospray analysis on a Micromass QTOF Ultima mass spectrometer (Waters Corporation). The QTOF was operated in a data dependent acquisition mode (DDA).

In DDA mode, a time-of-flight MS (TOFMS) survey scan was acquired (m/z 400-1900, 1.0 sec), with the three largest multiple charged ions (counts >50) in the survey scan sequentially subjected to MS/MS analysis. MS/MS spectra were accumulated for 1 sec (m/z 50-2500). The LC-MS/MS data was searched using the Mascot (Matrix Science Ltd, London, W1U 7GB UK) search engine against the Rodentia and Mus musculus databases as well as all entries in the NCBI non-redundant protein database.

2.10.2 Generation of Non-Fractionated Ovarian Peptides
For LC-MS/MS analysis of non-fractionated ovarian peptides, 8 whole ovaries were extracted into 2 mL of 2D rehydration buffer (see Section 2.9.1). Protein concentration was assayed using the 2D Quant kit (Amersham Biosciences) and the appropriate volume constituting 30 µg of protein extract transferred to a separate low-bind Eppendorf tube. Proteins were precipitated and tryptic peptides generated as outlined in Section 2.10.1. Peptides were vacuum centrifuged to dryness in a 96 well v-bottomed plate (Greiner Bio-One), resuspended in 10 µL of 0.1% formic acid and analysed using nano-scale reversed-phase liquid chromatography in conjunction with online electrospray ionisation (see Section 2.10.4).

2.10.3 Generation of IPG Strip-Fractionated Ovarian Peptides
An appropriate volume of the ovarian protein extract (see Section 2.9.1) to give 500 µg of protein was transferred to a separate low-bind Eppendorf tube. Proteins were precipitated and trypsin digested as outlined in Sections 2.10.1. At the completion of digestion, tryptic peptides were suspended in 250 µL 25 mM ammonium bicarbonate. Instead of vacuum centrifuging to dryness, the peptides were made up to a final volume of 460 µL, taking into account the addition of appropriate mass/volumes to achieve final concentrations of 8 M urea and 20 mM DTT. Colour was added to the sample by the addition of a few bromophenol blue crystals and carefully pipetted into a channel of an Immobiline DryStrip Reswelling Tray (Pharmacia Biotech/Pfizer. West Ryde, NSW, 2114 Australia). A 24 cm, pH 3 – 10 IPG strip (Amersham Biosciences) was then
carefully layered gel-side down into the channel and covered with mineral oil (Sigma-Aldrich). Strips were rehydrated for 16 h at RT before being subjected to isoelectric focusing. Isoelectric focusing was performed using an IPGphor focusing unit (Pharmacia Biotech/Pfizer) according to the following parameters:

1) Step and hold: 300 V for 900 volt h
2) Step 2) Gradient: 1000 V for 3900 volt h
3) Step 3) Gradient: 8000 V for 13,500 volt h
4) Step 4) Step and hold: 8000 V for 46,300 volt h
5) Step 5) Step and hold: 1000 V until strips were collected

The focused IPG strips were drained of mineral oil and cut into 8 × 3 cm segments. Each segment was transferred to a separate 2 mL Eppendorf tube and stored at -80°C until further use.

To recover peptides, tubes were thawed and each segment of IPG strip was diced into 5 mm sections whilst remaining inside the tube. 200 μL of 0.1% trifluoroacetic acid (TFA) in 50% acetonitrile was then added to each of the 8 tubes. Tubes were incubated for 20 min with vortexing every 5 min. Supernatants were extracted and the strip segments were then washed another 2 × to maximise recovery of peptides. The three elutes for each fraction (tubes 1-8) were pooled giving a total of 600 μL per fraction.

To remove contaminating mineral oil from the strip elutes, the peptide extracts were purified utilising StrataX reversed phase C18 columns (Phenomenex, Lane Cove, NSW, 2066 Australia) fixed to a vacuum system. StataX columns were conditioned with 2 × 1 mL of 60% acetonitrile in 0.1% formic acid. Columns were then equilibrated with 2 × 1 mL of 0.1% formic acid, and at no point was the fluid in any column allowed to drop to the level of the C18 solid phase. Each 600 μL strip fraction was diluted with 400 μL of 0.1% formic acid and added to a separate column with closed taps. Taps were partially opened to allow samples to slowly draw through the StrataX columns. Columns were then washed with 2 × 1 mL of 0.1% acetic acid to remove any mineral oil and contaminants. To elute peptides, columns were then treated with 2 × 1 mL of 60% acetonitrile in 0.1% acetic acid. Elutes from the 8 columns were collected and each vacuum centrifuged to dryness. Peptides from each of the fractions were then
resuspended in 10 µL of 0.1% formic acid and analysed using nano-scale reversed-phase liquid chromatography in conjunction with online electrospray ionisation (see Section 2.10.4).

2.10.4 LC-MS/MS Analysis
HPLC separation of peptide samples was achieved using a nano-MDLC system (Ettan MDLC, GE Healthcare, Piscataway, NJ, 08855-1327 USA). The system consisted of a trap (300 µm inner diameter × 5 mm length, C18) and separation column (75 µm inner diameter, 150 mm length), both of which were obtained from Agilent Technologies, Forest Hill, VIC, 3131 Australia. Elution of peptides from the separation column was achieved using a 0 – 60% acetonitrile gradient in 0.1% formic acid. Mass spectrometric analysis of the eluted peptides was performed on a linear quadrupole ion trap (LTQ; Thermo Scientific Inc. Waltham, MA, 02454 USA). A nano-ion spray source with 30 µm New Objective needle was used for online coupling of the MDLC and LTQ. The needle voltage was 1.6 kV and for each survey scan, of mass range 500 – 1400 amu; MS/MS data was obtained for the 3 most intense signals in the spectrum.

2.10.5 Interpretation of MS/MS data
To obtain protein identifications the collected MS/MS data was converted to TurboSequest generic format (*.dta). The resultant .dta files were searched against the latest Mus musculus IPI database at the time. The number of miscleavages was set to one, oxidation of methionine was selected and ion tolerance for peptide and fragment ions was set to 100.

Resultant protein identifications were filtered whereby for a peptide of charge of 1, acceptable Xcorr values were a minimum of 1.5, for a peptide of charge 2, Xcorr value was a minimum of 2.0, and for a peptide charge of 3, the Xcorr value was a minimum of 2.5.

It was desirable that a minimum of two peptides were detected for each particular protein, however for the purpose of screening GPI-anchored proteins, results with a single peptide match were also considered given the low abundance of protein and the
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fact that expression studies would be performed to confirm the presence of the protein within oocytes.

2.10.6 Fractionation of Oocyte Peptides: 2D LC-MS/MS Analysis

A protein extract was generated from 2346 denuded ZP-intact oocytes, as outlined in Section 2.9.1, and stored at -80°C. The sample was shipped on dry ice to APAF for 2D LC-MS/MS analysis.

Protein concentration for the sample was determined, after which proteins were reduced using DTT, alkylated with acrylamide and digested with sequencing grade trypsin (Promega). Peptides were loaded onto a PolyLC PolySulfoethyl A column fitted to an Agilent 1100 quaternary HPLC and separated by strong cation exchange (SCX) into 12 fractions. Individually each of the SCX fractions were desalted over a reverse phase peptide Captrap (Michrom Bioresources Inc. Auburn, CA, 95603 USA), which was then switched online with a C18 ProteCol column (SGE Analytical Science Pty Ltd. Ringwood, Victoria, 3134 Australia). Peptides were eluted from the C18 column over 120 min using an acetonitrile 3-step, linear gradient. Eluted peptides were analysed by a QStar XL MS/MS system (Applied Biosystems. Foster City, CA, 94404 USA).

During the MS/MS analysis, the reverse phase nanoLC eluent was subjected to positive ion nanoflow electrospray analysis in an information dependent acquisition mode (IDA). In IDA mode, a TOFMS survey scan was acquired (m/z 370-1600, 0.5 sec), with the three most intense multiple charged ions (counts >70) in the survey scan sequentially subjected to MS/MS analysis. MS/MS spectra were accumulated for 2 sec in the mass-to-charge range 100 – 1600.

The LC-MS/MS data were searched using Mascot (Matrix Science Ltd) against the Swissprot Mus musculus database.

2.11 Expression Sequence Tag Database Analysis

The identification of GPI-anchored proteins expressed on the surface of mouse oocytes was a primary aim for this project. As such, efforts to obtain identities for these important proteins were extended beyond the implementation of proteomics based techniques to draw upon molecular gene expression profiling and published EST
libraries. In October 2006, the Jackson Lab (Bar Harbour, Maine, 04609 USA) published the results of an extensive EST analysis performed on mouse oocytes. Generated from 12,000 oocytes, this impressive database provided a robust list of the genes transcribed within mouse oocytes and reported 4791 identified genes. Made available to the general public, the list of identified genes was consulted and cross-referenced against a list of all the mouse genes known to encode GPI-anchored proteins. The list of known GPI-anchored proteins was published on the Mouse Genome Informatics (MGI) website. At the time of consultation the list encompassed 116 murine GPI-anchored proteins, and effectively enabled GPI-anchored protein coding genes to be filtered from the oocyte EST database.

Upon identification of GPI-anchored protein-encoding genes, background literature for each the genes was consulted and the list of gene candidates refined. Genes for which previous studies had revealed knockout female mice to be fertile were eliminated. Alternatively, GPI-anchored protein-encoding genes for which knockouts were embryonically/neonatally lethal or yet to be performed were selected for investigation by characterisation studies. These characterisation studies involved:

(i) The detection of the GPI-anchored proteins in ovarian protein extracts by means of immunoblot analysis
(ii) Visualisation of ovarian expression by means of immunohistochemistry
(iii) Antibody based competitive inhibition of sperm-egg interaction studies.

During antibody inhibition studies, ZP-free oocytes were pre-incubated with antibodies against the identified GPI-anchored proteins for 30 min at 37°C. Oocytes were then transferred through 3 × 100 µL media washes before being utilised in sperm-egg binding and fusion bioassay (see Section 2.5). Resultant sperm-egg binding and fusion rates were analysed to determine the capacity of the GPI-anchored protein antibodies to block sperm-egg binding and/or fusion.
CHAPTER 3:

Characterisation of Sperm–Egg Interaction
CHAPTER 3 – CHARACTISATION OF SPERM-EGG INTERACTION

3.1 Introduction

Literature sources provide evidence suggesting that two primary classes of oolemmal protein fulfil important roles in sperm-egg interaction. These are the GPI-anchored proteins, where evidence indicates a role in sperm-egg binding (Coonrod et al., 1999a; Coonrod et al., 1999b), and the tetraspanins, for which evidence supports a role in sperm-egg fusion (Kaji et al., 2000; Le Naour et al., 2000; Miyado et al., 2000). The series of experiments outlined in this chapter were directed toward characterising murine sperm-egg interaction in an attempt to gain insight into the molecular machinery on the oocyte surface that is responsible for mediating this event.

During the early stages of this research, there remained some conjecture as to whether GV phase oocytes had limited capacity to bind and fuse with capacitated sperm. The idea that these immature eggs lacked the full capacity to be fertilised was an attractive notion, as it meant the egg membrane proteins that constitute the fertilisation complex, such as a possible GPI-anchored protein and tetraspanin, were either not expressed on the surface of GV eggs, or were present but in an inactive state. If this were indeed the case, then the comparison of functionally immature GV oocytes against fertilisation-competent MII oocytes would provide a means of highlighting the appearance of these important proteins. In light of this possibility, a series of experiments were undertaken to compare the ability of immature and mature oocytes to bind and fuse with sperm. In doing this however, it was not desirable to use ovulated MII oocytes as these mature oocytes have been exposed to, and possibly modified by, proteins present in oviductal fluid. GV oocytes isolated directly from PMSG-stimulated follicles within ovaries however have not been exposed to proteins present in oviductal fluid. The first step was therefore to develop an in vitro maturation protocol to enable the preparation of both immature and mature oocytes from an isolated pool of immature eggs.

This was made possible due to the fact that immature oocytes arrested at prophase of meiosis I, spontaneously resume meiosis when intracellular cAMP concentrations are reduced (Conti et al., 2002; Downs and Hunzicker-Dunn, 1995; Nebreda and Ferby,
During development, cAMP levels within the oocyte are maintained by surrounding granulosa cells that supply the oocytes with this second messenger through trans-zonal gap junctions. Upon ovulation, these gap junctions are lost and as cAMP levels decrease meiosis resumes. During in vitro studies, GV oocytes are aspirated from ovaries with their protective surrounding of cumulus cells intact. However, mechanical removal of these granulosa cells deprives the oocyte of its cAMP supply, and over a period of approximately 18 hours the oocyte completes meiosis I and once again arrests at metaphase of meiosis II ready for fertilisation. The primary means of cAMP degradation within oocytes to permit the resumption of meiosis is hydrolysis by the enzyme phosphodiesterase 3 (PDE3) (Masciarelli et al., 2004). Oocyte specific knockout of PDE3 results in female infertility, and is the result of females ovulating immature GV phase oocytes instead of mature MII phase oocytes. Fertility of the PDE3 knockout oocytes is restored in vitro by promoting the degradation of intracellular cAMP. However the presenters of these findings made no mention as to whether sperm were able to bind to the surface of untreated PDE3 knockout oocytes. It remained possible therefore that fertility in GV arrested oocytes was the result of a lack of expression of the necessary proteins in the membrane of GV eggs.

It was not possible to obtain PDE3 knockout oocytes for the purpose of IVF and proteomic studies, it was however possible to inhibit the action of PDE3 through the PDE3 inhibitor, milrinone. Isolating GV oocytes into medium supplemented with 1 μM milrinone provided a means of maintaining oocytes at the immature GV phase, effectively enabling a recreation of the above situation in which GV oocytes were reportedly infertile. Alternatively, milrinone could be washed out of the GV oocytes permitting the maturation process to resume in vitro. It was therefore possible during these investigations to obtain oocytes at three different developmental points, these were (i) immature GV oocytes, (ii) in vitro matured MII oocytes, and (iii) ovulated MII oocytes. The following series of investigations routinely refer to the use of oocytes from these three different developmental points in experiments designed to elucidate the biological and molecular basis of sperm-oolemmal interaction.
3.2 Experimental Rationale

In order to gain insight into the mechanisms that mediate sperm-egg interaction, we made use of the fact that bacterial PI-PLC has been found to release GPI-anchored proteins from the oolemma to effectively inhibit sperm-egg binding (Coonrod et al., 1999a; Coonrod et al., 1999b). However the authors of this study did not identify the oocyte GPI-anchored proteins released by PI-PLC. Thus PI-PLC has still not been exploited as a means of obtaining pools of GPI-anchored proteins potentially involved in sperm-egg interaction. Prior to application of PI-PLC in a series of proteomic studies, detailed in Chapter 4, it was firstly necessary to characterise the effect of PI-PLC on mouse oocytes in vitro so as to provide a ‘proof of principle’ for the technique, which ultimately forms the underlying basis for a major component of this research.

In addition, this research aimed to identify other novel factors that could be used to impact upon sperm-egg interaction in an attempt to further understand the molecular mechanisms involved. In an attempt to identify novel factors effecting sperm-egg interaction, a series of xenobiotic agents (including quinones) were screened for their possible capacity to modify egg membrane proteins in such a manner as to affect sperm-egg binding and/or fusion. For the purpose of this area of research, xenobiotic agents were chosen with known chemical properties such as thiol alkylation or oxidation as well as redox cycling. The decision to utilise chemical agents with thiol modifying properties was based on the hypothesis that sperm-egg binding and fusion events may closely parallel those of enveloped virus-host cell fusion, and as such be mediated at the molecular level through a thiol/disulphide exchange mechanism (Barbouche et al., 2003; Fenouillet et al., 2001; Gallina et al., 2002; Ryser et al., 1994). Additionally, studies in sea urchin had previously demonstrated a role for sea urchin dual oxidase 1 (Udx1), a redox active enzyme capable of generating hydrogen peroxide, in processes occurring within just moments of fertilisation, including the block to polyspermy (Wong and Wessel, 2005). As such, compounds with a capacity to generate ROS were also of interest and chosen for investigation.

The final series of experiments presented in this chapter represents an in depth characterisation of the effect of CD151 deletion on murine fertilisation. CD151 null mice have been subject to preliminary characterisation, with the study revealing only mild phenotypic abnormalities in several different tissues (Wright et al., 2004a). These
phenotypes included impaired migration of keratinocytes \textit{in vitro}, unstable haemostasis and hyperproliferative T lymphocytes. CD151 null mice on this genetic background are regarded as essentially normal suggesting that there is complementation of CD151 function by other tetraspanins, similar to the capacity of CD81 to substitute for CD9 in sperm-egg fusion (Rubinstein et al., 2006a). Although CD151 null females are fertile, breeding data revealed that CD151 null mice did not appear to reproduce as readily as wild type littermates. In light of this, and the previously demonstrated important roles for CD9 (Kaji et al., 2000; Le Naour et al., 2000; Miyado et al., 2000) and CD81 in fertilisation (Rubinstein et al., 2006a), investigations were warranted to further scrutinise the processes of sperm-egg interaction in these CD151 deficient mice. These studies have led to the discovery of a novel role for CD151 in murine fertilisation.

Thus the experimental aims for this series of investigations were to:

1) Investigate the role of GPI-anchored proteins in murine fertilisation by first confirming observations reported in the literature. Once confirmed, to expand these investigations by researching the effects of additional enzymes with related enzymatic activities.

2) To survey the impact of a series of xenobiotic compounds with the aim of identifying an agent with the capacity to disrupt sperm-egg binding and/or fusion. In the event that an agent(s) should successfully modulate sperm-egg interaction, attempt to understand how the agent(s) impacts on the oocyte and what it means when applied to the biological system.

3) Perform an in-depth analysis of mice lacking the tetraspanin CD151 with respect to reproductive physiology, and characterise the effect, if any, of CD151 deletion in mouse oocytes.
3.3 Results

3.3.1 In vitro Oocyte Maturation

3.3.1.1 In vitro Maturation: Germinal Vesicle to Metaphase II Oocytes.

Ovaries were excised from 6 – 8 wk old female Swiss mice 48 hrs post-intraperitoneal injection with 10 IU of PMSG and transferred to α-MEM. To prevent spontaneous maturation, α-MEM was supplemented with 1 μM of the PDE inhibitor milrinone. Ovarian follicles were aspirated using a 30 gauge needle effectively releasing CEOs. CEOs were collected and washed through 3 × 100 μL α-MEM droplets containing 1 μM milrinone. To obtain denuded oocytes, cumulus cells were then mechanically sheered away by repeated pipetting through a fine bore Pasteur pipette (bore diameter of approximately 80 μm). Once denuded, the immature status of oocytes was established by visual confirmation of a membrane-bound GV (see Figure 3.1A).

When incubated for 18 h in the presence of 1 μM milrinone, the characteristic GV of the immature oocytes remained intact (see Figure 3.1B). On the other hand, a population of oocytes that were washed through 3 × 100 μL droplets of α-MEM lacking milrinone and then incubated for 18 h were observed to have undergone spontaneous maturation. In addition to the lack of a GV, these oocytes were observed to have ejected the first polar body (see Figure 3.1C).
Figure 3.1 Effect of milrinone on *in vitro* maturation of denuded, zona-intact murine oocytes.
CEOs were obtained by aspiration from ovarian follicles into α-MEM supplemented with 1 μM milrinone. (A) The oocytes were denuded of cumulus cells using a fine bore Pasteur pipette. 50% of the oocytes were incubated for 18 h in the presence of 1 μM milrinone, whilst the remainder were washed and then incubated in unsupplemented α-MEM. (B) Incorporation of milrinone into the incubation media prevented GV breakdown. (C) Oocytes incubated in the absence of milrinone however underwent GV breakdown and polar body extrusion.
3.3.1.2 Sperm-Egg Binding & Fusion: Germinal Vesicle vs Metaphase II Oocyte

Having established an effective *in vitro* oocyte maturation protocol, it was possible to obtain both GV phase and MII phase oocytes from same original population of oocytes that had not been exposed to follicular fluid. GV phase and *in vitro* matured MII phase oocytes were then assayed for their capacity to bind and fuse with capacitated spermatozoa. In order to assay the binding and fusion capabilities of the oolemma, it was first necessary to remove the ZP. Following ZP removal by dissolution with acidified Tyrodes solution (pH 2.2), oocytes were incubated for 60 min to allow recovery of any surface proteins that may have been affected by the acid treatment. GV or *in vitro* matured MII oocytes were pre-loaded with 10 µg/mL DAPI, washed briefly and then injected into 100 µL droplets of capacitated mouse spermatozoa at 3.0 x 10⁵ sperm/mL for 60 min. Approximately 15 oocytes were added to each 100 µL sperm droplet. After washing to remove any loosely bound spermatozoa, eggs were mounted on slides ready for visualisation.

Upon viewing by phase contrast microscopy, it was observed that GV oocytes did indeed possess the capacity for sperm-egg binding (see Figure 3.2 A). In addition, epifluorescence microscopy revealed DAPI-labelled sperm nuclei present in the cytoplasm of GV phase oocytes (see Figure 3.2 B), thereby demonstrating the capacity for GV oocytes to effectively fuse with spermatozoa *in vitro*. As anticipated, MII oocytes also exhibited the capacity to both bind and fuse with capacitated spermatozoa (see Figure 3.2 C and D). Statistical analysis was not necessary as sperm-egg binding and fusion rates were not being compared between GV and MII oocytes, instead merely the capacity of both classes of oocytes to bind and fuse with sperm was being investigated.
Figure 3.2 Sperm-egg binding and fusion for GV and MII phase murine oocytes
Following ZP removal, GV and in vitro matured MII oocytes were pre-loaded with 10 µg/mL DAPI, washed and then incubated in 100 µL droplets of capacitated sperm at 3.0 x 10^5 cells/mL for 60 min. Eggs were washed, mounted on slides and viewed at 250 × magnification. GV phase oocytes clearly demonstrated a capacity to bind with sperm (A), as well as undergo successful fusion (B). MII oocytes also exhibited a capacity to both bind (A) and fuse (B) with the capacitated mouse spermatozoa.
3.3.2 **Oolemmal GPI-Anchored Proteins**

3.3.2.1 **Confirmation of PI-PLC-mediated GPI-anchored protein release**

Through hydrolysing a phospo-linkage in ethanolamine, the enzyme PI-PLC is known to provide an efficient means of cleaving GPI anchors, effectively releasing GPI-anchored proteins from the extracellular surface of live cells (Low and Finean, 1978). Two other such proteins with reported GPIase activity are GPI-PLD (Davitz et al., 1989; Huang et al., 1990) and ACE (Kondoh et al., 2005). In order to ascertain the effectiveness with which these enzymes cleave GPI-anchored oolemmal proteins, an assay was developed that monitored the release of the known oolemmal GPI-anchored protein CD55 (Sun et al., 1999). CD55, detected through the use of monoclonal goat anti-mouse CD55, was effectively released from oocytes treated with 5 U/mL PI-PLC (see Figure 3.3).

Relative to control oocytes, detection of CD55 on oocytes treated with 5 U/mL PI-PLC was almost completely abolished. However, treating oocytes with either 5 U/mL GPI-PLD or 5 U/mL ACE failed to produce any reduction in the CD55 signal; intensities of the CD55 signal for both these treatments were comparable to that observed for the control oocytes. Although the latter enzymes failed to reduce CD55 detection, for reasons that will be discussed, these studies represent the first reported attempts to characterise the effects of both GPI-PLD and ACE on live oocytes.
Figure 3.3 Confirmation of CD55 release from live ZP-free oocytes

ZP-free mouse oocytes were treated with PI-PLC, GPI-PLD or somatic ACE at 5 U/mL for 60 min. Oocytes were then washed and incubated in 1:50 goat anti-mouse CD55 (1° antibody) for 30 min at 37°C to label this known GPI-anchored protein. To detect labelling, oocytes were washed, incubated in 1:50 donkey anti-goat biotin (2° antibody) for 30 min and washed again. Oocytes were then incubated in 1:100 Streptavidin-FITC (3° conjugate) for a further 30 min. Oocytes were mounted on slides and viewed at 400 × magnification via confocal microscopy. The 1° antibody control, in which goat anti-mouse CD55 was omitted, and 2° antibody control, in which donkey anti-goat-biotin was omitted, revealed no non-specific labelling of oocytes by either the donkey anti-goat-biotin or the streptavidin-FITC. Untreated oocytes revealed strong labelling of CD55 on their cell surface. Incubation of oocytes in PI-PLC almost completely abolished CD55 detection on these oocytes, whilst incubation in GPI-PLD or ACE had no effect on CD55 detection.
3.3.2.2 Effect of GPI-anchored protein release on Sperm-Egg Interaction

Having established that PI-PLC treatment of live oocytes effectively cleaved the oolemmal GPI-anchored protein marker CD55, investigations were performed to confirm the inhibitory effect of the enzyme’s GPI cleaving activity on sperm-egg interaction. Following a 60 min pre-treatment of ZP-free oocytes with 0, 0.05, 0.5, 1.0 or 5.0 U/mL PI-PLC, oocytes were washed, loaded with DAPI and incubated with capacitated spermatozoa at $3 \times 10^5$ cells/mL for 60 min. Oocytes were then washed, mounted on slides and the rates of successful sperm-egg binding and fusion were assayed.

Treating oocytes with PI-PLC resulted in significant ($P = 0.015$), dose-dependent reductions in rates of sperm-egg binding, with inhibitory effects observed at and above PI-PLC concentrations of 0.5 U/mL (see Figure 3.4). Inclusion of the heat inactivated PI-PLC control confirmed a role for the enzyme’s GPIase activity in producing the observed inhibition. Thus, following heat denaturation to destroy the enzyme’s GPI-cleaving activity (95°C for 5 min), it was observed that 5 U/mL PI-PLC was unable to exert the inhibitory effect on sperm-egg binding and/or fusion ($P = 0.438$). 5 U/mL treatments of GPI-PLD and ACE were also assayed for their effects on sperm-egg interaction; however both enzymes failed to inhibit the rates of sperm-egg binding or fusion (data not shown). These results for GPI-PLD and ACE are consistent with the above report that both enzymes failed to cleave the GPI-anchored marker CD55 from the surface of live oocytes (see Figure 3.3).
Figure 3.4 Effect of PI-PLC treatment of oocytes on sperm-egg interaction

ZP-free oocytes were treated with PI-PLC at concentrations ranging from 0 to 5 U/mL for 60 min. Oocytes were examined for their ability to bind and fuse with spermatozoa using the sperm-egg binding and fusion bioassay. PI-PLC treatment of oocytes revealed effective inhibition of sperm-egg binding with significant levels of inhibition observed for PI-PLC concentrations ≥ 0.5 U/mL. Heat inactivation of PI-PLC prior to oocyte treatment abolished the inhibitory effect of PI-PLC treatment on sperm-egg interaction. Similar treatments with 5 U/mL GPI-PLD or 5 U/mL ACE failed to produce any inhibitory effects on sperm-egg binding and fusion rates (data not shown). (Data are mean ± sem, n = number of oocytes, *P ≤ 0.015; unpaired Student T-test).
3.3.3 Xenobiotic Modification of the Oolemma

3.3.3.1 Sperm-Egg interaction: Xenobiotic Screening

In an attempt to better understand the mechanisms involved in sperm-egg interaction, a series of xenobiotic agents was screened to determine whether exposure of oocytes to these compounds had the capacity to disrupt sperm-egg interaction. The compounds screened were specifically chosen because of their reported chemical properties including thiol alkylation, disulphide reduction or redox cycling. On the other hand duroquinone and hydroquinone were expected to be quite inert (Klassen, 2001; Lusini et al., 2002; McAmis et al., 2003; Nelson and Pearson, 1990).

Table 3.1 Chemical properties of xenobiotic agents

<table>
<thead>
<tr>
<th>Agent</th>
<th>Chemical Properties</th>
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<tbody>
<tr>
<td>Benzoquinone</td>
<td>Thiol alkylation, redox active</td>
</tr>
<tr>
<td>Duroquinone</td>
<td>Unable to alkylate, possibly redox active</td>
</tr>
<tr>
<td>Hydroquinone</td>
<td>Relatively inert analogue of Benzoquinone</td>
</tr>
<tr>
<td>Menadione</td>
<td>May be metabolised to redox active or thiol alkylation</td>
</tr>
<tr>
<td>Bis Maleimide</td>
<td>Thiol alkylation, non-redox Active</td>
</tr>
<tr>
<td>Iodoacetamide</td>
<td>Thiol alkylation</td>
</tr>
<tr>
<td>TCEP</td>
<td>Disulphide bond reduction</td>
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Pre-treatment of oocytes with hydroquinone and duroquinone failed to produce any significant effect on rates of sperm-egg binding or fusion relative to the DMSO-treated control oocytes. Menadione treatment of oocytes resulted in a 27% reduction in the observed rate of sperm-egg binding. Despite the observed reduction in rates of sperm-egg binding, menadione treatment did not affect rates of sperm-egg fusion (P = 0.064), with menadione treated oocytes fusing with sperm at rates comparable to that of control oocytes. Highly significant effects on rates of sperm-egg interaction were observed for two other compounds examined. Following pre-treatment with 10 µM benzoquinone, oocytes were observed to bind with sperm at a rate of just 18.6% of that observed for control oocytes (P < 0.0001). Similarly, oocytes pre-treated with 10 µM bis maleimide bound sperm at a rate equivalent to 25.9% of that observed for control oocytes (P < 0.0001). As a consequence of the drastically reduced incidence of sperm-egg binding, sperm-egg fusion rates for both benzoquinone and bis maleimide treated oocytes were both significantly reduced (P vales were both P < 0.00001), with an average of < 0.3 sperm/egg achieving successful fusion with these oocytes (see Figure 3.5).
Characterisation of Sperm-Egg Interactions

Figure 3.5 Effect of treating oocytes with xenobiotic compounds on sperm-egg binding and fusion

1000 × stocks of the test compounds were prepared in DMSO then diluted 1:1000 in BWW/PVA. ZP-free oocytes were then pre-incubated in the xenobiotic compounds at final concentrations of 10 µM for 20 min before being washed, loaded with 10 µg/mL DAPI and used to perform sperm-egg binding and fusion bioassays. Hydroquinone and duroquinone failed to produce any significant effects on rates of sperm-egg interaction relative to the vehicle control (1:1000 DMSO). Pre-incubation of oocytes in 10 µM menadione resulted in a slight but significant reduction in sperm-egg binding (P = 0.01) reducing the numbers of bound spermatozoa by 27% (control oocytes bound an average of 9.72 ± 0.89 sperm/egg; menadione treated oocytes bound an average of 7.0 ± 0.69 sperm/egg). Benzoquinone and bis maleimide both resulted in highly significant disruption of sperm-egg binding, reducing the numbers of bound spermatozoa by 81.4% and 74.1% respectively (benzoquinone treated oocytes bound an average of 1.80 ± 0.33 sperm/egg; bis maleimide treated oocytes bound an average of 2.51 ± 0.08 sperm/egg). P-values for both compounds were P < 0.0001 when compared to binding rates of the DMSO-treated control. Downstream of sperm-egg binding, benzoquinone and bis maleimide both induced significant reductions in average fusion rates. P-values for both compounds were again P < 0.0001 when compared to fusion rates of the control oocytes (Data are mean ± sem, n = number of oocytes, *P = 0.01, ***P < 0.00001; unpaired Student T-test).
3.3.3.2 Xenobiotic Dose Dependency Studies

In light of the finding that benzoquinone and bis maleimide possessed the capacity to radically inhibit sperm-egg binding and fusion; dose-dependency studies were performed to ascertain the effective concentrations at which these compounds induced their inhibitory effects. Oocytes were pre-incubated in benzoquinone or bis maleimide at final concentrations of 0, 0.1, 1.0 or 10 µM for 20 min prior to use in the sperm-egg binding and fusion bioassay (see Figure 3.6). Treatment of oocytes with 0.1 µM benzoquinone produced no effect on the rates of either sperm-egg binding or fusion relative to the DMSO control. Treatment with 1.0 µM benzoquinone appeared to slightly increase the rates of sperm-egg binding whilst fusion rates remained unaffected, however this increase was not statistically significant (P > 0.05). At the highest concentration investigated of 10 µM, benzoquinone drastically reduced sperm binding rates by 87.7%, consistent with previously observed results. Statistically the result was highly significant (P < 0.0001). Treatment of oocytes with 0.1 or 1.0 µM bis maleimide produced no effect on rates of sperm-egg binding relative to the control. At a final concentration of 10 µM however, bis maleimide again produced significant inhibition of sperm-egg binding, with binding rates reduced by 89%. Observations for 10 µM bis maleimide were again highly significant (P < 0.0001) and consistent with the previous results presented in Figure 3.4. The observed reduction in rates of sperm-binding for 10 µM benzoquinone and bis maleimide once again resulted in significant reductions in rates of successful sperm-egg fusion (P < 0.0003).
Figure 3.6 Dose dependencies of benzoquinone and bis maleimide induced inhibition of sperm-egg interaction

ZP-free oocytes were pre-treated with benzoquinone or bis maleimide at final concentrations of 0, 0.1, 1.0 or 10 µM for 20 mins. The oocytes were then washed and used to perform the sperm-egg binding and fusion bioassay. No significant differences were observed in rates of sperm binding or fusion upon treating oocytes with 0.1 µM benzoquinone. Interestingly, 1.0 µM benzoquinone appeared to slightly enhance rates of sperm binding relative to the control, however this increase was not statistically significant. At 10 µM, benzoquinone drastically reduced binding rates to just 12.3% of that observed for control oocytes. Bis maleimide produced no discernable effects on rates of sperm-egg binding at the 0.1 and 1.0 µM concentrations. Treatment of oocytes with 10 µM bis maleimide however resulted in significant inhibition of sperm binding, with the average rate of binding observed at just 11% of that for the control. Fusion rates for oocytes treated with the agents at 10 µM final concentrations were subsequently reduced also (Data are mean ± sem, n = number of oocytes, *P < 0.0003, ***P < 0.0001; unpaired Student T-test).
The results gathered so far indicated that it was indeed possible to impact upon sperm-egg interaction by exposing the oocytes to specific xenobiotics. The data demonstrated that pre-treatment of oocytes with benzoquinone or bis maleimide were both capable of significantly inhibiting the eggs capacity to bind sperm, with menadione capable of producing less severe effects. Dose dependency studies revealed that sub-micromolar concentrations of benzoquinone and bis maleimide were both too low to impact sperm-egg binding and fusion rates, with effects only observed at the 10 µM concentration. Taken together these findings raised the possibility that the xenobiotic agents may only be influencing sperm-egg interaction by compromising oocyte viability. To address this possibility, oocyte viability studies were performed utilising both the eosin and trypan blue exclusion tests.

Following 20 min exposure of oocytes to an array of xenobiotic compounds at final concentrations of either 1 or 10 µM, oocytes were transferred into droplets of either eosin or 0.2% trypan blue and incubated for 10 min. Oocytes were removed from the membrane impermeable dyes, transferred through a series of three washes and visualised at full magnification under a dissecting microscope. The xenobiotics examined included benzoquinone (1.0 and 10 µM), hydroquinone (1.0 and 10 µM) duroquinone (10 µM), menadione (1.0 and 10 µM), bis maleimide (1.0 and 10 µM), and iodoacetamide (10 µM). Of the 8-10 oocytes visualised for each treatment, all oocytes were found to have remained viable, as evidenced by the lack of red (eosin) or blue (trypan blue) cytoplasmic staining, with the exception of 1 oocyte which stained blue from the 1.0 µM benzoquinone treatment. In light the fact that oocytes from all other treatments remained 100% viable, this particular oocyte is believed to have been compromised during handling in the early stages of the assay.
Table 3.2 Effect of xenobiotic treatment on oocyte viability
Assessment of oocyte viability by means of eosin and trypan blue exclusion tests demonstrated that 100% of the oocytes remained viable for each of the agents examined, with the exception of 1.0 µM benzoquinone, in which only 1 oocyte stained with trypan blue.

<table>
<thead>
<tr>
<th>Xenobiotic</th>
<th>Concentration [µM]</th>
<th>Eosin Exclusion Test</th>
<th>Trypan Blue Exclusion Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMSO Control</td>
<td>N/A</td>
<td>Unstained 0</td>
<td>Unstained 0</td>
</tr>
<tr>
<td>Benzoquinone</td>
<td>1.0</td>
<td>9</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>9</td>
<td>0</td>
</tr>
<tr>
<td>Hydroquinone</td>
<td>10.0</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>Duroquinone</td>
<td>10.0</td>
<td>9</td>
<td>0</td>
</tr>
<tr>
<td>Menadione</td>
<td>1.0</td>
<td>9</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>9</td>
<td>0</td>
</tr>
<tr>
<td>Bis maleimide</td>
<td>1.0</td>
<td>9</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>9</td>
<td>0</td>
</tr>
<tr>
<td>Iodoacetamide</td>
<td>10.0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

3.3.3.3 Xenobiotic Time Dependency Studies
Results of the viability study presented in Table 3.2 demonstrated that the observed inhibition of sperm-egg interaction upon treating oocytes with benzoquinone or bis maleimide was a genuine phenomenon and not merely the result of induced oocyte cell death. Further studies were thus warranted to characterise the time frame of exposure required for the xenobiotics to induce their inhibitory effects. By assaying the ability of oocytes to bind and fuse with sperm after only very brief (2 min) or the previously used 20 min of exposure to the xenobiotics, it was envisaged that insight into the modes of action of the compounds might be obtained. Observing effective inhibition after just 2 mins exposure to the compounds may indicate that rapid chemical changes such as thiol alkylation were responsible for the observed effect, whilst the requirement for longer incubations times may indicate enzymatic-based mechanisms are important, such as redox cycling.

Time dependency studies were performed whereby oocytes were assayed for their capacity to bind and fuse with sperm after 2 min or 20 min exposure to either 10 µM benzoquinone or 10 µM bis maleimide. A collection of oocytes was also exposed to 10 µM iodoacetamide at both time points. Iodoacetamide was previously demonstrated to have no effect on sperm-egg interaction and therefore served as a negative control. It was observed that 2 min exposure of oocytes to the xenobiotic compounds failed to influence rates of either sperm-egg binding or fusion relative to the DMSO-treated
Characterisation of Sperm-Egg Interactions

control oocytes. At the 20 min pre-treatment time point however, benzoquinone and bis maleimide both induced highly significant reductions (P < 0.0001) in rates of sperm-egg binding. Oocytes pre-treated for 20 min with benzoquinone or bis maleimide successfully bound capacitated spermatozoa at rates of just 13.3% and 28.7% respectively of that observed for the control oocytes. As anticipated, oocytes pre-treated for 20 min with 10 μM iodoacetamide revealed sperm-egg binding rates comparable to control levels. Due to time limitations imposed by the staggered IVF assays, fusion rates were not assayed during this particular series of experiments.

Figure 3.7 Time dependency of xenobiotic induced inhibition of sperm-egg interaction
Oocytes were pre-treated for 2 or 20 min with 10 μM benzoquinone, 10 μM bis maleimide or 10 μM iodoacetamide. Oocytes were then washed, loaded with 10 μg/mL DAPI and rates of sperm-egg binding and fusion assayed. None of the compounds tested affected sperm-egg interaction after 2 min of oocyte exposure. After 20 min exposure however, benzoquinone and bis maleimide reduced binding rates by 86.7 and 71.3% respectively. Oocytes pre-treated for 20 min with 10 μM iodoacetamide exhibited binding rates comparable to that of the control (Data are mean ± sem, n = number of oocytes, *P < 0.0001; unpaired Student T-test).
3.3.3.4 Effect of Xenobiotics on Surface Thiol Status

To attempt to understand the mechanism of action of the xenobiotic compounds, characterisation studies were performed to ascertain the effect of the compounds, if any, on the status of egg surface thiols. Visualisation of the egg surface thiols was achieved using the fluorescent probe AlexaFluor 488 C₅ Maleimide. The maleimide constituent provided a thiol reactive moiety, which was reportedly more thiol selective than alternative thiol conjugating agents such as iodoacetamide (Molecular Probes), whilst AlexaFluor 488 constituted a fluorochrome enabling detection of the probe by confocal microscopy.

Treatment of oocytes with 10 µM concentrations of hydroquinone and duroquinone did not affect the labeling intensity of AlexaFluor 488 C₅ Maleimide observed on the oocytes membranes. Interestingly, treatment of oocytes with 10 µM menadione resulted in cytoplasmic detection of the AlexaFluor signal. Relative to control oocytes, AlexaFluor labeling intensity appeared to be reduced for oocytes incubated in the presence of 10 µM bis maleimide. Incubation of oocytes in 10 µM benzoquinone however, almost completely abolished the ability of AlexaFluor to label the membrane of oocytes. In contrast, pre-treatment of oocytes with 1 mM TCEP resulted in enhanced AlexaFluor labeling of the oocyte membranes. A total of 28-30 oocytes were analysed for each xenobiotic treatment across 4 replicate experiments. Dual representative images of observed thiol labeling patterns appear in Figure 3.8.
Characterisation of Sperm-Egg Interactions

Figure 3.8 AlexaFluor detection of egg surface thiols following xenobiotic exposure

ZP-free oocytes were treated with benzoquinone, hydroquinone, duroquinone, menadione or bis maleimide, at 10 µM final concentrations, or 1 mM TCEP for 20 min. Oocytes were then washed and incubated for 10 min in droplets of medium containing 10 µM AlexaFluor 488 C₅ Maleimide. Oocytes were again washed before mounting on slides and viewing at 250 × magnification by confocal microscopy. Hydroquinone and duroquinone did not affect AlexaFluor labeling relative to control oocytes. Menadione resulted in cytoplasmic signaling whilst bis maleimide produced a reduction in labeling intensity. Benzoquinone essentially abolished membrane labeling of the oocytes, whilst treatment with 1 mM TCEP greatly enhanced membrane labeling. A total of 28-30 oocytes were analysed per treatment. Representative images are shown.
3.3.3.5 Effect of PI-PLC on Surface Thiol Status

Given that treatment of oocytes with 10 μM benzoquinone or bis maleimide affected sperm-egg binding (as opposed to sperm-egg fusion) in a similar manner to GPI-anchored protein release with PI-PLC, studies were performed to ascertain whether treatment with PI-PLC resulted in the loss of a substantial level of surface thiol detection.

In previous studies, oocytes had been labelled with antibodies directed against CD55 detection. For the purpose of this series of investigations however, following the release of GPI-anchored proteins with 1 U/mL PI-PLC, oocytes were incubated in the presence of 10 μM AlexaFluor 488 for 20 mins. Results of this labelling revealed that the release of GPI-anchored proteins from the egg surface appeared to have no effect on the level of surface thiols detected.

![Figure 3.9 Effect of PI-PLC mediated GPI-anchored protein release on egg surface thiols](image)

ZP-free oocytes were treated with 1 U/mL PI-PLC for 60 min to release egg membrane proteins as previously optimized. Oocytes were then washed and incubated for 20 min in droplets of medium containing 10 μM AlexaFluor 488 C3 Maleimide. Oocytes were again washed before mounting on slides and viewing at 250 x magnification by confocal microscopy. Control oocytes revealed strong labeling of the oocyte surface by the fluorescent thiol-detecting probe. No apparent difference in surface thiol labeling was observed between untreated oocytes and those for which GPI-anchored proteins had been released by means of PI-PLC treatment.
3.3.3.6 Effect of Xenobiotics on Cortical Granule Extrusion

To investigate the mode of action of benzoquinone and bis maleimide, the effect of various xenobiotics on cortical granule extrusion was investigated. Zona-free oocytes were incubated in the presence of 10 µM benzoquinone, bis maleimide or hydroquinone for 20 min. Positive and negative controls were included to induce and suppress cortical granule exocytosis respectively. For the positive control a collection of oocytes were incubated in 2.0 mM strontium chloride for 20 min, while the negative controls were incubated in the absence of strontium chloride or xenobiotics. Oocytes were then washed and incubated in the presence of FITC-conjugated Lycopersicon Esculentum lectin (LEL) at 5 µg/mL.

Despite repeated attempts to optimise this assay, variation between replicates in addition to technical difficulties associated with manipulating lectin-coated oocytes meant that meaningful data was unable to be obtained. The study does however represent an additional attempt to understand the mechanism of benzoquinone and bis maleimide mediated inhibition of IVF.

3.3.4 Oocyte Tetraspanins

The tetraspanins CD9 and CD81 have previously been demonstrated to play important roles in murine sperm-egg interaction (Kaji et al., 2000; Le Naour et al., 2000; Miyado et al., 2000; Rubinstein et al., 2006a). The existence of a strain of mice deficient in another tetraspanin protein CD151 presented an opportunity to investigate whether it too was important in sperm-egg interaction. Although fertile, breeding data for CD151 null mice (see Table 3.3) suggested that the mice do not reproduce as efficiently as their wild type counterparts.
Table 3.3 Breeding statistics for wild type and CD151 null female mice over a 12 month period

<table>
<thead>
<tr>
<th>Mouse Strain</th>
<th>Number of Litters</th>
<th>Animals Born</th>
<th>Animals Weaned</th>
<th>Pre-weaning Mortality</th>
<th>Average Litter Size</th>
<th>Offspring per female per month</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild Type</td>
<td>1494</td>
<td>7977</td>
<td>7540</td>
<td>5%</td>
<td>5.34</td>
<td>3.55</td>
</tr>
<tr>
<td>CD151−/−</td>
<td>142</td>
<td>705</td>
<td>655</td>
<td>7%</td>
<td>4.96</td>
<td>2.29</td>
</tr>
</tbody>
</table>

These breeding data warranted closer examination of the processes of fertilisation in these animals, in order to ascertain whether the reduced fecundity was the result of defective sperm-egg fusion, as is the case with CD9 deficient females.

3.3.4.1 Histological comparison of Wild Type and CD151 Null Ovarian Structure

Analysis of ovarian tissue sections revealed an increased prevalence of atretic follicles in superovulated CD151 null ovaries. In addition to the presence of these atretic follicles, it was observed that corpora lutea were substantially smaller in the CD151 null ovaries. In addition to their smaller size, tissue comprising these corpora lutea appeared friable. Finally, closer examination of the tissue layers revealed possible detachment of follicular basement membrane from stromal layers (see Figure 3.10).
Figure 3.10 Histological comparison of wild type and CD151 null ovaries
Ovaries were excised from 6-8 week old Swiss mice following superovulation and fixed in 4% paraformaldehyde, embedded in paraffin wax and 5 µm sections cut. Section were de-waxed and stained with haematoxylin and eosin using standard procedures (Hutt et al., 2006). Comparison of histological sections revealed smaller corpora lutea in ovaries from CD151 null mice. In addition, tissue comprising corpora lutea of CD151 ovaries appeared friable. CD151 null ovaries also exhibited an increased prevalence of atretic bodies as well as signs of basement membrane detachment.
3.3.4.2 CD151 Expression in Oocytes and Ovarian Sections

Although CD151 null female mice are fertile, previous histological data suggested that CD151 deletion had adverse effects on the murine ovary. It was not known however whether these effects were attributable to overall poorer health of the animals or the removal of CD151 action within the ovary itself. As a result, efforts were made to attempt to confirm the expression of CD151 within normal murine ovaries. Microarray analysis of ovarian transcripts revealed expression of the CD151 gene in both neonatal and adult ovaries.

Figure 3.11 Detection of CD151 gene expression in neonatal and adult murine ovaries

Neonatal and adult ovaries were excised from Swiss outbred mice and snap frozen. RNA was then extracted using Trizol reagent. Gene expression analysis was performed using CodeLink™ Whole Genome Array Slides and 10 µg of *in vitro* amplified RNA. Slides were scanned with an Axon scanner and data analysed with proprietary CodeLink™ Expression Analysis Software and relative expression levels of genes of interest were determined. The gene encoding CD151 was successfully detected in neonatal and adult ovaries. In addition, CD9 and the CD9 binding partner EWI-F were also successfully detected. Error bars are not provided as data represents a single replicate. This CodeLink™ analysis was performed by Janet Holt for purpose of investigating CXCR4/SDF1 expression (Holt et al., 2006).
CD151 was therefore successfully detected at the gene level, however limited success was also achieved in detecting CD151 at the protein level. The antibody LAI-2 was against recombinant preparations of murine CD151, with rabbit as the host species. The antibody was used to perform immunoblot analysis of CD151 expression in whole ovaries isolated from wild type or CD151 deficient mice. The antibody revealed non-specific labelling of protein bands present in both wild type and null ovarian extracts. Despite this non-specific binding, it was observed that LAI-2 successfully labelled a protein band of the appropriate molecular weight for CD151, 29 kDa, that was present in wild type ovarian protein extracts but absent in CD151 null protein extracts.

Figure 3.12 Detection of CD151 protein expression in wild type and CD151 adult ovaries
SDS protein extracts of wild type and CD151 null ovaries were separated by SDS-PAGE then western transferred to nitrocellulose membrane. Membrane were blocked in 3% BSA then probed with rabbit anti-mouse CD151 (LAI-2) according to standard immunoblotting procedures. Upon ECL visualisation, it was observed that LAI-2 recognised a band of approximately 28-30 kDa in the wild type ovarian protein extracts that was absent from CD151 null ovarian protein extracts. Non-specific protein labelling was also evident (not shown).
3.3.4.3 **Sperm-Egg Interaction: Wild Type vs CD151 Null Oocytes**

Using the sperm-egg binding and fusion bioassay, ZP-free oocytes from CD151 deficient mice (C57Black genetic background) were examined for their capacity to bind and fuse with capacitated wild type sperm. After being preloaded with DAPI, wild type or CD151 null oocytes were co-incubated with capacitated wild type sperm for 60 min. Once washed to remove and loosely attached sperm, rates of sperm binding and fusion were assayed for each egg via phase contrast and fluorescence microscopy respectively. With an average of 8.4 ± 1.77 sperm/egg bound for the wild type oocytes versus 9.1 ± 0.95 sperm/egg bound for the CD151 nulls oocytes, CD151 deletion had no apparent effect on sperm-egg binding rates *in vitro* (P = 0.346). CD151 deletion did however result in significant reduction in rates of sperm-egg fusion (P = 0.0001). An average of 3.0 ± 0.91 sperm/egg fused for wild type oocytes, while an average of only 0.73 ± 0.17 sperm/egg fused with CD151 null oocytes. These findings represent a reduction in oocyte fusibility of 75.4%.

This investigation was repeated using CD151 deficient mice on a FVBN genetic background. On the FVBN genetic background, CD151 deletion had no significant effect on sperm-egg binding rates (P<0.487). On average 6.50 ± 0.58 sperm/egg and 6.47 ± 0.73 sperm/egg bound to wild type and CD151 null oocytes respectively. Fusion rates were however significantly reduced in that 2.87 ± 0.24 sperm/egg fused with wild type oocytes compared to 1.72 ± 0.19 sperm/egg for CD151 null oocytes (P = 0.0003). Fusibility of oocytes from CD151 null mice of FVBN genetic background was therefore reduced by 39.9% relative to wild type oocytes.
Figure 3.13 Effect of CD151 deletion on sperm-egg binding and fusion

ZP-free oocytes were prepared from wild type and CD151 null mice of C57Black and FVBN genetic background. The oocytes were loaded with 10 µg/mL DAPI and then incubated with capacitated mouse spermatozoa for 60 min. Oocytes were washed, mounted on slides and rates of binding and fusion assayed. (A) For C57 Black mice, no significant difference was observed in rates of sperm binding between wild type and CD151 null oocytes (P = 0.346). Of the spermatozoa bound to wild type oocytes, 35.6% achieved successful membrane fusion. In contrast, only 8.04% of sperm bound to CD151 null oocytes achieved successful membrane fusion (P = 0.0001). Fusion rates were therefore reduced by 75.4% in CD151 null oocytes from C57Black mice. (B) Deletion of CD151 from FVBN females revealed no effect on sperm-egg binding rates in that no significant difference was evident between average binding rates for wild type and CD151 null oocytes (P = 0.487). Of sperm bound to wild oocytes, 44.2% achieved successful membrane fusion. In contrast, only 26.5% of sperm achieved successful membrane fusion upon binding to CD151 null oocytes (P = 0.0001), equalling a reduction in oocyte fusibility of 39.9%. (Data are mean ± sem, n = number of oocytes, *P = 0.0003, **P = 0.0001; unpaired Student T-test).
3.3.4.4 CD9 Expression: Wild Type vs CD151 Null Oocytes & Ovarian Sections

Having observed the effect of CD151 deletion on sperm-oocyte fusion, possible mechanisms by which CD151 deletion may influence oocyte fusibility were investigated. Wright and colleagues reported that CD9 expression was significantly reduced on epidermal keratinocytes from CD151 null mice (Wright et al., 2004a). A similar situation in the oocyte could account for the observed reduction in fusibility of the CD151 null oocytes. As such, CD151 null oocytes were investigated for possible altered CD9 expression levels. Quantitative confocal microscopy was used to compare CD9 expression levels on the cell surface of wild type and CD151 null oocytes. With confocal settings maintained, no discernable difference was observed in CD9 expression levels on CD151 null oocytes compared to oocytes from wild type females. In light of this unexpected finding, expression levels of CD81 were also examined. However CD81 could not be detected on the surface of either wild type or CD151 null oocytes with the reagents available.
Figure 3.14 Expression of CD9 and CD81 live wild type and CD151 null oocytes
ZP-free oocytes were isolated from wild type and CD151 null mice. Oocytes were incubated for 30 min in the presence of rat anti-mouse CD9 (KMC8 clone) or biotin-tagged hamster anti-mouse CD81 at final dilutions of 1:100 in α-MEM. Oocytes were transferred through 3 × 100 μL washes before being incubated for a further 30 min in either 1:100 goat anti-rat-FITC or 1:100 streptavidin-FITC. Oocytes were again washed before mounting on slides and viewing at 250 × magnification by confocal microscopy. CD9 was successfully detected on the surface of both wild type and CD151 null oocytes. Intensities of the fluorescent labeling for these oocytes revealed no apparent difference in CD9 expression levels between wild type and CD151 null oocytes. CD81 could not be detected on wild type or CD151 null oocytes.
CD9 localisation was also visualised by immunohistochemistry. Consistent with successful CD9 labelling of live oocytes, CD9 expression was again detected on the surface of oocytes within ovarian sections from both wild type and CD151 null females. CD81 expression was not analysed by means of immunohistochemistry given that inability to detect CD81 on the surface of live oocytes with the reagents available.

Figure 3.15 Expression of CD9 on wild type and CD151 null ovarian sections
Ovaries were excised from wild type and CD151 null mice, washed in PBS and fixed in 4% paraformaldehyde in PBS. Ovaries were then embedded in paraffin and 5 µm sections cut. Standard immunohistochemistry procedures were applied using rat anti-mouse CD9 at 1:100 dilution as the primary antibody and alkaline phosphatase-conjugated goat anti-rat IgG at 1:250 dilution as the secondary antibody. Positive labelling (▼) was observed around the periphery of oocytes from both wild type and CD151 null ovaries.
3.4 Discussion

The overall purpose of the research described in this chapter was to identify robust model systems that could be used to monitor the biochemical changes in the oolemma associated with the sperm-oolemmal binding and fusion. As previously stated, some conjecture existed at the commencement of this project as to whether immature GV phase oocytes had a limited capacity to bind to and fuse with capacitated spermatozoa. A reduced ability of immature oocytes to bind and fuse with sperm was an attractive notion, as it would provide a rationale for comparing the surface proteome of fertilisation incompetent GV oocytes with their fertilisation competent MII counterparts. To address this possibility, an *in vitro* oocyte maturation system was developed to enable the preparation of both immature GV and mature MII oocytes from a single pool of isolated oocytes. The technique was reliant on observations that immature oocytes undergo spontaneous maturation as a result of decreasing intracellular cAMP levels (Conti et al., 2002; Downs and Hunzicker-Dunn, 1995; Nebreda and Ferby, 2000; Qian et al., 2001). Within the ovary, oocytes are supported by the surrounding granulosa cells. However, they are also developmentally inhibited by the delivery of cAMP from the granulosa cells to the oocytes by means of trans-zonal gap junctions. As a result, oocytes are maintained in an arrested state at prophase of meiosis I. However, ovulation or the aspiration of the oocytes from the ovary, results in the loss of these junctions and, as intracellular cAMP levels decrease, meiotic division resumes until the oocytes again arrest at metaphase of meiosis II. During these *in vitro* investigations high intracellular cAMP levels were maintained artificially within isolated oocytes by inclusion of milrinone in the incubation medium. As milrinone is a PDE3 inhibitor, the degradation of cAMP was prevented and intracellular levels of cAMP remained sufficiently high to maintain meiotic arrest, as evidenced by the presence of the GV within the oocytes incubated in milrinone (see Figure 3.1).

Oocytes from both the GV and MII stages of development were then assessed for their capacity to be fertilised by capacitated spermatozoa. Representative images of the results for these sperm-egg binding and fusion assays are presented in Figure 3.2. The results of this study provided conclusive evidence that immature GV phase oocytes do indeed possess the capacity to both bind and fuse with capacitated spermatozoa. It was observed that capacitated spermatozoa readily bound to the membranes of immature GV phase oocytes as well as their matured MII counterparts (see Figure 3.2 A and C).
In addition, through the use of DAPI in conjunction with epifluorescence microscopy, successful sperm-egg membrane fusion events were visualised for both GV and MII phase oocytes (see Figure 3.2 B and D). The major difference between oocytes at different stages of maturation is that only mature oocytes had the competence to decondense the sperm nuclei following fusion with the oolemma.

The findings, which clearly demonstrated the fertilising capacity of both GV and MII oocytes, had important implications for the future direction of this project, as it meant that the proteins involved in sperm-egg binding and fusion were already expressed on the surface of immature oocytes. A proteomic comparison of membrane proteins expressed in immature and mature oocytes would thus not be an appropriate model for identifying constituents of the oolemma involved in binding and fusion.

With a proteomic comparison of GV and MII oocytes no longer able to provide a means of discriminating which oolemmal proteins were responsible for mediating fertilisation, an alternative approach was adopted based on previous studies emphasising the importance of GPI anchored proteins in sperm-egg interaction. Thus, *in vitro* studies published by Coonrod et al., 1999 indicated that enzymatic cleavage of GPI-anchored proteins from the oolemma surface profoundly disrupted sperm-egg interaction (Coonrod et al., 1999a; Coonrod et al., 1999b). This finding was later confirmed in 2003 through *in vivo* studies which demonstrated that oocyte specific deletion of genes encoding proteins necessary for GPI anchor biosynthesis resulted in severely diminished fertility in female mice (Alfieri et al., 2003). Despite strong evidence suggesting that a GPI-anchored protein(s) was implicated in gamete interaction at the level of sperm-egg binding, the identity of this important player in the molecular basis of sperm-egg interaction has never been elucidated. As such, the characterisation of egg membrane GPI-anchored proteins was deemed to be of high priority, with the project ultimately aiming to identify the specific GPI-anchored protein(s) mediating sperm-egg interaction and their subsequent characterisation.

In addition to the importance of GPI-anchored proteins, relevant literature has identified the importance of the tetraspanins as mediators of sperm-egg interaction. Simultaneous publications from three separate laboratories reported severely affected fertility of female mice deficient in CD9 (Kaji et al., 2000; Le Naour et al., 2000; Miyado et al.,
Further investigation later revealed that expression of the exogenous tetraspanin CD81 could partially rescue infertility in the CD9 deficient oocytes (Kaji et al., 2002). Infertility of CD9 knockout mice was reported to be attributable to a lack of fusibility of the oocytes from these mice. So whilst GPI-anchored proteins appeared to be important in sperm-egg binding, tetraspanins appeared to be important in the subsequent events of membrane fusion. Taken together, these two classes of proteins provided a system of exoplasmic cell fusion where binding and fusion events were separated, not unlike that observed for envelope virus-host cell fusion.

The bacterial derived enzyme PI-PLC specifically hydrolyses the inositol moiety of the GPI-anchor to achieve effective release of GPI-anchored proteins from host cell membranes. Coonrod and colleagues (Coonrod et al., 1999a; Coonrod et al., 1999b) had previously utilised PI-PLC to cleave GPI-anchored proteins from the surface of live eggs during their initial discovery into the importance of GPI-anchored proteins in sperm-egg interaction. Since this research was published in 1999, additional enzymes such as GPI-PLD and ACE have been discovered to possess similar GPIase activities. Previous investigations on HeLa cells, utilising both GPI-PLD and PI-PLC, revealed that GPI-PLD was the more efficient enzyme of the two when it came to cleaving the complement of GPI-anchored proteins tethered to the membrane of these cells (Elortza et al., 2006). However GPI-PLD and ACE had not been utilised to investigate the role of GPI-anchored proteins on the egg surface during sperm-egg interaction. As such, a series of studies were performed to: (i) determine the most efficient means of cleaving GPI-anchored proteins from oocyte membranes, and (ii) confirm the inhibitory effect of GPI-anchor protein release on sperm-egg interaction.

To determine the effectiveness with which the three enzymes PI-PLC, GPI-PLD and ACE cleaved GPI-anchored proteins from the surface of oocytes, an assay was developed whereby effective GPI-anchored protein release could be ascertained by monitoring expression of the known egg GPI-anchored protein, CD55 (Alfieri et al., 2003). The results of these studies, which are summarised in Figure 3.3, revealed that PI-PLC was indeed effective in releasing GPI-anchored proteins from the membranes of live eggs. Through the use of confocal microscopy and immunocytochemical detection of CD55, it was observed that incubating ZP-free mouse oocytes in 5 U/mL PI-PLC resulted in almost complete abolition of fluorescent CD55 detection. Whilst PI-PLC
produced dramatic inhibition of the CD55 signal, it was observed that both GPI-PLD and ACE failed to result in a loss of CD55 expression. This was unexpected given that GPI-PLD was previously reported to cleave a more comprehensive complement of GPI-anchored proteins from the surface of HeLa cell membranes than PI-PLC (Elortza et al., 2006). Further investigation revealed that the lack of GPIase activity observed upon treating live oocytes was due to the fact that GPI-PLD requires the presence of a detergent. Like many other eukaryotic phospholipases, evidence suggests that GPI-PLD action is restricted by the physical state of the phospholipid bilayer in which the substrate GPI-anchored protein is embedded (Low and Huang, 1991). Solubilisation of membranes with detergents therefore enhances access of GPI-PLD to these substrates.

Since oocytes were to be subjected to sperm-egg binding and fusion assays following GPI-anchor protein cleavage, destroying the cells by protein extraction was obviously not an option. GPI-PLD was therefore not suitable for use in the context of this project. In regards the GPIase activity previously reported for ACE (Kondoh et al., 2005), analysis of the effect of this enzyme on live oocytes revealed no effect on CD55 detection relative to non-treated control oocytes. Both the somatic and testicular isoforms of ACE are reported to possess GPIase activity (Kondoh et al., 2005), however once again the authors report this activity based on assays where GPI-anchored proteins were suspended in detergent solutions. It is therefore possible that somatic ACE, as analysed in these experiments, requires presence of a detergent, similar to GPI-PLD, and as such failed to cleave GPI-anchored proteins from live oocytes.

Based on results of the CD55 assay, it was apparent that bacterial derived PI-PLC represented the most effective means of cleaving GPI-anchored proteins from the surface of live oocytes and therefore best served the needs of this project. Therefore, bacterial derived PI-PLC was used throughout the remainder of these investigations to achieve GPI-anchor protein cleavage.

Having established the efficacy of the PI-PLC treatment, experiments were performed to ascertain whether GPI-anchor protein release by PI-PLC was indeed effective in inhibiting sperm-egg interaction. Preliminary experiments were conducted whereby oocytes were pre-incubated in 5 U/mL PI-PLC prior to being utilised in sperm-egg binding and fusion assays. Under these conditions, treatment of oocytes with 5 U/mL
PI-PLC, gave effective inhibition of sperm-egg binding and fusion rates. Following on from these positive results, dose dependency studies were performed to determine the optimum PI-PLC concentration required to produce the desired inhibitory effect. During these studies, the results of which appear in Figure 3.4, oocytes were pre-treated with either 0 (control), 0.05, 0.5, 1.0 or 5.0 U/mL PI-PLC for 60 min prior to being subjected to the sperm-egg binding and fusion assay. The results of this study yielded three important findings. Firstly, that 1 U/mL PI-PLC treatment was sufficient to induce a level of inhibition comparable to that of the maximum observed for the 5 U/mL treatment. This meant that 1 U/mL PI-PLC could be used for future studies to achieve effective inhibition while providing significantly greater economical use of the enzyme. Secondly, that since heat denaturation of the enzyme resulted in loss of the inhibitory effect; the result could genuinely be attributed to the action of the enzyme. Thirdly and most importantly, PI-PLC dependent inhibition of sperm-egg interaction occurred at the level of sperm-egg binding. This latter finding is important as it provides some initial insight into the role of the GPI-anchored proteins in sperm-egg interaction and identifies these important proteins as most likely fulfilling a role in sperm-egg recognition and binding, as opposed downstream events such as membrane fusion.

Based on the effectiveness with which PI-PLC was able inhibit rates of sperm-egg binding, it was believed that the identification of GPI-anchored proteins on the egg surface would constitute a major advancement in understanding the molecular basis of sperm-egg interaction. As such the identification of these important proteins by either proteomic or bioinformatics-based techniques was deemed a major priority for this research project.

Following collection of this series of data confirming the importance of GPI-anchored proteins in sperm-egg interaction, characterisation studies were expanded in an attempt to gain further insight into other factors that effect sperm-egg interaction. The approach utilised was to expose oocytes to a range of xenobiotic (mainly quinone) reagents in the expectation that one of more of these compounds may exhibit the capacity to influence sperm-egg interaction. In the event that a compound was discovered to either positively or adversely affect sperm-egg interaction, it was anticipated that understanding the chemical properties of that compound and how it affected the biology of the oocytes
would provide further insight into the oocyte’s mechanisms mediating sperm-egg interaction.

The array of xenobiotic agents screened in these experiments included hydroquinone, benzoquinone, duroquinone, menadione, bis maleimide, iodoacetamide and TCEP. The experimentation itself entailed incubating ZP-free oocytes in media containing one of these compounds for 20 min, then assessing the impact of the exposure on rates of sperm-egg binding and fusion. The study, which represented an attempt to broaden the current state of knowledge as to the factors that affect sperm-egg interaction, was successful in identifying two compounds with the capacity to strongly influence sperm-egg interaction, and a third compound with marginal impact.

In the data presented in Figure 3.5, it was observed that a 20 min exposure of oocytes to 10 µM benzoquinone or 10 µM bis maleimide resulted in drastic inhibition in rates of sperm-egg interaction. In both instances, this inhibition occurred at the level of sperm-egg binding, in that relative to the DMSO control, binding rates for benzoquinone and bis maleimide treatments were reduced by 81.4% and 74.1% respectively. In addition, it was revealed that treatment of oocytes with 10 µM menadione resulted in a 27% reduction in the rates of sperm-egg binding. Having identified compounds with a capacity to strongly inhibit sperm-egg binding rates, a series of experiments were performed to further characterise these effects.

Preliminary studies utilised the xenobiotic reagents at 10 – 25 µM. To gain a better understanding of the levels at which these compounds were active in inducing inhibition, dose dependency studies were performed, the results of which are presented in Figure 3.6. The effect of pre-treating oocytes with benzoquinone and bis maleimide were analysed at 0, 0.1, 1.0 and 10 µM. At 0.1 and 1.0 µM, bis maleimide exhibited no effect sperm-egg interaction. At a final concentration of 10 µM however, bis maleimide again induced significant inhibition in the rate of sperm-egg binding. Similarly, pre-incubating oocytes in 0.1 and 1.0 µM benzoquinone had no effect on sperm-egg binding rates. At final concentrations of 10 µM however, benzoquinone was again observed to dramatically inhibit rates of sperm-egg binding relative to oocytes of the DMSO control.
The fact that benzoquinone and bis maleimide suddenly induced their inhibitory effects at $\geq 10 \, \mu M$ in a seemingly ‘all or nothing’ manner raised concerns that the observed inhibitory effects may in fact be attributable to the agents killing the oocytes. To address this concern, two different oocyte viability assays were performed. The eosin dye exclusion test and trypan blue dye exclusion test enable visual discrimination between viable and non-viable cells. Non-viable cells, which have lost the ability to regulate the flow of substances across their membranes, stain either red (eosin) or blue (trypan) during these viability tests as the dyes are able to permeate the cell membranes. Results of the viability studies, which appear in Table 3.1, demonstrated quite conclusively that oocytes remained viable following exposure to xenobiotics at concentrations of up to 10 $\mu M$.

The positive results of the viability studies therefore alleviated concerns that the inhibition in sperm-egg binding observed for benzoquinone and bis maleimide treatments were simply the result of oocyte death. Continuing with the characterisation studies, investigations were undertaken to determine the time dependency of the xenobiotic effects. By examining the period of time required for the xenobiotics to induce their inhibitory effects, it was believed that some insight may be obtained as to their modes of action. At this stage it was postulated that the xenobiotics were inhibiting sperm-egg interaction possibly through alkylating essential thiol residues within the egg surface proteins mediating sperm-egg binding, or by stimulating the generation of various ROS, which might then have exerted deleterious effects on the molecular players involved. Thiol alkylation is a rapid process whilst the accumulation of ROS as a result of redox cycling requires a greater amount of time. As such, time dependency studies in which the inhibitory effects of benzoquinone and bis maleimide were observed after only brief exposure to the agents may suggest mechanisms such as thiol alkylation were responsible, while the requirement for longer periods of incubation would suggest redox cycling or other enzymatic based processes were involved.

Figure 3.7 illustrates the results of the time dependency studies during which oocytes were incubated for either 2 or 20 min in the presence of 10 $\mu M$ benzoquinone or bis maleimide, both of which had previously demonstrated inhibitory effects at this concentration. Also included at both time points was a 10 $\mu M$ iodoacetamide treatment,
which was incorporated as a negative control. During this assay, oocytes were removed from the xenobiotic containing media after just 2 or 20 min and briefly washed, DAPI loaded and injected into droplets of capacitated mouse sperm under standard IVF conditions. As can be seen, none of the three xenobiotic agents exhibited any inhibitory effect on rates of sperm-egg interaction after just 2 min of oocyte exposure. Upon increasing the exposure time to 20 min however, benzoquinone and bis maleimide again resulted in a significant inhibition of sperm-egg binding rates. Conversely, as anticipated, 10 µM iodoacetamide had no effect on binding rates after 20 min of exposure.

Based on the previously outlined rationale for the time dependency studies, the results presented in Figure 3.7 therefore provided some support that an enzymatic-based process such as redox cycling was the underlying mechanism attributable to the observed inhibition. This was unexpected given that while benzoquinone is capable of both thiol alkylation and redox cycling, bis maleimide is a thiol alkylating agent incapable of redox cycling (Fickentscher and Kohler, 1976; Phelps and Walker, 1999). The results presented in Figure 3.7 therefore suggest that the initial rationale for the time dependency studies was incorrect, and that alkylation of protein thiol groups requires longer periods of time then initially anticipated. Evidence supporting this oversight may be obtained upon looking to current proteomic techniques, such as 2D PAGE and trypsin digestion. Protocols for these techniques incorporate protein alkylation steps and typically allocate 15 – 60 min for the completion of the process. It remained possible therefore that thiol alkylation was occurring, but that the process required more then two minutes to reach a biologically detrimental level.

To obtain a more conclusive answer, experiments were undertaken to directly visualise the effect of various xenobiotic agents, including benzoquinone and bis maleimide, on egg surface thiols. For the purpose of these experiments, the thiol reactive probe AlexaFluor 488 C5 maleimide was employed. This probe is essentially a thiol alkylating agent with an attached fluorochrome to enable visual detection of labelling. Using this probe, the effect on surface thiol status after 20 min incubation in 10 µM benzoquinone, hydroquinone, duroquinone, menadione or bis maleimide was examined, as well as 20 min incubation in 1 mM of the thiol reducing agent TCEP.
The results of this study are summarised in Figure 3.8, where fluorescent labelling represents successful detection of thiol residues. In what could be regarded as an exciting find itself, untreated oocytes were found to express considerable levels of cell surface thiols, as evidenced by strong labelling across a majority of the cell surface. The arc of unlabelled egg surface represents the amicrovillar region where the polar body was extruded and where sperm are unable to bind. As a proof of concept, it can be seen that reducing egg surface thiols with 1 mM TCEP prior to incubation in AlexaFluor 488 resulted in a significant increase in the level of thiol detection. With confidence in the ability to quantitatively detect surface thiols, it was observed that incubation of oocytes in 10 μM benzoquinone resulted in almost complete abolition of thiol detection. This result was to be expected and is consistent with benzoquinone’s known thiol alkylating properties (Hughes et al., 2006). In addition, it was observed that while bis maleimide did not completely abolish AlexaFluor labelling, pre-incubating oocytes in the xenobiotic compound did reduce the level of cell surface thiol detection. Alternatively, hydroquinone and duroquinone, both of which had no effect on sperm-egg binding (see Figure 3.5), produced no effect on the level of cell surface thiol labelling. Agents with a capacity to inhibit rates of sperm-egg binding were therefore correlated with a capacity to impact upon egg surface thiol status, while those with no effect on sperm-egg binding had no effect of egg surface thiols.

Deviating from this paradigm however were the results obtained for menadione. Pre-incubating oocytes in 10 μM menadione significantly effected rates of sperm-egg binding (see Figure 3.5), however the impact was not as profound as that observed for benzoquinone and bis maleimide. Looking at results of the surface thiol labelling, it was seen that oocytes pre-incubated in menadione continued to exhibit cell surface AlexaFluor labelling, and in doing so appeared to deviate from the paradigm that inhibitory agents were correlated with thiol alkylating activity. However the AlexaFluor profile for menadione was unique in that it resulted in strong cytoplasmic labelling of the oocytes. In this regard, it is believed that menadione impacted upon sperm-egg interaction through mechanisms distinctly different from that involved in benzoquinone and bis maleimide inhibition. The fact that menadione’s impact on sperm-egg interaction was not as pronounced as that observed for benzoquinone and bis maleimide may be indicative of an alternative mode of action.
The mode of action of menadione remains yet to be elucidated, however it is important to consider that within oocytes menadione may be metabolised into thiol alkylating or redox active forms (see Table 3.1). The fact that Figure 3.8 demonstrates no menadione effect on egg surface thiol labelling suggests that the latter may be involved.

The discovery that benzoquinone and bis maleimide possessed the capacity to modify the oocyte in such a manner as to adversely effect sperm-egg interaction was a novel finding. Equally exciting was the discovery that agents with a capacity to inhibit sperm-egg interaction exhibited a correlative capacity to negatively impact upon egg surface thiol labelling.

Observations up until this point had revealed that both PI-PLC treatment and xenobiotic exposure of oocytes specifically affected sperm-egg binding as opposed to sperm-egg fusion. The question was therefore raised as to whether the two inhibitory techniques, PI-PLC treatment and xenobiotic exposure, were in fact both targeting the same subset of proteins. It was considered highly plausible that the PI-PLC-sensitive GPI-anchored protein(s) implicated in sperm-egg binding might contain thiol/disulphides that were essential to its receptor function. If this was indeed the case, one could anticipate that loss of egg surface GPI-anchored proteins may be reflected in a loss of surface thiol detection.

The results of this study are summarised in Figure 3.9. As these results and previously outlined results illustrate, surface thiol detection on untreated oocytes is very strong. Diminished thiol detection in response to PI-PLC treatment would indeed have been an exciting find. However, looking at the results presented in Figure 3.9, this was clearly not the case as 1 U/mL PI-PLC appeared to have no effect on the AlexaFluor 488 labelling intensity. Although disappointing, the result does not eliminate the possibility that a thiol containing GPI-anchored protein is implicated in sperm-egg binding. Since GPI-anchored proteins represent only a small fraction of membrane proteins on any cell type and are unlikely to be the only thiol containing class of proteins on egg surface. It therefore remains possible that a thiol containing GPI-anchored protein was released by PI-PLC, but that its release went undetected due to high levels of global thiol labelling.
A second postulated mechanism for benzoquinone and bis maleimide action was that these reagents might disrupt sperm-oolemmal interaction by inducing the vitelline block to polyspermy secondary to cortical granule extrusion. Cortical granule extrusion is an important step in the block to polyspermy and following successful fertilisation functions to render the egg membrane refractory to any further sperm binding. The cortical granules reside within the cortex of MII oocytes, just under the plasma membrane. Successful fusion with a spermatozoon stimulates calcium oscillations and this influx of calcium results in fusion of the cortical granules with the overlying egg plasma membrane (Schuel, 1985). As the cortical granules fuse with the membrane they release their contents onto the surface of the egg. The contents are known to modify the properties of the zona pellucida such that sperm are no longer able to bind and penetrate this structure (Yanagimachi, 1994b). However the cortical reaction also functions to deposit large quantities of egg and embryo-abundant peptidylarginine deiminase-like (ePAD) protein on the egg surface. ePAD, a 75 kDa protein, is thought to assist in preventing polyspermy as well as promoting implantation during embryo development (Wright et al., 2003), and will be addressed again in Chapter 4 during the results of proteomic analyses.

Unfortunately the assay to visualise the effects of the xenobiotics on cortical granule extrusion proved unreliable and problematic. Results of the investigations as to whether xenobiotic exposure of oocytes induced cortical granule extrusion therefore remain inconclusive at this stage.

Although the exact mechanism of action for benzoquinone and bis maleimide has not yet been clarified, the results of these investigations have already provided some insight to the possible mechanisms mediating sperm-egg interaction. AlexaFluor 488 studies have clearly highlighted the fact that thiol residues exist on the surface of oocytes and that these residues are susceptible to both alkylation and oxidation by compounds such as benzoquinone. Previous studies in the field of envelope virus fusion with host cell membranes have implicated thiol/disulphide exchange (Barbouche et al., 2003; Fenouillet et al., 2001; Gallina et al., 2002; Ryser et al., 1994) as well as high-energy cross-strand disulphide bonds in membrane fusion (Wouters et al., 2004). This observation, taken together with that fact that binding and fusion between sperm and egg appear to be separate events mediated by different classes of proteins, for review
see (Primakoff and Myles, 2007), supports the notion that gamete membrane fusion mechanisms may parallel those of envelope virus-host cell membrane fusion.

The investigations discussed thus far have highlighted the importance of GPI-anchored proteins as likely oolemmal receptors for sperm-egg binding and report on the novel use of benzoquinone and bis maleimide to affect sperm-egg interaction. However, also of primary concern to this project was a class of proteins known as tetraspanins.

Tetraspanins as previously described are a family of proteins that span the plasma membrane of eukaryotic cells four times. Both the N- and C-termini of this class of protein reside within the cell giving rise to two extracellular loops known as EC1 and EC2 (Boucheix et al., 1991). While extracellular loop EC1 is small, EC2 is large and contains domains that are both conserved and variable between different tetraspanins (Seigneuret et al., 2001). Of key interest to investigators researching tetraspanins are the 4 to 6 conserved extracellular cysteine residues linked into 2 or 3 disulphide bonds.

Within the field of mammalian reproductive biology, the tetraspanin CD9 recently generated great interest following simultaneous publications by three separate laboratories identifying infertility in CD9 deficient female mice (Kaji et al., 2000; Le Naour et al., 2000; Miyado et al., 2000). Rubinstein et al., 2006 later demonstrated that this infertility could be partially rescued by inducing exogenous expression of another tetraspanin CD81 (Rubinstein et al., 2006a). In light of the redundancy demonstrated by tetraspanins in both the egg and other biological systems, it was plausible that other tetraspanins may be expressed on the surface of eggs and that these tetraspanins may also be implicated in sperm-egg interaction.

During the course of this project, the opportunity arose to acquire a strain of C57 mice deficient in the tetraspanin CD151. Although the effect of CD151 deletion on these mice had already been characterised and only minor abnormalities reported (Wright et al., 2004a), staff from the animal services unit claimed that the animals were reportedly ‘harder to breed’ then their wild type littermates. Generally this would be attributed to embryonic lethality associated with a gene deletion resulting in death of the homozygous mutant offspring, however CD151 deletion was not embryonically lethal (Wright et al., 2004a). Analysis of the breeding data for CD151 null females presented
in Table 3.1 supported these claims in that compared to wild type females, slight decreases were observed for the average litter size and the number of offspring per female per month. With apparent breeding problems existing in these animals and the previously outlined importance of CD9 and CD81 in female fertility (Kaji et al., 2000; Le Naour et al., 2000; Miyado et al., 2000), further experiments were warranted to more closely examine the reproductive biology of these females.

Prior to investigating the events of sperm-egg interaction in CD151 null oocytes, experiments were performed to visually compare the histology of ovaries from wild type and CD151 null females. The results of this analysis, which are presented in Figure 3.10, were exciting in that ovaries from CD151 deficient females were found to be histologically abnormal. Compared to wild type ovaries, it was observed that CD151 null ovaries exhibited smaller corpora lutea and a greater incidence of atretic follicles occurred throughout the cortex and medulla regions. Unlike wild type ovaries, which exhibited good follicular cellular continuity and organisation, follicles within CD151 null ovaries appeared friable and in particular basement membrane detachment was evident between follicles and stromal layers. Overall, ovaries from CD151 deficient females exhibited a less ordered, less consistent structure and oocytes did not appear as healthy as their wild type counterparts.

Despite having documented abnormalities in the CD151 deficient ovaries, it was not known whether CD151 was even expressed in wild type murine ovaries and whether the protein could therefore even be accountable for the observed affects following its deletion. To determine whether CD151 was indeed expressed in the murine ovary, studies were performed to attempt to detect CD151 at both the gene and protein level. A CodeLink™ microarray analysis successfully detected CD151 transcript within both neonatal and adult ovaries (see Figure 3.11). To provide further evidence supporting CD151 expression within murine ovaries, CD151 protein was detected by means of immunoblot analysis (see Figure 3.12). The antibody LAI-2 was raised in rabbits against recombinant preparations of murine CD151. During immunoblot analysis, LAI-2 recognised a protein band of the correct molecular weight for CD151 within protein extracts from wild type ovaries. Furthermore, 28-30 kDa protein band recognised by LAI-2 was absent in the protein extract prepared from CD151 null ovaries.
Taken together, the CodeLink™ microarray data and the immunoblot analysis provided strong evidence supporting the expression of CD151 within murine ovaries. Following on from these results, experiments were conducted to access the direct effects, if any, of CD151 deletion on sperm-egg interaction. Assessing the effect of CD151 deletion on sperm-egg interaction was performed through use of the sperm-egg binding and fusion bioassay whereby wild type or CD151 null oocytes were incubated with capacitated wild type sperm. Once again exciting results were obtained.

These exciting results, which appear in Figure 3.13, demonstrated that on average, deletion of CD151 had no effect on rates of sperm-egg binding: both wild type and CD151 null oocytes binding an average of approximately 9 sperm per oocyte. However through the use of DAPI to visualise membrane fusion, it was observed that substantial differences existed in the rates of sperm-oocyte fusion. Upon examining the wild type oocytes, it was observed that approximately 35% of sperm bound to the egg had achieved successful membrane fusion. In comparison, it was observed that only approximately 7% of sperm bound to oocytes from CD151 deficient mice had achieved successful membrane fusion. Thus, unlike the release of GPI-anchored proteins or xenobiotic exposure in this instance the proteins implicated in sperm-egg binding remained unaffected whilst those involved in the downstream events involving membrane fusion were clearly compromised.

The observation that oocytes from CD151 deficient mice exhibited impaired rates of sperm-egg fusion was a significant finding. As was the case with oocytes from CD9 and CD81 deficient mice, oocyte fusibility once again appeared to be affected as a result of tetraspanin deletion. Documenting reduced fusibility in oocytes from CD151 deficient females raised the series of obvious questions: was fusogenicity affected because CD151 interacted with sperm proteins directly? Or was formation of an egg receptor complex disrupted as a result of CD151 deletion? Given that CD9 and CD81 have already been implicated in oocyte fusibility and that the expression of one tetraspanin has been reported to affect the expression of other tetraspanins, the question was also raised: was reduced fusibility of oocytes from CD151 deficient females attributable to CD151 deletion affecting CD9 or CD81 expression?
Although the antibody LAI-2 yielded encouraging results in the immunoblot detection of CD151, the antibody, which was produced in house by an associated laboratory, was extremely problematic and difficult to optimise. At the time of writing, there remained no reliable commercially available antibodies for the detection of murine CD151. As a result, obtaining confocal imagery of CD151 expression on live ZP-free oocytes proved impossible. Ideally a CD151 antibody would have been valuable in attempting to competitively inhibit sperm-egg fusion on wild type oocytes. Similar to use of the CD9 antibody JF9 to inhibit to sperm-egg interaction (Chen et al., 1999), effective inhibition of oocyte fusibility by a CD151 antibody could have been regarded as evidence of a trans interaction, whereby CD151 interacted directly with proteins on the sperm surface. Although an effective CD151 antibody was not available to perform such analyses, antibodies were however available to assess affects on tetraspanin co-expression.

During the course of these investigations, decreased CD9 expression as a result of CD151 deletion was deemed to be the most plausible explanation for the observed affect on oocyte fusibility. Confocal imagery of CD9 expression levels on wild type and CD151 null oocytes was therefore expected to reflect this. However the results presented in Figure 3.14 did not support this hypothesis. On wild type oocytes, CD9 expression appeared strong with labelling observed across the entire surface of the cells, with exception of the amicrovillar region, as anticipated. Comparison of wild type CD9 labelling with that observed for CD151 null oocytes under quantitative conditions demonstrated that CD9 expression remained high despite CD151 deletion. Attempts to label CD81 in a similar fashion failed to detect CD81 on wild type or CD151 null oocytes.

As a means of confirming unaltered CD9 expression on wild type and CD151 null oocytes, CD9 expression was again visualised using wild type and CD151 null ovarian sections. Results of the ovarian immunohistochemistry, Figure 3.15, confirmed results previously observed for CD9 labelling on live oocytes in that CD9 expression was detected on both wild type and CD151 null ovarian sections. In both cases, labelling was observed around the periphery of the oocytes, consistent with CD9’s known distribution as an integral membrane protein.
The fact that CD151 deletion resulted in reduced oocyte fusibility with no apparent effect on CD9 expression has important ramifications. The findings suggested that CD151 was itself involved mediating oocyte fusibility. The precise mechanism by which CD151 may be mediating oocyte fusibility is yet to be identified, possible modes of action of CD151 are however explored in-depth during the final discussion.

In summary, investigations to develop robust models for investigating the molecular mechanisms underpinning sperm-egg interaction have centralised on three main areas. The first was the use of PI-PLC to release GPI-anchored proteins from the egg membrane that appear to be implicated in sperm-egg binding. The effect of cleaving GPI-anchored proteins on fertilisation was a phenomenon originally observed by Coonrod and colleagues. The results presented herein corroborate this effect and elaborate the first ever application of other GPIase enzymes to this system. PI-PLC was determined to be the optimal enzyme for use on live oocytes and represented a viable means of obtaining pools of GPI-anchored proteins for proteomic analysis.

The second investigative focal point was the use of xenobiotics to influence sperm-egg interaction. Several xenobiotic agents were screened in the hope that one or more of these compounds may exhibit a capacity to affect sperm-egg interaction. It was anticipated that an understanding of a particular compound’s chemical properties and the way in which it might have modified oocyte surface protein structure and function would help unlock as yet unknown aspects of oocyte biology. As a result of this approach, two different compounds were discovered to possess the capacity to dramatically inhibit sperm-egg interaction, with a third producing mild effects. All 3 compounds inhibited at the level of sperm-egg binding and while understanding of their chemical properties have not yet yielded any insights into the underlying mechanisms, they do support the notion that binding and fusion are mediated by separate events and emphasised the potential involvement of thiol/disulphide exchange in the former. In doing so, the results obtained from these studies strengthen the emerging parallel between fertilisation and envelope virus-host cell fusion.

Finally, as sperm-egg binding was being modulated upon by GPI-anchored protein studies and xenobiotic analyses, tetraspanin studies delved into downstream events of membrane fusion. In what represents an exciting and novel finding, CD151 deletion
disrupted sperm-egg fusion and in doing so reiterated a growing consensus that binding and fusion are mediated by separate mechanisms. It was seen that CD151 deletion did not affect CD9 co-expression. This finding suggests that CD151 itself may be involved in mediating sperm-egg fusion.
CHAPTER 4:

Proteomics and Bioinformatics
CHAPTER 4: PROTEOMICS AND BIOINFORMATICS

4.1 Introduction

The term ‘proteome’ describes the entire complement of proteins encoded by an organism’s genome (Wilkins et al., 1996). Proteomics is the study of the function of these proteins, and in recent times the field has seen a surge in both the popularity of this experimental approach as a means to obtaining biologically relevant data, as well as in advances in the technology itself. Despite advances made towards the development of better proteomic techniques, there remain many variables within the “protein world” that present challenges in relation to the acquisition of meaningful data. These often unavoidable problems include limited and variable sample material, sample degradation, vast dynamic ranges in protein abundance, a myriad of post-translational modifications, boundless tissue types, variation generated by differing developmental and temporal expression patterns, as well as disease and drug related alterations (Tyers and Mann, 2003). Despite these challenges, proteomics in its early stages already represents an excellent means of generating large-scale data sets for protein identification, protein-protein interactions, protein composition of specific organelles as well as the protein profiling of pathological conditions in a search for new diagnostic tools (Tyers and Mann, 2003).

The experimental advantages afforded by proteomics should not be used as a stand alone means of resolving biological issues, but rather in conjunction with other powerful experimental techniques such as microarray-based expression profiles, phenotypic profiling at both the cellular and organism level, as well as systems biology. The ability to integrate these and other such techniques whereby the strength of one complements the strength of another will only serve to enhance our ability to build comprehensive data sets of gene structure and function, and determine where these genes fit in relation to the biology of entire systems.
4.2 Experimental Rationale
The overall goal of this project was to characterise the factors important in murine sperm-egg interaction and expand on the current state of knowledge by identifying the important proteins implicated in this event. Outlined in the ensuing chapter are the results of the experimental techniques that were applied in order to progress from simply understanding that a particular class of protein is important in sperm-egg interaction, such as GPI-anchored proteins (see Section 3.3.2), through to obtaining “real” protein identities able to be characterised through in-depth functional studies. In order to ascertain which proteins are important in sperm-egg interaction, this project has utilised a proteomics based approach directed toward large-scale identification of proteins within complex mixtures of cell extracts, as well as the identification of proteins within fractionated samples of interest.

The murine oocyte has been the focus of studies utilising proteomic techniques in the past. One such example is the attempt made by Coonrod et al., 1999 to investigate the role of GPI-anchored proteins in sperm-egg interaction. The study provided strong evidence correlating a loss of egg surface GPI-anchored proteins with decreased capacity for the oocytes to be bound by sperm (Coonrod et al., 1999b). The study advanced to the point of visualising the two dimensional SDS-PAGE profile of GPI-anchored proteins released from the surface of eggs, however the identities for these important proteins were never obtained, and as such the GPI-anchored proteins implicated sperm-egg interaction remain yet to be identified. To resolve this issue, the experimental techniques applied within this chapter were directed towards enabling the proteomic analysis of murine oocyte proteins. To maximise the relevance of the data obtained, protein fractionation techniques were applied to purify protein classes of interest, such as surface proteins and GPI-anchored proteins, from whole egg lysates.

Due to the fact that the ultimate endpoint for this series of experiments was to obtain successful protein identifications, the studies performed revolve around the use of nanoflow LC-MS/MS. With that in mind, the experimental aims for these series of investigations were to:

1) Effectively fractionate a pool of whole egg lysate into GPI-anchored proteins, egg surface proteins and remaining egg lysate proteins.
2) Subject these pools of proteins to standard proteomic techniques enabling their visualisation and the collection of mass-to-charge ratio data.

3) Process this mass-to-charge ratio data to compile lists of protein identities for the fractionated protein samples.

4) Utilise bioinformatics analysis of mouse gene databases to complement the obtained proteomic data.
4.3 Results

4.3.1 SDS-PAGE Profiling of Protein Samples

4.3.1.1 Visualisation of Oolemmal Surface Proteins: GV vs MII Oocytes

Through utilising the strength and stability of the biotin-streptavidin interaction, it was possible to purify egg surface proteins from whole egg lysates. This technique was employed to enable the comparison of oolemmal protein profiles from immature GV oocytes against both in vitro matured MII oocytes and ovulated MII oocytes. ZP-free oocytes (GV phase, in vitro matured MII phase or ovulated MII phase) were incubated in the presence of 1 mg/mL Sulfo-NHS-LC-Biotin for 30 mins at 37°C. Following extraction of whole egg proteins (0.5% Triton X-100 in PBS), biotin-tagged surface proteins were purified by magnetic separation using M280 Streptavidin Dynabeads. Following elution from the magnetic beads, the biotin-tagged surface proteins, along with the unbound lysates and the final bead wash, were separated by SDS-PAGE and electroblotted onto nitrocellulose membranes. Membranes were blocked and then probed using streptavidin-HRP to enable visualisation of the biotin tagged proteins (see Section 2.9.3).

Biotin-tagged proteins were observed for the bead elute lanes only of both the GV phase and in vitro matured MII phase protein extracts, demonstrating that all biotin-tagged proteins were successfully purified from the whole egg extract. It was observed that GV phase and in vitro matured MII oocytes yielded very similar oolemmal protein profiles. In both cases, a multitude of proteins ranging from approximately 40 – 200 kDa were observed. In comparison the profile of oocyte surface proteins observed for ovulated MII oocytes varied significantly from the profile observed for GV and in vitro matured MII phase oocytes. Biotin labelling revealed that a large number of additional proteins were present on surface of ovulated oocytes compared to the non-ovulated oocyte (see Figure 4.1).
Surface proteins of ZP-free oocytes at 3 different developmental time points were labelled using Sulfo-NHS-LC-Biotin. Whole oocyte proteins were then extracted using 0.5% Triton X-100 in PBS. Biotin-labelled surface proteins were then purified from the whole egg lysate using M280 Streptavidin Dynabeads. After being eluted off the beads (95°C for 5 mins in SDS loading buffer), the purified surface proteins as well as unbound lysate and final bead wash fractions were separated by SDS-PAGE (7.5% gel) and electroblotted to nitrocellulose membrane. The membrane was then blocked (5% skim milk powder in TBS) and probed with streptavidin-HRP. For the Triton X-100 control, biotin-labelled proteins were not detected in any of the 3 fractions. For the GV phase egg extract, no labelled protein bands were observed in either the unbound or final wash fractions. A series of protein bands ranging in molecular weight from approximately 45 – 200 kDa were however released from the beads following boiling. Similarly, no labelled protein bands were observed for the unbound or final wash fractions of the in vitro matured MII phase egg extract, however a series of biotin labelled proteins was again observed in the bead elute fraction. These proteins again ranged from approximately 45 – 200 kDa, and revealed an SDS-PAGE profile markedly similar to that observed for the GV phase profile. Streptavidin-HRP detection of biotin labelled proteins from ovulated MII phase eggs revealed a greater number of protein bands than that observed for the GV and in vitro matured MII oocytes. Biotin labelled surface proteins from ovulated eggs ranged from approximately 20 – 210 kDa. Many of these protein bands did not correlate with bands observed for the GV and in vitro matured MII profiles.

Figure 4.1 Comparison of oolemmal protein profiles for GV, in-vitro matured MII and ovulated MII phase oocytes

Surface proteins of ZP-free oocytes at 3 different developmental time points were labelled using Sulfo-NHS-LC-Biotin. Whole oocyte proteins were then extracted using 0.5% Triton X-100 in PBS. Biotin-labelled surface proteins were then purified from the whole egg lysate using M280 Streptavidin Dynabeads. After being eluted off the beads (95°C for 5 mins in SDS loading buffer), the purified surface proteins as well as unbound lysate and final bead wash fractions were separated by SDS-PAGE (7.5% gel) and electroblotted to nitrocellulose membrane. The membrane was then blocked (5% skim milk powder in TBS) and probed with streptavidin-HRP. For the Triton X-100 control, biotin-labelled proteins were not detected in any of the 3 fractions. For the GV phase egg extract, no labelled protein bands were observed in either the unbound or final wash fractions. A series of protein bands ranging in molecular weight from approximately 45 – 200 kDa were however released from the beads following boiling. Similarly, no labelled protein bands were observed for the unbound or final wash fractions of the in vitro matured MII phase egg extract, however a series of biotin labelled proteins was again observed in the bead elute fraction. These proteins again ranged from approximately 45 – 200 kDa, and revealed an SDS-PAGE profile markedly similar to that observed for the GV phase profile. Streptavidin-HRP detection of biotin labelled proteins from ovulated MII phase eggs revealed a greater number of protein bands than that observed for the GV and in vitro matured MII oocytes. Biotin labelled surface proteins from ovulated eggs ranged from approximately 20 – 210 kDa. Many of these protein bands did not correlate with bands observed for the GV and in vitro matured MII profiles.
4.3.1.2 Visualisation of Oocyte GPI-Anchored Proteins

GPI-anchored proteins are a small subset of oolemmal proteins of great significance to this project. It was previously demonstrated that cleavage of GPI-anchored proteins from egg surfaces could be achieved by incubating oocytes in PI-PLC (see Figure 3.3), and that this cleavage resulted in dose-dependent inhibition of sperm-egg binding (see Figure 3.4). In light of these findings, the complement of GPI-anchored proteins released from ZP-free mouse oocytes was analysed by SDS-PAGE. For this purpose, ZP-free oocytes were incubated in BWW/PVA containing 1 U/mL PI-PLC for 60 min. The oocytes were then removed and the supernatant media droplets containing any released GPI-anchored proteins were collected. As a control, a collection of oocytes were ‘mock treated’ whereby oocytes were incubated in the absence of PI-PLC. Proteins present in the supernatant from this mock treatment therefore represented proteins spontaneously released into the culture media by untreated oocytes. Supernatants for both the mock treatment and PI-PLC treatment were collected. Any proteins present within these supernatants were recovered from the BWW/PVA solution by means of chloroform-methanol precipitation and resuspended in SDS loading buffer.

Upon separation by SDS-PAGE, it was observed that untreated oocytes spontaneously released a host of proteins into the egg culture media. Comparing the profile of spontaneously released mock treatment proteins against those present in the PI-PLC treatment, it was however observed that a total of seven protein bands were specific to the PI-PLC treated sample. With the exception of band 5, which was the correct molecular weight for the exogenous PI-PLC itself (35 kDa and previously visualised to migrate as a single band), these bands specific to the PI-PLC treated sample were therefore believed to represent GPI-anchored proteins released from the egg surface as a result of PI-PLC cleavage of their GPI anchors. Excluding the band of PI-PLC itself, these six protein bands exhibited approximate molecular weights of 110, 60, 55, 45, 27 and 17 kDa (see Figure 4.2).

To obtain an approximate indication of the relative abundances of the visualised proteins, a BSA gradient ranging from 500 – 5 ng of protein was also run on the gel. Of the six visualised bands attributable to GPI-anchored proteins, 5 revealed protein quantities <5 ng, with band 7 revealing an approximate quantity of 25 ng.
Figure 4.2 SDS-PAGE profile of proteins released from mock-treated or PI-PLC treated ZP-free oocytes

Approximately 400 ZP-free oocytes were treated with 1 U/mL PI-PLC in a 50 µL BWW/PVA droplet for 60 mins or mock treated whereby PI-PLC was omitted. Oocytes were removed and the supernatant media droplets were collected. The supernatant media droplets were subjected to chloroform-methanol precipitation. Resultant precipitates were then resuspended in 20 µL of SDS extraction buffer and loaded onto 7.5% SDS-PAGE gels. Following separation, proteins were silver stained for visualisation purposes. To aid in determination of protein quantification, a dilution series of BSA ranging from 500 ng down to 5 ng was included. A total of 12 protein bands were observed within the supernatant of PI-PLC treated oocytes. Of these 12 protein bands, only 7 were specific to the PI-PLC treatment group in that the remaining 5 bands were also observed within the media droplet from untreated oocytes. Protein bands specific to the PI-PLC treated sample exhibited approximate molecular weights of 110, 60, 55, 45, 35, 27 and 17 kDa.
4.3.1.3 **Fractionation of Oolemmal Surface Proteins from Whole Egg Lysates**

Although immunoblot detection of biotin tagged surface proteins was successful in enabling the comparison of surfaced proteins from GV and MII oocytes, the technique did not enable the visualisation of proteins that remained in the whole oocyte lysate given these intracellular proteins were not biotin tagged. As such, oolemmal proteins were again fractionated from whole egg lysates of GV phase oocytes by means of M280 Streptavidin Dynabead purification, however following separation of the resultant fractions by SDS-PAGE, gels were silver stained to enable the visualisation of all proteins.

Silver staining revealed a large number of proteins in the unbound fraction of the egg extract. These proteins constitute the non-biotin labelled proteins present in the whole egg lysate. Proteins present in this fraction spanned the entire range of the molecular weight range visualised of 19.3 – 206 kDa.
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Figure 4.3 SDS electrophoresis of biotin-labelled egg surface proteins and unlabelled cytosolic proteins from whole egg extracts

Surface proteins of ZP-free oocytes were labelled using Sulfo-NHS-LC-Biotin prior to extraction of whole egg proteins in PBS + 0.5% Triton X-100. Biotin-labelled surface proteins were then purified from the whole egg lysate using washed M280 Streptavidin Dynabeads. Unbound fractions were extracted and stored, after which the beads were washed 4 times by resuspension in Dulbeccos PBS. Finally beads were resuspended in SDS loading buffer and boiled for 5 mins to release any biotin labelled proteins bound to the beads. The resultant fractions were separated by SDS-PAGE (7.5% gel) and the gel silver stained. The PBS + Triton X-100 control revealed that a small number of proteins were released into the bead elute fraction following boiling of control beads in SDS. No protein bands were present for the unbound or final wash fractions. For beads incubated in the egg extract, a series of bands ranging in molecular weight from approximately 19 – 210 kDa were observed for the unbound fraction. None of the non-biotin labelled proteins from the unbound fraction carried through to the final wash in that no protein bands were eluted from the beads during the final wash. A seemingly different subset of protein bands ranging from 19 – 200 kDa was then eluted from the beads following boiling of the Dynabeads incubated in the egg extract.
4.3.1.4 Comparison of Oocyte Protein Profiles

Through the use of biotin labelling of the egg surface, it was possible to separate oocyte surface proteins from those proteins that constitute the remainder of the oocyte. Further fractionation of the GPI-anchored protein subclass of oocyte surface proteins was achieved through use of the bacterial enzyme PI-PLC. A direct comparison of these three fractions of oocyte proteins appears below.

Figure 4.4 Comparison of SDS-PAGE protein profiles of GPI-anchored protein, oocytes surface proteins and oocytes lysates fractionated from whole oocytes

GPI-anchored proteins (→) were cleaved from the surface of ZP-free oocytes and collected. Following biotin labelling of surface proteins, oocyte proteins were extracted using 0.5% Triton X-100. Surface proteins were then fractionated from the whole oocyte lysate by means of M280 Streptavidin Dynabeads. The proteins present in all 3 fractions were then separated by 1D SDS-PAGE and visualised by silver staining. Each protein fraction exhibited a distinctly different profile of protein bands.
4.3.2. **LC-MS/MS Analysis of Known Protein Standards**

4.3.2.1 *DeCyder* MS Visualisation of Peptide Detection Limits

Prior to beginning LC-MS/MS based attempts to identify proteins within potentially complex mixtures of protein fractions, it was first necessary to investigate the detection limits of the nanoflow chromatograph and mass spectrometer to be utilised. In order to achieve this, a solution was prepared containing a mix of 4 known proteins (BSA, α-Casein, β-Casein and Carbonic Anhydrase) at a final concentration of 1 mg/mL each. This preparation, which was trypsin digested, served as a protein standard and was used to: (i) ascertain the quantity of original starting material required in order to achieve successful detection of peptides, and (ii) demonstrate what level of peptide detection was required in order to achieve successful identification of all proteins represented within a peptide mixture.

Utilising *DeCyder* MS software, it was possible to construct virtual peptide elution and detection profiles, whereby one can gain a visual appreciation of the time point at which a particular peptide eluted from the LC column as well as the mass/charge ratio of that particular peptide as detected by the MS. Analysis of $4 \times 1$ ng, $4 \times 100$ ng and $4 \times 1 \mu$g of protein revealed that peptides could be detected with as little as $4 \times 1 \mu$g, however confident peptide detection was achieved at $\geq 100$ ng of protein.
A solution containing a mix of 4 known proteins (BSA, α-Casein, β-Casein and Carbonic Anhydrase) at a final concentration of 1 mg/mL each was prepared and trypsin digested. Aliquots of the tryptic peptides containing $4 \times 1$ ng, $4 \times 100$ ng or $4 \times 1$ µg of the proteins were then analysed by LC-MS/MS. **DeCyder** MS peptide profiles were generated and the peptide detection limits visualised. **DeCyder** MS analysis of a peptide mix containing 1 ng of each of the four proteins revealed approximately 5 – 10 peptide spots. The detected peptides eluted from the C18 column after 53 – 60 min of the LC run, and were barely detectable above background ion trace levels recorded for the 0.1% formic acid only blank. At $4 \times 100$ ng, a multitude of peptides spots were observed at levels that partially suppressed the background ion trace. This background ion trace was almost completely suppressed in the analysis of $4 \times 1$ µg of peptide mix, with a large number of protein spots detected across the course of the LC elution run.
4.3.2.2 **Sequest Protein Identities**

The *DeCyder* MS analysis of known protein standards demonstrated that peptides were detectable within peptide samples that contain as little as 4 ng of peptide sample (1 ng of each of 4 protein standards). However the detection of peptide spots does not guarantee successful identification of any or all of the proteins digested to produce a peptide. To determine the quantities of original protein sample that would be required in order to obtain successful protein identifications, the LC-MS/MS raw data obtained for the known peptide mixes was further analysed using Sequest software to attempt to obtain protein identities for each of the protein quantities analysed. For the purpose of the known standards, the search was performed against the bovine peptide fingerprint database as the known proteins utilised as standards were of bovine origin.

When the peptide mix containing 100 ng of each of the 4 known proteins was analysed in-house by 1D LC-MS/MS, α-casein, β-casein and BSA were successfully identified, carbonic anhydrase however was not successfully identified. The commercially available proteins utilised as standards are known to contain small quantities of contaminating proteins. One of these proteins, haemoglobin, was also identified within the 4 × 100 ng sample. Within the 4 × 1 µg sample, carbonic anhydrase was once again absent from search results, however α-casein, β-casein and BSA were again successfully identified. In addition, numerous other contaminants were detected with haemoglobin again amongst the search results.

When a peptide mix generated from a protein sample containing just 1 ng of each of the four known proteins was analysed, α-casein was the only protein of the 4 known standards successfully identified (see Table 4.1).
Table 4.1 Protein identities obtained for known protein standards analysed by LC-MS/MS
Protein identities mapped by Sequest against the bovine database for the different quantities of peptide mix analysed by LC-MS/MS.

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<th>Obtained Protein Identifications</th>
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<tr>
<td>4 x 100 ng Peptide Mix</td>
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<td>Casein alpha-S2 [Bos taurus]</td>
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<tr>
<td></td>
<td>Albumin [Bos taurus]</td>
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<td></td>
<td>Hemoglobin, beta [Bos taurus]</td>
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<td>4 x 1 µg Peptide Mix</td>
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<tr>
<td></td>
<td>Hemoglobin, beta [Bos taurus]</td>
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<td>CPXV1.38 protein [Cowpox virus]</td>
</tr>
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<td>E Chain E, Trypsin Complex with Bowman-Birk Inhibitor</td>
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</table>

4.3.3 LC-MS/MS Analysis of Oocyte Lysate Proteins

4.3.3.1 DeCyder MS Peptide Profile of Oocyte Lysate

Having demonstrated that effective peptide detection and subsequent protein identification could be achieved with sub-microgram quantities of starting material, efforts were commenced to sequence proteins contained within the oocyte lysate fraction. This lysate was anticipated to contain a complex mixture of proteins that are implicated in all aspects of oocyte biology.

The ion trace presented in Figure 4.6A suggests that peptides were successfully detected during the 1D LC-MS/MS analysis of the egg lysate proteins. DeCyder MS analysis of the raw data revealed that peptides were indeed detected as a large number of discrete spots representing peptides were visualised (see Figure 4.6B).
Figure 4.6 *DeCyder* MS peptide profile of oocyte lysate

Following biotinylation of oolemmal surface proteins, ZP-free oocyte proteins were extracted using 0.5% Triton X-100 in PBS. Oolemmal surface proteins were fractionated using M280 Streptavidin Dynabeads. Proteins from the remainder of the oocyte lysate were recovered by chloroform-methanol precipitation and trypsin digested. The tryptic digest was analysed by LC-MS/MS. For each MS scan, MS/MS data was obtained for the 3 most intense ions. The ion trace (A) illustrates the relative abundance of peptide ions detected and the time point at which they were eluted off the column. Following MS detection of these eluted peptides, *DeCyder* MS software was utilised to generate virtual peptide profiles (B). Peptides from the oocyte lysate were successfully detected, and clearly visible on a *DeCyder* MS profile. Relative to the blank, a vast number of peptide spots were visualised, the majority of these peptides eluted from the column within 25 – 60 mins of the 80 min LC run.

Raw data obtained from the 1D LC-MS/MS analysis of the oocyte lysate fraction was subjected to bioinformatics analysis (Sequest) to convert peptide mass-to-charge ratio data into meaningful protein identities. These protein identities are presented in Table 4.2.
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### 4.3.3.2 Sequest Protein Identities

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4.3.4 LC-MS/MS Analysis of Affinity-Purified Surface Proteins

4.3.4.1 DeCyder MS Peptide Profile of Oolemmal Surface Proteins

As previously outlined, in an attempt to gain insight into the important proteins embedded in and tethered to the oocyte membrane that mediate sperm-egg interaction, attempts were made to fractionate surface proteins from those of the remaining egg lysate (see Figure 4.6, Table 4.2). Presented in Figure 4.7 are results of the 1D LC-MS/MS analysis performed on oocyte proteins isolated under conditions to enrich and purify surface proteins.

The ion trace for the surface protein fraction revealed detection of a large number of peptides as they were eluted off the nanoflow LC column. Multiple large peaks were observed across the elution time course. These peaks were eluted following retention times of approximately 20, 25, 30 and the 35 minutes (see Figure 4.7A). Visual representation of this data as DeCyder MS virtual peptide profile revealed a vast number of peptide spots. Unlike the peptide profile for peptides of the egg lysate (see Figure 4.6B) where peptides were primarily detected after 45 mins elution, the majority of peptides from the surface protein fraction appeared to have eluted within the first 45 mins of the LC run.
Proteomics and Bioinformatics

Figure 4.7 DeCyder MS peptide profile of oolemmal surface proteins
Following biotin labelling of egg surface proteins, proteins from ZP-free oocytes were extracted using 0.5% Triton X-100 in PBS. Oolemmal surface proteins were fractionated using M280 Streptavidin Dynabeads. Proteins in the purified oolemmal fraction were recovered by chloroform-methanol precipitation and trypsin digested. The tryptic digest was analysed by LC-MS/MS. For each MS scan, MS/MS data was obtained for the 3 most intense ions. The ion trace (A) illustrates the relative abundance of peptides and the time point at which they were eluted off the column. Following MS detection of these eluted peptides, DeCyder MS software was utilised to generate virtual peptide profiles (B). Peptides from the digested oolemmal proteins swamped background ion trace levels and were readily detectable. The DeCyder MS profile revealed that the majority of the peptides eluted within 18 – 40 mins of the 80 min LC run.
4.3.4.2 Sequest Protein Identities

As previously performed for results of the egg lysate fraction, LC-MS/MS results for the surface protein fraction were subjected to sequence analysis using Sequest software. The obtained proteomic identifications are presented in Table 4.3 below.

Table 4.3 Protein identities obtained for the oolemmal surface protein fraction analysed by LC-MS/MS

Surface proteins were fractionated from oocyte lysates by means of biotin labelling and streptavidin Dynabead purification. Proteins were then trypsin digested and the peptides subjected to LC-MS/MS analysis. MS/MS data was analysed using Sequest software and yielded 40 proteomic identifications.

<table>
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<th>Score</th>
<th>Accession</th>
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<td>38094836</td>
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<td>10.2</td>
<td>6753362</td>
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<td>10.2</td>
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<td>similar to zinc finger protein 352 [Mus musculus]</td>
<td>8.1</td>
<td>38075802</td>
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<td>38</td>
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<td>40</td>
<td>potassium channel tetramisation domain contain</td>
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4.3.5 LC-MS/MS Analysis of GPI-Anchored Proteins

In-house sequencing of the oocyte surface protein fraction revealed a total of 40 proteomic identifications. Although these identifications represented successful proteomic analysis, it was apparent that known egg membrane proteins of interest such as CD9 were absent amongst the search results. This was believed to be attributable to in-house sequencing methods failing to detect the lower abundance proteins. Since the results presented in Figure 4.2 had previously demonstrated that GPI-anchored proteins were present in very low abundance (<5 ng), the decision was made to utilise the professional sequencing service offered by APAF in attempt to maximise identification of these important proteins.

4.3.5.1 Mascot Protein Identifies obtained by APAF

Proteins present in the GPI-anchored protein sample were analysed by APAF via 1D LC-MS/MS analysis. The obtained mass spectrometry data was then analysed by Mascot to obtain protein identifications. During this Mascot sequence analysis, the significance threshold for the returned protein identifications was $P < 0.05$ (see Section 2.10.1).
Table 4.4 Mascot protein identities obtained for the GPI-anchored protein fraction by APAF.

Mascot protein identities for the GPI-anchored protein fraction were obtained by APAF (p<0.05). Analysis was performed against the murine database alone as well as the rodent database as a whole. Searching against the mouse database revealed a total of 18 protein identities. Broadening the search to include all rodent databases a total of 20 protein identities were obtained. None of the proteins identified were known to be GPI-anchored.

<table>
<thead>
<tr>
<th>Protein ID</th>
<th>Mouse Identity</th>
<th>Rodent Identity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>arginine deiminase-like protein</td>
<td>arginine deiminase-like protein</td>
</tr>
<tr>
<td>2</td>
<td>unnamed protein product</td>
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</tr>
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<td>3</td>
<td>lactate dehydrogenase B chain</td>
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</tr>
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<td>4</td>
<td>keratin Kb40</td>
<td>Type II keratin kb1</td>
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<td>5</td>
<td>keratin complex 2</td>
<td>keratin 10</td>
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<td>6</td>
<td>unnamed protein product</td>
<td>keratin Kb40</td>
</tr>
<tr>
<td>7</td>
<td>keratin, type I cytoskeletal 10</td>
<td>similar to keratin 6 alpha</td>
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<td>unnamed protein product</td>
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</tr>
<tr>
<td>9</td>
<td>TI-225 Ubiquitin</td>
<td>keratin, type I cytoskeletal 10</td>
</tr>
<tr>
<td>10</td>
<td>Type II keratin Kb37</td>
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</tr>
<tr>
<td>11</td>
<td>Ubiquitin carboxy-terminal hydrolase</td>
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<tr>
<td>12</td>
<td>unnamed protein product</td>
<td>Type II keratin Kb37</td>
</tr>
<tr>
<td>13</td>
<td>unnamed protein product</td>
<td>ubiquitin thiolesterase</td>
</tr>
<tr>
<td>14</td>
<td>transducin-like enhancer protein 6</td>
<td>Chain B, Peroxiredoxin</td>
</tr>
<tr>
<td>15</td>
<td>BC031593 protein</td>
<td>heat Shock protein 90</td>
</tr>
<tr>
<td>16</td>
<td>keratinocyte associated protein 1</td>
<td>transducin enhancer protein 6</td>
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<td>18</td>
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<td>pancreatic trypsin 1</td>
</tr>
<tr>
<td>20</td>
<td></td>
<td>unnamed protein product</td>
</tr>
</tbody>
</table>

Upon obtaining the APAF sequencing results presented in Table 4.4, each of the resultant protein identities was investigated to further identify any GPI-anchored proteins. Based on accession numbers (data not shown), current literature was explored to investigate known protein functions. In addition, amino acid sequences were obtained for each protein, this amino acid was then analysed using online GPI-anchor predicting software (http://mendel.imp.ac.at/gpi/gpi_server.html). Results of the literature searches and GPI-anchor protein predicting software revealed that none of the proteins identified by APAF were GPI-anchored.

4.3.6 Protein Analysis by Peptide Fractionation

Due to the lack of success in identifying known egg surface proteins such as CD9 by in-house sequencing and GPI-anchored proteins by APAF sequencing, attempts were made to enhance the number of successful proteomic identities obtained by means of separating peptides into 8 fractions, as opposed to separating proteins into 3 fractions. Peptide fractionation was to be achieved by isoelectric focusing on a pH 3 – 10 IPG
strip. Prior to utilising precious oocyte protein however, investigations were conducted to compare the number of successful protein identities obtained from 500 µg of fractionated peptides against that of 30 µg of unfractionated peptides from the same ovarian protein extract.

4.3.6.1 LC-MS/MS Analysis of Unfractionated Whole Ovaries
Ovaries had been collected during previously outlined sperm-egg binding and fusion experiments. These ovaries had been decapsulated, washed and stored at -80°C until further required. After thawing, 8 ovaries were again washed in PBS before transferring 2D rehydration buffer. Ovaries were homogenised and proteins extracted at 4°C for 60 mins on a rotary mixer. After assaying protein content, 30 µg of this protein sample was extracted, recovered by chloroform-methanol precipitation and then digested overnight with sequencing grade trypsin (Promega). The trypsin buffer was evaporated to dryness before peptides were resuspended in 0.1% formic acid. The entire sample was then analysed by LC-MS/MS analysis.
Whole ovaries were extracted into 2D rehydration buffer and the protein concentration determined. 30 µg of the protein extract was chloroform-methanol precipitated then trypsin digested overnight at 4°C on a rotary mixer. After drying, the peptides were resuspended in 0.1% formic acid and the entire sample analysed by 1D LC-MS/MS. For each MS scan, MS/MS data was obtained for the 3 most intense ions. The ion trace (A) illustrates the relative abundance of peptides and the time point at which they were eluted off the column. Following MS detection of these eluted peptides, DeCyder MS software was utilised to generate virtual peptide profiles (B). Large quantities of peptides eluted from column after 35 min of the 70 min LC run. Beyond 35 mins, the level of peptide detection remained very high up until 65 mins retention time. The abundance of these peptides was clearly visualised using DeCyder MS, which revealed a vast number of peptide spots eluted during the 35 – 65 mins time points.

1D LC-MS/MS analysis of the whole ovary protein extract revealed extensive levels of peptide detection. Sequest software was again utilised to translate these peptide detections into protein identifications.
4.3.6.2 Sequest Protein identities obtained for unfractionated Whole Ovary
Sequest analysis was performed on raw data obtained for the 30 μg of unfractionated ovarian protein extract. With filtering limits applied as outlined in Section 2.10.5 (+1<1.5: +2<2.0: +3<2.5), a total of 722 proteins were successfully identified. A complete list of the proteins identified from 30 μg of unfractionated whole ovaries can be found in Appendix A, Section A.1 (pages 1 – 16 of Appendix A).

4.3.6.3 LC-MS/MS Analysis of Fractionated Whole Ovaries
A protein extract was prepared as previously outlined whereby whole ovaries were extracted into 2D rehydration buffer (see Section 2.9.1). 500 μg of this protein extract was chloroform-methanol precipitated then trypsin digested. Rather than being directly analysed by LC-MS/MS however, the peptides were separated according to their isoelectric points using on an IPG strip. The strip was then cut into 8 × 3 cm segments and the peptides from each segment were eluted as separate fractions (see Section 2.10.3). LC-MS/MS analysis was then performed on peptides contained within each of the 8 fractions.

Ion trace and DeCyder MS profiles were visualised for each of the 8 fractions. A comparison of the abundance of ions detected for each fraction during the LC-MS/MS run is provided in Figure 4.9. Very few peptides appeared to have been detected for fractions 3, 5 and 7 as the ion trace levels remained relatively low for these fractions throughout the duration of the 70 min LC-MS/MS run. Each of these fractions did reveal a large peak in ion detection approaching the completion of the run, however this peak is attributable to the unavoidable elution of polyethylene glycol (PEG) from the LC system at high range acetonitrile concentrations and is therefore non-specific. In instances where little or no ion trace is detected for the sample being analysed, this PEG signal ultimately serves as the maximum source of ions and is therefore is assigned the maximum abundance for that sample. This PEG signal is however minimised by the detection of sample ions and was non-existent in the remaining fractions for which greater levels of ions were detected.
Ovarian proteins were trypsin digested and the resultant peptides separated into 8 fractions by isoelectric focusing (24 cm IPG strip, pH 3 – 10). LC-MS/MS analysis was then performed on each of the 8 fractions. Using BioWorks software, the ion trace recorded for each of the fractions was visualised. Each graph illustrates the relative abundance of ions (Y-axis) detected by the MS as peptides eluted off the C18 column over the 70 min LC run (X-axis). Incremental values on each axis are too small to be legible, however this is irrelevant as all graphs feature the same minimum (0) and maximum (100) values on both axis thus enabling visual comparison of ion abundance for each fraction. Fractions 3, 5 and 7 exhibited low level ion traces throughout the duration of the LC-MS/MS run. Ions were however detected in greater abundance for the remaining 5 fractions, of which fractions 1 and 8 in particular exhibited the greatest levels of ion detection.

MS/MS data was acquired for each fraction and once again utilised to obtain protein identifications based on peptide mass-to-charge ratios. The number of protein identifications obtained for each IPG strip segment varied considerably. Based on predicted pI values calculated for a vast range of previously obtained peptide sequences, the majority of peptides, and thus resultant protein identifications, were anticipated to
focus within fractions 5 and 6. This was not the case however as the final number of proteins identified for each fraction varied considerably with no apparent trend (see Figure 4.10). Fractions 1 – 4 yielded 38, 188, 22 and 104 protein identifications respectively, whilst 170, 85, 3 and 552 protein identifications were obtained from fractions 5 – 8 respectively. The total number of proteins identified across the 8 fractions was therefore 1162. A complete list of proteins identified within each fraction can be found in Appendix A, Section A.2 (pages 17 – 49 of Appendix A).

Figure 4.10 Final numbers of proteins identified within whole ovary peptide fractions

Peptides from whole ovaries were isoelectrically focused then eluted into 8 different fractions. LC-MS/MS data was acquired for peptides contained within each of the 8 fractions. For each MS scan, MS/MS data was obtained for the 3 most intense ions. The captured MS/MS data was analysed to obtain protein identifications. Fractions 1 – 4 yielded 38, 188, 22 and 104 proteins identifications respectively, whilst fractions 5 – 8 yielded 170, 85, 3 and 552 proteins identifications respectively. Total number of proteins identified was 1162, however the individual values that constitute this total were highly variable. Graphical representation of individual fraction results revealed no apparent trend in the distribution of peptides, and thus protein identifications, across the pH 3 – 10 gradient.

4.3.7 Analysis of Oocyte Proteins by 2D LC-MS/MS

Proteomic analysis of unfractionated peptides generated from 30 µg of whole ovary extract yielded a total of 722 protein identifications. The ion trace and DeCyder MS profile for this 30 µg of sample revealed high levels of peptide detection throughout the LC-MS/MS run (see Figure 4.8). Alternatively, the separation of 500 µg of peptides from whole ovaries into 8 separate fractions prior to LC-MS/MS yielded a total of 1162
protein identifications. Although a larger total number of proteins were identified using the peptide fractionation technique, a significant amount of peptide sample was believed to have been lost during elution of the peptides from the IPG strip segments following isoelectric focusing. This was evident in the fact that peptides recovered from segment 8 yielded 552 protein identifications, whilst adjacent to this segment 7 yielded only 3 protein identifications. The percentage of peptide recovery from each fraction therefore appeared to be highly susceptible manual handling of each fraction. To eliminate this human error, it was apparent that a 2D fractionation protocol where peptides were separated using 2D nanoflow LC, as opposed to isoelectric focusing, was a more efficient approach.

For this purpose, a total of 2346 oocytes were collected for use in proteomic analysis. Oocytes were denuded of cumulus cells but left ZP-intact. After extensive rounds of purification, the oocytes were extracted into oocyte lysate buffer containing protease inhibitors. Complete dissolution of the oocytes and their ZP into the extraction buffer was visually confirmed. The total of 2346 oocytes were collected across 4 separate isolations from a total of 100 Swiss mice. The solubilized sample was then stored at -80°C and sent to APAF on dry ice for protein quantification and 2D LC-MS/MS analysis.

The 2346 oocytes gave a total protein pool of approximately 250 µg. During APAF handling of the sample, this 250 µg of oocyte protein was reduced with DTT, alkylated with acrylamide then digested with trypsin. NanoLC electrospray MS/MS was performed using an Agilent 1100 nanoLC system in conjunction with a QStar XL MS/MS system. As outlined in the Section 2.10.6, this process involved SCX separation of the peptides into 12 fractions. Each of these fractions was then individually subjected to C18 nanoLC whereby peptides were eluted from the C18 column according to their hydrophobicity in response to increasing acetonitrile concentration. Eluted peptides were subjected to positive ion nanoflow electrospray analysis, where for each 0.5 second MS survey, the 3 most intense ions were subjected to further MS/MS analysis. LC-MS/MS data was then searched using Mascot (Matrix Science, London, UK) against the Swissprot database.
2D LC-MS/MS analysis of the oocyte protein extract by performed APAF yielded a total of 337 protein identifications \((P < 0.05)\). The complete list of proteins identified appears in Table B1 of Appendix B. Protein identifications obtained by APAF were analysed using GoMiner software (Genetic and Bioinformatics Group), and where information was available resultant proteins were categorised according to their roles within biological processes, cellular components and molecular function. It was possible for individual proteins to appear in multiple categories, as applicable to their known cellular distribution and function.

Of the oocyte proteins identified, 121 constituted cellular components, 127 were involved in biological processes and 133 fulfilled roles in molecular functions. GoMiner categorisation of oocyte proteins according to molecular function (see Figure 4.11B) revealed a total of 24 proteins possessing oxidoreductase activity. Within this group of oxidoreductases, 5 enzymes possessed protein disulphide oxidoreductase activity and were identified as the protein disulphide isomerase isoforms A1, A3, A4, A5 and A6 (PDIA1, PDIA3, PDIA4, PDIA5, PDIA6) as well as glutaredoxin-related protein 5 (GLRX5).

The cellular component category was of greatest relevance to this project (see Figure 4.11C). Within this category, intracellular proteins constituted the major class of proteins identified with 106 successful identifications obtained. Organelle-derived proteins were second most abundant with 90 proteins successfully identified, followed by proteins of the membrane, for which 36 successful identifications were obtained. As a testament to the validity of the 2D LC-MS/MS sequencing and GoMiner classifications, zona pellucida proteins 1, 2 and 3 (ZP1, ZP2 and ZP3) were all successfully identified within the oocyte protein extract and appropriately categorised as extracellular matrix. Of the 337 identities obtained, none of the proteins were categorised as GPI-anchored proteins following GoMiner analysis.

An important note was that of the 337 protein identities obtained by APAF, GoMiner was able recognise and categorise just 140 of the proteins when their gene name was searched against the *Mus musculus* database. A total 190 proteins are therefore not represented in the categorisation results of Figure 4.11.
Figure 4.11 Categorisation of oocyte protein results recognised by GoMiner software analysis

Peptides generated from whole mouse oocyte protein extracts were analysed by 2D LC-MS/MS. MS/MS data was analysed using Mascot against the *Mus musculus* database to obtain protein identifications. The obtained list of protein identifications was categorised according to known molecular structure and function using GoMiner software. Of the 337 protein identifications obtained, 147 were recognised by GoMiner. Proteins that were recognised were implicated in a wide variety of biological processes, possessed a broad range of molecular functions and were constituents of assorted cellular components including the oocyte membrane. The majority of proteins recognised were intracellular proteins, followed by those comprising organelles and the membrane.

Due to the fact that GoMiner was unable to categorise 190 of the 337 oocyte proteins identified, additional measures were utilised to screen the APAF protein results for GPI-anchored proteins.

The Mouse Genome Informatics website (http://www.informatics.jax.org/) is dedicated to providing an online, up-to-date resource that enables access to integrated data on the genetics, genomics, and biology of the laboratory mouse. Genes submitted into the database are tagged for all known properties such as structure, function and expression, as well as a host of additional properties. One such option that may be searched is gene
ontology, within which ‘GPI-anchor binding’ is an available category. Selecting the ‘GPI-anchor binding’ category yields a list of all the known GPI-anchored proteins expressed within the laboratory mouse at that point in time. At the time of this study, 116 genes encoding GPI-anchored proteins had confirmed expression within the laboratory mouse in one or more tissues.

The 337 oocyte protein identifications obtained from 2D LC-MS/MS analysis of whole oocytes were manually cross-referenced against the list of known murine GPI-anchored proteins on the MGI website. However none of the obtained oocyte protein identifications could be confirmed as GPI-anchored.

4.3.8 Bioinformatic Identification of Oocyte GPI-Anchored Proteins

Although 2D LC-MS/MS analysis proved effective in obtaining a large number of oocyte protein identifications, none of the proteins for which information was available appeared to be GPI-anchored. The lack of GPI-anchored proteins among the search results suggested that the proteins were again present in insufficient quantity within the fractions to qualify for MS/MS analysis given that only the 3 most intense ions are selected for MS/MS analysis for each MS scan. To complement proteomic data however, efforts were also made to identify oocyte GPI-anchored proteins at the gene expression level.

4.3.8.1 Mouse Oocyte EST Library

In October 2006, the following study was published by members of the Jackson Laboratory, Bar Harbour, Maine, USA:

**Title:** Cracking the egg: molecular dynamics and evolutionary aspects of the transition from the fully grown oocyte to embryo. Genes & Dev., Oct 2006; 20: 2713 – 2727.

**Authors:** Alexei V. Evsikov, Joel H. Graber, J. Michael Brockman, Ales Hampl, Andrea E. Holbrook, Priyam Singh, John J. Eppig, Davor Solter, and Barbara B. Knowles.
The study represents an extensive EST analysis performed on fully grown mouse oocytes, with the intention of documenting the changes in gene transcription that occur during the transition of oocyte to embryo. Generated from approximately 12,000 oocytes, this impressive database provides a robust list of the genes transcribed within mature mouse oocytes, and of significant to this project are the reported names of 4790 identified genes (Evsikov et al., 2006). Made publicly available, the published gene list presented an excellent opportunity to gain insight into the downstream proteins one may expect to be expressed within fully grown oocytes.

4.3.8.2 GPI-Anchored Proteins Identified within Mouse Transcriptome

Reported in the EST database by Evsikov et al, 2006 were 4790 identified genes. Amongst this list were genes encoding proteins that are known to fulfil functions in a vast multitude of cellular processes, including enzymes, metabolic proteins, cytoskeletal proteins, membrane proteins, receptor proteins, extracellular proteins, as well as genes from many more aspects of cellular biology. Within this list was undoubtedly a host of genes known to encode murine GPI-anchored proteins. In order to gain insight into which GPI-anchored proteins may be mediating murine sperm-egg interaction, it was firstly necessary to identify which genes within the database encoded GPI-anchored proteins. It was not feasible to perform background checks on the protein structures encoded by all 4790 genes, as such the list of 116 known GPI-anchored proteins on the MGI website was again utilised and cross-referenced against the list of 4790 genes identified by the EST analysis.

Of the 116 known GPI-anchored proteins published on the MGI website, transcripts for 11 of these genes were detected by Evsikov et al., 2006 within the mouse oocytes during the EST analysis. The number of ESTs detected and the rank of each gene within the overall transcript pool can be seen in Table 4.5.
Table 4.5 Genes encoding GPI-anchored proteins reportedly expressed in murine oocytes

Genes identified from the Evsikov et al., 2006 study were cross-referenced against the list of known GPI-anchored proteins published on the MGI website. Cross-referencing identified 11 genes within the EST analysis results that encoded GPI-anchored proteins.

<table>
<thead>
<tr>
<th>GPI-AP Rank</th>
<th>No. of ESTs</th>
<th>Total Transcript Rank</th>
<th>Protein ID</th>
<th>Abbreviation</th>
<th>Investigation Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4</td>
<td>877</td>
<td>CD160</td>
<td>CD160</td>
<td>No knockout generated</td>
</tr>
<tr>
<td>2</td>
<td>4</td>
<td>972</td>
<td>Ly6/Plaur domain containing 3</td>
<td>LYPD3</td>
<td>No knockout generated</td>
</tr>
<tr>
<td>3</td>
<td>2</td>
<td>2003</td>
<td>CD55</td>
<td>DAF</td>
<td>Knockout fertile</td>
</tr>
<tr>
<td>4</td>
<td>2</td>
<td>2122</td>
<td>Lipoprotein lipase Retinoic acid early response element</td>
<td>LPL</td>
<td>Knockout embryonically lethal</td>
</tr>
<tr>
<td>5</td>
<td>2</td>
<td>2400</td>
<td>Contactin 2</td>
<td>RAET1C</td>
<td>No knockout generated</td>
</tr>
<tr>
<td>6</td>
<td>1</td>
<td>3329</td>
<td>Contactin 4</td>
<td>CNTN2</td>
<td>Knockout fertile</td>
</tr>
<tr>
<td>7</td>
<td>1</td>
<td>3330</td>
<td>Contactin 6</td>
<td>CNTN4</td>
<td>No knockout generated</td>
</tr>
<tr>
<td>8</td>
<td>1</td>
<td>3331</td>
<td>GDNF Family receptor alpha 1</td>
<td>CNTN6</td>
<td>Knockout fertile</td>
</tr>
<tr>
<td>9</td>
<td>1</td>
<td>3619</td>
<td>RAET1C</td>
<td>GFRA1</td>
<td>Knockout embryonically lethal</td>
</tr>
<tr>
<td>10</td>
<td>1</td>
<td>3657</td>
<td>Glypican 2</td>
<td>GPC2</td>
<td>No knockout generated</td>
</tr>
<tr>
<td>11</td>
<td>1</td>
<td>3722</td>
<td>Hyaluronidase 2</td>
<td>HYAL2</td>
<td>No knockout generated</td>
</tr>
</tbody>
</table>

Of the 11 genes encoding GPI-anchored proteins found expressed within mouse oocytes, some had already been subject to extensive investigation, such as CD55, whilst very little was known about others, such as CD160. Gene knockout studies have already been performed for CD55, Lipoprotein Lipase (LPL), Contactin 2 (CNTN2), Contactin 6 (CNTN6) and GDNF family receptor α1 (GFRα1). Of these, knockout females were found to be fertile for CD55, CNTN2 and CNTN6, whilst knockout of the genes LPL and GFRα1 were found to be embryonically lethal. Based on findings presented in the existing literature, CD160, Ly6/Plaur domain containing protein 3 (LYPD3), LPL, RAET1C, CNTN4, GFRα1, Glypican 2 (GPC2) and Hyaluronidase 2 (HYAL2) therefore represented the list of gene products that remained potential candidates for fulfilling a role in murine sperm-egg interaction.
4.4 Discussion

The body of research presented within this chapter aimed to progress from simply understanding that a particular class of protein was important in mediating sperm-egg interaction, through to obtaining actual identities of candidate proteins around which characterisation studies could be based.

Conjecture as to whether immature GV phase oocytes possessed the capacity to bind and fuse with sperm led to proposals that a comparison of surface proteins of GV and MII oocytes may be effective in identifying proteins on the MII oocyte implicated in sperm-egg interaction. To effectively highlight proteins of importance, GV oocytes would therefore have needed to lack the ability to bind and fuse with sperm whilst MII oocytes remained competent to be fertilised. The results presented in Figure 3.2 of the previous chapter however, demonstrated quite clearly that GV phase oocytes possess the capacity to not only bind capacitated spermatozoa, but also undergo effective membrane fusion. In this regard, a visual comparison of surface proteins from GV and MII oocytes was therefore expected to yield similar protein profiles given that the proteins mediating sperm-egg interaction were obviously already expressed on oocytes at both these developmental stages.

To confirm this, a visual comparison of GV and MII surface proteins was performed by means of biotin-labelling surface proteins from both GV and MII oocytes, then comparing the separation profiles of the biotin-labelled proteins following SDS-PAGE. The results of study are presented in Figure 4.1, where it can be seen that biotin labelled surface proteins were effectively purified from whole oocyte lysates for both GV and MII oocytes. The lack of labelled protein bands within the ‘unbound’ or ‘final wash’ fractions for both GV and MII oocytes demonstrated that the M280-streptavidin Dynabeads utilised to purify the biotin-labelled surface proteins were effective in sequestering all the labelled egg surface proteins. Elution of these biotin-labelled proteins followed by SDS-PAGE separation revealed that the profile of surface proteins was essentially identical between GV phase and in vitro matured MII phase oocytes. This finding was consistent with previous observations that GV phase oocytes already possess the surface proteins necessary to accommodate effective sperm binding and fusion. The results therefore reiterated expectations that a proteomic comparison of
GV and MII oocytes would not highlight the appearance of proteins mediating sperm-egg interaction on the MII oocyte.

As a result of the previously outlined findings, the means by which the proteins involved in sperm-egg interaction would ultimately be identified was altered. It was therefore decided that obtaining lists of the protein identities contained within fractionated pools of protein classes of interest was the appropriate direction to take. Within the resultant lists of proteins, any candidates that represented potential sperm binding and/or fusion receptors, or potential members of a receptor complex, could then be further investigated by means of characterisation studies.

In order to maximise the chances of obtaining proteomic identification of proteins implicated in sperm-egg interaction, the decision was made to separate oocyte proteins into specific fractions. These fractions included (i) GPI-anchored proteins, (ii) oocyte surface proteins, and (iii) proteins of the remaining egg lysate. GPI-anchored proteins were fractionated from the remainder of the oocyte by means of PI-PLC. After PI-PLC mediated hydrolysis of GPI anchors on live oocytes, proteins that had been released into the egg culture media were collected and stored. The oocytes were then washed and incubated in the presence of Sulfo-NHS-Biotin to label the remaining egg surface proteins. Following extraction of the whole oocyte proteins into 0.5% Triton X-100, these biotin labelled surface proteins were purified from the remaining lysate by means of M280 Streptavidin Dynabeads.

The silver stained gel depicted in Figure 4.2 illustrates the successful acquisition of oocyte GPI-anchored proteins. A comparison of the proteins spontaneously released from untreated (mock treated) oocytes against those released in the presence of 1 U/mL PI-PLC revealed 7 protein bands that were not spontaneously released by oocytes into their surrounding medium. Since PI-PLC itself had been previously observed to migrate as a single 35 kDa protein (band 5), the remaining 6 bands represented the effective release and acquisition of a pool of egg surface GPI-anchored proteins.

Efforts were originally made to excise and sequence these bands by 1D LC-MS/MS analysis, however the approach failed to yield meaningful proteomic identifications (mainly keratin contamination: data not shown). This is believed to be have been attributable to the extremely low abundance of the GPI-anchored proteins, as evidenced
in Figure 4.2. Comparing the intensity of the GPI-anchored proteins against those of the BSA gradient it could be seen that with the exception of band 7, all the GPI-anchored proteins appeared to present in no greater quantity then 5 ng. The quantity of GPI-anchored protein sample that was visualised by SDS-PAGE constituted 100% of a protein pool obtained from approximately 400 oocytes. The low abundance of proteins would have important ramifications for proteomic analysis and was kept in mind at all times.

At the same time as GPI-anchored proteins were being pooled, oocytes were being used to generate pools of surface proteins as well as proteins of the remaining lysate. Biotin labelling of cell surface proteins was employed as a means separating oocyte cell surface and membrane proteins from those the remainder of the lysate. Purification of proteins by biotin labelling takes advantage of the strong interaction between biotin and streptavidin and is common practice in many laboratories. Looking at Figure 4.3, it can be seen that utilising magnetic Dynabeads coated with streptavidin provided an effective means of separating surface proteins from those of the remainder of the oocyte lysate. The fact that no protein bands were present in the final wash indicates that the beads had been effectively washed of any proteins bound non-specifically, and provides assurance that those proteins eluted off the beads following this final wash were indeed biotin labelled. The complement of surface proteins purified by biotin labelling differed considerably from those of the remaining egg lysate upon separation by SDS-PAGE. This was particularly the case at high range molecular weights (>100 kDa), as it can be seen in the visual comparison provided in Figure 4.4 that multiple high molecular weight bands existed in the surface protein fraction that were absent from the remaining lysate.

As well as highlighting the differences in the SDS-PAGE profiles between the biotin labelled proteins and those of the remaining lysate, Figure 4.4 demonstrated the successful fractionation of oocyte proteins into GPI-anchored proteins, membrane and surface proteins and those of the remaining lysate. This fractionation was anticipated to enhance sequencing of the proteins contained within each fraction.

Prior to committing precious oocyte sample to LC-MS/MS analysis however, sequencing trials were conducted to test the sensitivity of the instruments using known
protein standards. The trials were performed to gain insight into the quantity of starting material that was required in order to obtain effective protein identifications, and utilised a protein standard mix containing equal quantities of BSA, \(\alpha\)-casein, \(\beta\)-casein and carbonic anhydrase. The *DeCyder* MS profiles presented in Figure 4.5 demonstrate increasing levels of peptide detection as the quantity of protein standard starting material was increased. Table 4.1 correlates the abundance of ions detected with the number of proteins successfully identified when 1, 100 or 1000 ng of peptide for each protein standard was analysed using the LC-MS/MS. When just 1 ng worth of peptide for each of the four proteins was analysed, \(\alpha\)-casein was the only protein standard successfully identified. When 100 ng of peptide for each protein was analysed however, \(\alpha\)-casein, \(\beta\)-casein and BSA were all successfully identified. With three of the four proteins successfully identified, results of the LC-MS/MS trials therefore demonstrated that successful identification of proteins within unknown samples was probable for proteins present in quantities \(\geq\)100 ng.

Having obtained an insight into the quantities of starting material required for effective protein identification, sequencing efforts were commenced using the remaining oocyte lysate fraction. Oocytes contain a large volume of cytoplasm, and proteins from the cytoplasm were expected to constitute the majority of the cells proteinaceous material. Fractionation of surface proteins away from this cytoplasmic material was therefore expected to yield better sequencing coverage of proteins from the surface fraction as well as provide a relatively large pool of oocyte protein on which to trial LC-MS/MS on complex mixtures of proteins.

In-house LC-MS/MS analysis of peptides generated from the remaining lysate fraction delivered encouraging results. Figure 4.6A illustrates the abundance of peptide ions detected by the MS as they were eluted from the C18 column during nanoLC. A better appreciation for the level of peptide detection was however obtained by performing a *DeCyder* MS analysis of the results. The *DeCyder* MS comparison of peptide ions detected for the lysate sample against those detected during a blank LC-MS/MS run clearly highlights peptides spots attributable to proteins that were present in the lysate fraction.
Utilising Sequest software, the mass-to-charge ratio for these peptides ions was translated into protein identifications. Analysis of MS/MS data against the Swissprot *Mus musculus* database yielded 102 protein identifications. The primary identification during these searches was egg and embryo abundant peptidylarginine deaminase-like protein, or ePAD. ePAD localises to the cortical granules present in the cytoplasm underlying the egg membrane (Wright et al., 2003), and in light of the high abundance of this protein within the oocyte cytoplasm the results were exactly as expected. Other expected protein identifications included lactate dehydrogenase and heat shock proteins, which are both prevalent in the oocyte cytoplasm (Calvert et al., 2003). Although numerous other interesting protein identifications were obtained, individual identifications will not be discussed further as the results fulfilled their primary purpose of demonstrating the efficacy of the oocyte lysate LC-MS/MS analysis.

Having experienced success with the in-house LC-MS/MS analysis of the oocyte lysate fraction, proteins of the oocyte surface fractions were also committed to in-house LC-MS/MS analysis. The ion trace of Figure 4.8A depicts successful ion detection during the LC-MS/MS run, but once again it is the comparison of the DeCyder MS profile for the surface protein fraction against that of the blank LC-MS/MS run that highlights detection of proteins that were present in the fractionated sample (see Figure 4.8B).

Sequest software was again utilised to analyse MS/MS data obtained for the surface protein fraction. A total of 40 proteins were identified, the names of which appeared in Table 4.3. Results of the surface protein analysis were promising in that unlike the lysate analysis, ePAD was not detected among the sample. The fact that ePAD was not detected within the surface protein fraction was encouraging, as the lack of ePAD identification suggested that biotin had not managed to label intracellular proteins. This provided assurance that those proteins isolated by means of streptavidin Dynabead purification were proteins embedded in or tethered to the oocyte membrane. The lack of ePAD among the surface protein fraction results also indicated that oocytes were healthy and viable at the time of biotin labelling, as oocyte membrane integrity must have remained intact.

It was also reassuring to note that amongst the list of identified proteins were a host of proteins known to localise to the membrane of cells. Most notably these included a G-
protein coupled receptor, a tumour cell antigen and a potassium channel. Overall however, results of the membrane protein analysis were disappointing. This was due to the fact that proteins such as CD9, which is known to be expressed on the surface of oocytes and implicated in sperm-egg interaction (Kaji et al., 2000; Le Naour et al., 2000; Miyado et al., 2000), failed to be identified. It was possible that CD9 had not been identified within the membrane fraction as the CD9 structure may not have been compatible with the biotin labelling used to facilitate purification. However if this were indeed the case, CD9 would therefore be expected to have been identified within the remaining lysate fraction, which it was not. The result suggested that although membrane and surface proteins were indeed being purified, the majority were not present in sufficient quantity to enable successful identification.

Results of the surface protein fraction analysis demonstrated that comprehensive protein identification using the in-house LC-MS/MS system was unlikely to be achieved for low abundance protein samples. In light of this, the decision was made to utilise the professional services of APAF for proteomic analysis of the GPI-anchored protein sample. It was already known that proteins of the GPI-anchored protein sample were present in low abundance, however the SDS-PAGE analysis presented in Figure 4.2 indicated that the sample contained only approximately 20 proteins. As such it was hoped that peptides from GPI-anchored proteins would be selected for MS/MS analysis despite their low abundance and that protein identifications would be obtained. Results of the LC-MS/MS sequencing of the GPI-anchored protein sample performed by APAF are presented in Table 4.4. Using Mascot to search peptide mass-to-charge ratio data against the Mus musculus database, a total of 18 proteins were successfully identified within the GPI-anchored protein sample (P<0.05). When the search was expanded to include all rodent databases, this figure increased to 20 successful protein identifications.

Of the 20 identifications obtained however, none were revealed to be GPI-anchored proteins. This was confirmed by means of both consulting the relevant known literature as well as screening the amino acid sequence for each protein using GPI anchor predicting software. In light of the lack of GPI-anchored proteins, it is believed that the protein identifications that were obtained represent those proteins that were spontaneously released from the oocyte into the incubation media as visualised in
Figure 4.2. It does however remain possible that the proteins identified may have been associated with GPI-anchored proteins liberated from the oocytes surface.

Due to the lack of success in identifying known egg membrane proteins such as CD9 by in-house sequencing and GPI-anchored proteins by APAF sequencing, it was decided to attempt to enhance the success rate of proteomic identification by implementing two major changes in the approach proteomic analysis of the murine oocyte. Firstly, to avoid the possibility that GPI-anchored proteins were being lost as a result of ZP removal, oocytes were to be solubilised whilst zona-intact and without using PI-PLC to release GPI-anchored proteins into the egg culture media. All the egg membrane proteins, including GPI-anchored proteins, should therefore be intact at the time of protein extraction. Secondly, rather than separating protein classes of interest into three fractions then generating tryptic peptides for analysis from each fraction, a pooled oocyte protein extract was to be trypsin digested as a whole. Peptides contained within this tryptic digest could at that stage then be analysed using LC-MS/MS, or if need be, be separated into 8 fractions by means of isoelectric focusing with each fraction then individually analysed by LC-MS/MS.

It was not known however, whether fractionation of peptides by isoelectric focusing would even yield a greater number of successful protein identifications for the whole oocyte sample. As such, trial analyses were performed utilising whole ovary protein extracts to compare the number of successful identifications obtained for LC-MS/MS analysis of unfractionated peptides against the number of identifications obtained for fractionated peptides. Data obtained for the unfractionated ovarian peptides appears in Figure 4.8 and represents the analysis of 30 µg of whole ovary protein extract. The first important observation to note was extremely high abundance of peptide ions detected, which remained very consistent beyond the 38 min time point of the LC-MS/MS run (see Figure 4.8A). This high level of detection translated into a large number of peptide spots when visualised by means of DeCyder MS analysis (see Figure 4.8B). Peptides generated from 30 µg of ovarian sample were detected in such high abundance that the background ion traces evident in for blank LC-MS/MS run control were completely silenced.
Such intense peptide detection was encouraging and upon Sequest analysis the LC-MS/MS data translated into 722 successful proteomic identifications. The obtained protein identities were filtered according to charge and Xcorr as outlined in section 3.3.6.2, and due to the extensiveness of the list are presented in Table A.1 of Appendix A. Since the object of the ovarian peptide analysis was simply to compare the effectiveness of unfractionated peptide analysis against fractionated peptide analysis, little will be said regarding these proteomic identifications except that proteins were observed from a variety of subcellular localisations, including histones and homeobox proteins from the nucleus of the cells, all the way through to proteins such as α-integrin and plasma membrane calcium transporting ATPase 2 of the cell membrane.

LC-MS/MS analysis of 30 µg of unfractionated peptides therefore revealed high levels of peptide detection and yielded 722 protein identifications. It was hoped however that through the use of IPG strips to separate these peptides into 8 fractions according to each peptides isoelectric point that the number of proteins ultimately identified would be enhanced. This peptide fractionation was achieved through the use of a 24 cm, pH 3 – 10 IPG strips, onto which peptides generated from 500 µg of ovarian extract were loaded. Upon completion of the isoelectric focusing, the strip was cut into 3 cm segments and the peptides contained within each segment eluted as separate fractions. Upon LC-MS/MS analysis of the peptides within each fraction however, it was observed that the level of peptide detection for each fraction was generally low compared to the previously analysed 30 µg unfractionated peptide sample. Fractions 3, 5, and 7 in particular, revealed very low level peptide ion abundances (see Figure 4.9), with the peptide ions failing to silence the characteristic peak of background noise typically observed after approximately 70 min of a blank LC-MS/MS run. In the event that the 500 µg of peptides fractionated evenly across the 8 fractions, each fraction would be expected to contain approximately 60 µg of protein. Although peptides were not expected to fractionate evenly across the pH range, each fraction was expected to contain at least 20 µg of peptide with fractions 5 and 6, which together span the physiological pH ranges of 6.5 – 8.25, expected to contain up to 100 µg each. The series of ion traces illustrating the peptide abundance detected for each fraction however do not support this, and although some instances of high peptide abundance were detected for fractions 1, 4 and 8, overall peptide sample appears to have been lost. This loss of
peptide was also visually evident upon evaporation of the samples. Vacuum evaporation of the 30 µg non-fractionated sample resulted in a clearly visible peptide pellet. Following fractionation of 500 µg of peptide however, none of the 8 fractions revealed peptides comparable in size to that of the 30 µg pellet.

Nonetheless, upon Sequest analysis successful protein identifications were obtained for peptides from each of the 8 factions. As illustrated in Figure 4.10, fractions 1 – 4 yielded 38, 188, 22 and 104 protein identifications respectively, whilst fractions 5 – 8 yielded 170, 85, 3 and 552 respective protein identifications. Together the fractions therefore yielded a total of 1162 successful protein identifications. Despite the apparent loss of considerable quantities of peptide, peptide fractionation by means of IPG strip therefore yielded 1.6X as many protein identifications as analysis of unfractionated peptides.

Interestingly it was observed that the majority of protein identifications were obtained from fraction 8, which as can be seen in Figure 4.10 corresponds to a segment of the IPG strip spanning a pH range of approximately 9.12 – 10.0. It was expected that the majority of proteomic identifications would be obtained from fractions 5 and 6, which spanned pH ranges of 6.5 – 7.37 and 7.37 – 8.25 respectively. These expectations were based on the use of pH predicating software, which upon analysis of thousands of peptide sequences revealed an average pH of approximately 6.6. The fact this was not the case raised a series of possibilities: (i) the algorithm utilised by the pH predicting software to predict isoelectric points was incorrect, (ii) variable quantities of peptide (and thus downstream identifications) were lost during elution of the peptides from the individual IPG strip segments, (iii) that peptides were lost as a result of running off the ends of the strip, or (iv) that peptide isoelectric points deviated from those predicted as a result of the buffer they were solubilised in (2D rehydration buffer).

The fact that the number of identifications obtained from fraction 8 was vastly greater than any of the other fractions suggests that a considerable percentage of the peptides had indeed focused within the pH 9.12 – 10.0 range of the IPG strip. Since this fraction was on the positive end of the IPG strip it is reasonable to assume that a percentage of peptides had indeed been lost off the positive end of the IPG strip. Similarly, although
only 38 proteins were identified from fraction 1, a percentage of peptides are also believed to have been lost off the negative end of the IPG strip. During normal use of IPG strips, focused proteins and peptides would be recovered from the strip through electrophoretic migration into a polyacrylamide gel. For the purpose of these investigations however, peptides were not subjected to an electrophoretic force, and instead had to be recovered by means of washing peptides out of the individual segments of the strip. Although every effort was made to keep experimental handling consistent, this introduced the possibility that more peptides would be eluted from one fraction of the strip than that of another fraction. The fact that the number of proteins identified for each strip was highly variable supports this.

Irrespective of the ultimate reasons for peptide loss, fractionation of peptides by isoelectric focusing contained multiple stages at which substantial quantities of peptide could potentially be lost. As such, although the technique provided an overall greater number of protein identifications, it was deemed non-suitable for the analysis of the low abundance oocyte proteins. Fractionation of peptides by isoelectric focusing did however demonstrate the benefits of pre-fractionating peptides generated from complex biological mixtures, and for this reason it was decided to utilise 2D nanoflow LC-MS/MS for the analysis of the whole oocyte sample. Pre-fractionation of peptides by means of a SCX meant that the peptides generated by tryptic digestion would not be lost off the ends of IPG strips or through failure to elute from the IPG strip segments themselves, and although fractionation by SCX was not expected to be 100% efficient, the approach was anticipated to provide the minimal amount of peptide loss in order to achieve the necessary fractionation.

Due to the fact that 2D LC-MS/MS was not available in-house, the undigested protein extract from whole oocytes was sent to APAF for analysis. Following the necessary preliminary processing (reduction, alkylation and trypsin digestion), peptides generated from the sample were passed through a SCX column for separation into 12 discrete fractions. Each of the fractions was then individually analysed by C18 LC-MS/MS (see Section 2.10.6), and following Mascot analysis of the data, together the fractions yielded a total of 337 proteomic identifications. To assure the legitimacy of the proteomic results, sequencing was performed by APAF in accordance to a 95% confidence interval ($P < 0.05$). As a testimony to the validity of the results, zona
pellucida proteins 1, 2 and 3 were all observed amongst the proteomic identifications. Although a host of interesting proteins were observed amongst the results, such as four different isoforms of PDI, the results as they stood were simply a list and did not provide great insight as to which proteins present on the egg surface mediate sperm-egg interaction. As such, the list of gene names for the 337 identified oocyte proteins was analysed using GoMiner software.

As a tool for categorising genomic and proteomic data based on gene ontology, GoMiner provided an effective means of expressing results of 2D LC-MS/MS analysis in a format that enabled proteins of interest to easily be identified. GoMiner achieved this through categorising the proteins according to their known molecular functions, the biological processes they are involved in, and the sub-cellular components they comprise (see Figure 4.11). Once again referring to ZP proteins 1, 2 and 3, it was seen that ZP1, ZP2 and ZP3 all localised to the extracellular matrix precisely as anticipated.

Through utilising GoMiner it became apparent that the vast majority of proteins identified were of intracellular origin (see Figure 4.11C). This was to be expected given that oocytes contain a large cytoplasm that stores the proteins necessary for the initial stages of embryonic development following fertilisation. Proteins comprising organelles represented the next most abundant class of proteins followed by those of the membrane. Of primary significance to the project however, were any proteins categorised by GoMiner as GPI-anchored proteins. Within GoMiner, any recognised GPI-anchored proteins can be visualised upon navigating to: Molecular Function > binding > lipid binding > phospholipid binding > phosphoinositide binding > GPI anchor binding. Unfortunately however, navigating to this subcategory for results of the 2D LC-MS/MS analysis revealed no GPI-anchored protein were identified within the list. However it was noted that GoMiner was only able to categorise 147 of the 337 oocyte proteins identified. This was due to the fact that data the remaining 190 protein identities were not registered with the mouse database against which the results were analysed.

It remained possible therefore, that GPI-anchored proteins of interest were indeed among the 190 identifications that were unable to be categorised by GoMiner. To address this possibility and ensure that no proteins were overlooked, the entire 337
Protein identifications were cross-referenced against the database of all known murine GPI-anchored proteins. This list of murine GPI-anchored proteins was available online at the MGI website and at the time of cross-referencing the database reported that a total of 116 GPI-anchored proteins were known to be expressed within mice at that point in time. Unfortunately however, none of the 337 identified oocytes proteins appeared in the list of known murine GPI-anchored proteins. Although a variety of interesting proteins were identified, the 2D LC-MS/MS analysis of whole murine oocytes performed by APAF failed to yield candidate proteins that could be pursued through further characterisation.

Although a proteomic approach failed to provide insight into the identity of oocyte GPI-anchored proteins that may be mediating sperm-egg interaction, for reasons that will be addressed in the final discussion, alternative means of identifying oocyte GPI-anchored proteins were more productive and focused on identifying candidates at the level of gene transcription. Although the detection of a particular gene transcript within a cell type does not denote the presence of the encoded protein, the publication of an oocyte EST library in which over 4700 genes were identified represented an excellent opportunity to gain insight into the candidate GPI-anchored proteins that were potentially mediating sperm-egg interaction. Evsikov et al., 2006 made the complete list of 4790 genes available to the general public. To identify the GPI-anchored proteins within this list of genes, the 116 genes known to encode GPI-anchored proteins published on the MGI website were cross-referenced for their presence within the results of the Evsikov EST study. Cross-referencing the two databases resulted in the successful identification of 11 genes encoding GPI-anchored proteins within the oocyte EST library. These GPI-anchored protein encoding genes are listed in Table 4.5 in order of rank (based on the number of oocyte transcripts), and include CD160, Ly6/Plaur domain-containing protein 3, CD55, lipoprotein lipase, retinoic acid early response element, contactins 2, 4 and 6, GDNF family receptor alpha 1, glypican 2 and hyaluronidase 2.

As previously stated, the body of research presented in this chapter aimed to identify candidate proteins that may be implicated in sperm-egg interaction, the primary focus of which was the identification of oolemmal GPI-anchored proteins. It was anticipated that these identities would be obtained through proteomic analysis of the oocyte, and that
functional studies would then be based around their identification. Whilst GPI-anchored candidates were unable to be identified at the protein level, the aims of this chapter were ultimately achieved with the identification of 11 GPI-anchored protein candidates at the gene transcription level. Whilst existing literature was expected to preclude some of these genes as candidates as mediators of sperm-egg interaction, these 11 candidates thus served as the basis of the functional studies outlined in the following chapter.
CHAPTER 5:

Characterisation of Candidate Proteins
CHAPTER 5: CHARACTERISATION OF CANDIDATE PROTEINS

5.1 Introduction
A variety of experimental techniques may be employed to gain insight into the role of a particular protein within a biological system. Two commonly used approaches are antibody based detection studies and gene knockout analyses. The generation of gene knockout organisms, such as mice or *Drosophila*, can be expensive and requires considerable amounts of time to generate and validate the molecular constructs required for ultimately achieving gene deletion within a host organism. In addition, gene deletion may ultimately prove to be embryonically lethal, in which case researchers are unable to observe the resultant phenotype of the manipulated organism. Some level of characterisation of a given gene product can however be achieved using antibody-based technologies. Through the use of antibodies raised against specific target proteins, researchers are able to gain insights into the distribution of target proteins across a range of tissues, as well as the developmental time points at which the proteins are expressed within these tissues. Furthermore, when quantitative conditions are employed as in confocal microscopy, researchers may compare expression levels of proteins of interest in experimental situations between control and treatment samples.

In addition to providing insight into the spatial and temporal distribution of proteins, antibodies may also be employed as competitive inhibitors of protein function, and in doing so provide evidence as to the role of the target protein within a biological system. The monoclonal antibody JF9 for example, binds to the tetraspanin CD9 on the surface of eggs and competitively inhibits sperm-egg binding and fusion in a dose-dependent manner (Chen et al., 1999). In this instance JF9 appears to bind to a site within the structure of CD9 that is sufficient to sterically hinder the interaction of CD9 with other important proteins on either the sperm or egg surface that are essential for successful fertilisation. A single antibody clone may therefore provide valuable insight as to the importance of a particular protein within a biological system.

The use of antibodies to investigate protein function does however have limitations. Successful inhibition (or activation) of a protein in response to antibody challenge is
dependent upon the antibody binding to the target protein at an amino acid epitope that is essential for the protein to perform its function, or appropriately located so as to sterically hinder the interaction of the target protein with its binding partner. So whilst JF9 successfully inhibits sperm-egg interaction, another antibody raised against CD9 that binds to a different site within this molecule may produce no discernable affect. Therefore, while antibody trials are indeed a valuable approach to obtaining initial evidence as to the role of a particular protein within a biological system, the limitations of this approach need to be kept in mind.

5.2 Experimental Rationale

Experiments outlined in this chapter were performed to investigate the candidate proteins identified from the results of chapter 4 for their potential roles as mediators of sperm-egg binding and fusion. Although gene deletion would have been the ultimate means to ascertain the importance of these proteins within sperm-egg interaction, the generation of knockout mice for each protein of interest was not feasible within the remaining time frame of this project. As such, antibody based detection studies were employed to characterise the candidate proteins deemed to represent potential mediators of sperm-egg binding and fusion. For this purpose, a series of antibody based detection studies were performed in an effort to: (i) detect the proteins within whole ovarian protein extracts using immunoblotting, and (ii) detect the proteins on the surface of eggs using immunohistochemistry.

Furthermore, similar to the application of antibody JF9 to investigate the role of CD9 in sperm-egg binding and fusion (Chen et al., 1999), antibodies against the identified proteins of interest were also utilised as an initial means of investing whether the protein candidates were implicated in sperm-egg interaction.

The experimental aims for the series of investigations outlined in this chapter were to:

1) Filter the results of the previous chapter to generate a list of GPI-anchored protein candidates that could potentially be involved in sperm-egg interaction.

2) Utilise antibody based analysis to confirm the presence (or absence) of each protein candidate within ovarian extracts and on the surface of oocytes.
3) Ascertain whether antibodies against the protein candidates had the capacity to inhibit sperm-egg binding and/or fusion.
5.3 Results

5.3.1 Characterisation of Expression of GPI-Anchored Protein Candidates

5.3.1.1 Filtering of GPI-Anchored Protein Candidates

Results of the previous chapter yielded a total of 11 GPI-anchored protein identifications for investigation (see Table 5.1). Of the 11 identified GPI-anchored proteins identified, not all were potential candidates as mediators of sperm-egg binding and fusion.

<table>
<thead>
<tr>
<th>GPI-AP Rank</th>
<th>No. of ESTs</th>
<th>Total Oocyte Transcript Rank</th>
<th>Protein ID</th>
<th>Abbreviation</th>
<th>Investigation Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4</td>
<td>877</td>
<td>CD160</td>
<td>CD160</td>
<td>No knockout generated</td>
</tr>
<tr>
<td>2</td>
<td>4</td>
<td>972</td>
<td>Ly6/Plaur domain containing 3</td>
<td>LYPD3</td>
<td>No knockout generated</td>
</tr>
<tr>
<td>3</td>
<td>2</td>
<td>2003</td>
<td>CD55</td>
<td>DAF</td>
<td>Knockout fertile</td>
</tr>
<tr>
<td>4</td>
<td>2</td>
<td>2212</td>
<td>Lipoprotein lipase</td>
<td>LPL</td>
<td>Knockout embryonically lethal</td>
</tr>
<tr>
<td>5</td>
<td>2</td>
<td>2400</td>
<td>Retinoic acid early response element</td>
<td>RAET1C</td>
<td>No knockout generated</td>
</tr>
<tr>
<td>6</td>
<td>1</td>
<td>3329</td>
<td>Contactin 2</td>
<td>CNTN2</td>
<td>Knockout fertile</td>
</tr>
<tr>
<td>7</td>
<td>1</td>
<td>3330</td>
<td>Contactin 4</td>
<td>CNTN4</td>
<td>No knockout generated</td>
</tr>
<tr>
<td>8</td>
<td>1</td>
<td>3331</td>
<td>Contactin 6</td>
<td>CNTN6</td>
<td>Knockout fertile</td>
</tr>
<tr>
<td>9</td>
<td>1</td>
<td>3619</td>
<td>receptor alpha 1</td>
<td>GFRA1</td>
<td>Knockout embryonically lethal</td>
</tr>
<tr>
<td>10</td>
<td>1</td>
<td>3657</td>
<td>Glypican 2</td>
<td>GPC2</td>
<td>No knockout generated</td>
</tr>
<tr>
<td>11</td>
<td>1</td>
<td>3722</td>
<td>Hyaluronidase 2</td>
<td>HYAL2</td>
<td>No knockout generated</td>
</tr>
</tbody>
</table>

Of the protein identifications obtained, previous studies had demonstrated that female mice deficient in CD55, CNTN2 and CNTN6 remained fertile (Fukamauchi et al., 2001; Sun et al., 1999; Takeda et al., 2003). Therefore, these proteins were eliminated from subsequent characterisation studies. In addition, the proteins CNTN4 and GFRα1 were also eliminated. CNTN4 was eliminated on the premise that CNTN4 function was closely related to the function of CNTN2 and CNTN6. Since the knockout studies performed for both CNTN2 and CNTN6 revealed that females were fertile in both instances (Fukamauchi et al., 2001; Takeda et al., 2003), it was deemed highly probable that CNTN4 function was not involved in murine sperm-egg interaction. Knockout studies had however been performed for GFRα1, and deletion of this gene was found to be neonatally lethal (Tomac et al., 2000). Although neonatal lethality meant that fertility of the knockout animals could not be assessed, GFRα1 has a very well characterised role in neural tissue. In addition, deletion of the closely related protein GFRα3, revealed that knockout females were fertile (Nishino et al., 1999). Based on these reports it was again deemed highly unlikely that GFRα1 was implicated in sperm-egg interaction.
The remaining proteins, which consisted of CD160, LYPD3, LPL, RAET1C, GPC2 and HYAL2, were all selected for further investigation by in-depth characterisation studies. Unfortunately antibodies were unable to be sourced for LYPD3 and RAET1C. However antibodies were secured for the remaining candidates (CD160, LPL, GPC2 and HYAL2).

Table 5.2 Antibodies sourced for the characterisation of GPI-anchored proteins of interest

<table>
<thead>
<tr>
<th>GPI-AP Target</th>
<th>Antibody ID</th>
<th>Clone</th>
<th>Species &amp; Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD160</td>
<td>CD160</td>
<td>Monoclonal (7H1)</td>
<td>Raised in Rat against Mouse CD160 (biotinylated)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Monoclonal (2C7)</td>
<td>Raised in Rat against Mouse CD160 (biotinylated)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Monoclonal (CL1-R2)</td>
<td>Raised in Mouse against Human CD160</td>
</tr>
<tr>
<td>Lipoprotein Lipase</td>
<td>LPL.A4</td>
<td>Monoclonal</td>
<td>Raised in Mouse against human LPL. Also reacts with recombinant human and bovine LPL</td>
</tr>
<tr>
<td>Glypican 2</td>
<td>GPC2</td>
<td>Monoclonal</td>
<td>Raised in Rat against Mouse GPC2</td>
</tr>
<tr>
<td>Hyaluronidase 2</td>
<td>HYAL2</td>
<td>Polyclonal</td>
<td>Raised in Mouse against recombinant HYAL2</td>
</tr>
</tbody>
</table>

The procured antibodies were utilised as a means to (i) detect their respective GPI-anchored protein targets within ovarian lysates and sections, so as to confirm the expression of the candidates, and (ii) to investigate the role of each protein in sperm-egg interaction through assaying the ability of the antibody to inhibit or block sperm-egg binding and/or fusion.
5.3.1.2 Characterisation of CD160 Expression

CD160, a 20.55 kDa protein, was identified as a candidate GPI-anchored protein that may be implicated in murine sperm-egg interaction. Within both mice and humans CD160 exists as a multimeric GPI-anchored glycoprotein expressed on the surface of circulating cytotoxic lymphocytes (Bensussan et al., 1993). The protein possesses a single IgG-like domain and exhibits broad specificity for major histocompatibility complex (MHC) class Ia and Ib molecules (Maeda et al., 2005). Engagement of CD160 triggers natural killer (NK) cell-mediated cytotoxicity (Le Bouteiller et al., 2002) as well as cytokine production (Barakonyi et al., 2004).

UniGene (http://www.ncbi.nlm.nih.gov/UniGene/) online EST profile viewer revealed highly restricted expression of the CD160 gene within murine tissue. Limited expression of the CD160 transcript was detected in mammary gland, liver, thymus and spleen, for which just 6, 27, 31 and 50 transcripts per million (TPM) were detected respectively. Analysis of CD160 expression by developmental stage however, revealed high level detection of CD160 transcripts within the oocyte and unfertilised ovum, with 199 and 193 TPM detected respectively. Following fertilisation, CD160 expression declines (72 TPM in the pre-implantation embryo) and is no longer detectable by implantation (see Figure 5.1). Based upon the surveyed EST data presented in Figure 5.1, CD160 expression is clearly highly restricted, with expression largely limited to the oocyte/ovum. However, regardless of its detected intensity, the presence the CD160 transcript within oocytes does not denote the presence of the protein. In order to confirm the expression of CD160, immunoblot analyses were performed utilising anti-CD160 antibodies to detect the presence of the protein within ovarian protein extracts.
Characterisation of Candidate Proteins

Figure 5.1 Distribution of CD160 transcript within different tissues and developmental stages

Expression data for the gene CD160 was collected by means of EST analysis across a broad range of murine tissues and developmental stages. CD160 expression is highly restricted with transcripts detected in only mammary gland (6 TPM), liver (27 TPM), thymus (31 TPM) and spleen (50 TPM). However during development, CD160 expression is high within the oocyte (199 TPM) and unfertilised ovum (193). Following fertilisation, CD160 expression drops rapidly to just 68-72 TPM detected in the zygote and pre-implantation embryo. Beyond implantation, CD160 expression remains non-detectable until birth, at which point low levels of CD160 transcript are detected in neonates and juveniles.

Following a series of immunoblot investigations, CD160 appeared to have been successfully detected with ovarian protein extracts. It was observed that the 2 different antibody clones utilised (7H1, 2C7), each identified a protein band of the expected molecular weight (20 kDa) for CD160. In addition, the detected band was absent from the brain protein extract, which was utilised as a negative control based upon a lack of detection of the CD160 transcript within this tissue.
Figure 5.2 Immunoblot detection of CD160 within ovarian protein extracts
SDS protein extracts were prepared from decapsulated whole ovaries and mouse brain tissue. The protein content for each extract was quantified before equal quantities of each sample (4µg) were separated by SDS-PAGE (10% gel). Proteins were either silver stained for visualisation, or electrophoretically transferred to nitrocellulose membrane. Nitrocellulose membranes were blocked (3% BSA) and then probed with the CD160 antibody clones 7H1, 2C7 and CL1-R2 at 1:2500 dilution in TBS-T. Appropriate secondaries (streptavidin-HRP or goat anti-mouse IgG-HRP) were applied at 1:3000 dilution before blots were washed and developed. The silver stain demonstrated that equal quantities of protein were loaded from each protein extract. The antibody clones 7H1 and 2C7 both identified a protein band of approximately 20 kDa within the ovary extract. This band was absent in the brain extract. The anti-CD160 clone CL1-R2 failed to identify a protein band of the correct molecular weight for CD160 within either protein extract.

The silver stain gel pictured in Figure 5.3 demonstrated equal protein loading of brain and ovarian protein samples was achieved. As equal loading was achieved, these gel loading volumes were used repeatedly to produce a series of immunoblots with which to investigate the remaining GPI-anchored protein antibodies. As such, the image of the same silver stained gel features in each of the following immunoblot figures.
5.3.1.3 Characterisation of HYAL2 Expression

HYAL2, also known as Hyaluronidase-2 precursor, Hyaluronoglucosaminidase-2 and LUCA-2, is a 473 amino protein that belongs to a large family of proteins known as hyaluronoglucosaminidases. Despite being a member of this family, HYAL2 exhibits very low hyaluronidase activity (Danilkovitch-Miagkova et al., 2003), and is actually better known for its role as a receptor for the jaagsiekte sheep retrovirus (Dirks et al., 2002; Rai et al., 2001). Analysis of HYAL2 gene expression revealed detection of the HYAL2 transcript across a variety of murine tissues, including the oocyte.

![Breakdown by Tissue](image)

**Breakdown Developmental Stage**

Expression data for the gene HYAL2 transcript was collected by means of EST analysis across a broad range of murine tissues and developmental stages. HYAL2 exhibited expression within 24 of the 33 tissues examined. In addition, HYAL2 transcript was detected at 9 of the 11 developmental stages investigated, including the oocytes where detection was relatively low, at just 49 TPM, compared to the endocrine system where over 250 TPM were detected.

*Figure 5.3 Distribution of HYAL2 transcript within different tissues and developmental stages*
Although the level of HYAL2 transcript detected within the oocytes was reportedly low, antibody based attempts were made to detect the protein itself via immunoblot. The expected molecular weight of the HYAL2 protein is 53.6 kDa. During immunoblot analysis, polyclonal mouse anti-HYAL2 detected numerous proteins across a variety of molecular weights. Within the observed series of bands however, was a protein band of approximately 54 kDa that appeared in both ovary replicates. Reviewing the results of the EST analysis, it was observed that in addition to the oocyte, HYAL2 transcript was detected in brain tissue. Consistent with detection of the transcript in the brain tissue, it was observed that a protein band of molecular weight equal to that observed for the ovary replicates was detected in the brain protein extract also (see Figure 5.4).
Figure 5.4 Immunoblot detection of HYAL2 within ovarian protein extracts
SDS protein extracts were prepared from decapsulated whole ovaries and mouse brain tissue.
The protein content for each extract was quantified before equal quantities of each sample (4µg) were separated by SDS-PAGE (10% gel). Proteins were either silver stained for visualisation, or electroblotted to nitrocellulose membrane. Nitrocellulose membranes were blocked (3% BSA) and then probed with the mouse anti-HYAL2 at 1:2500 dilution in TBS-T. The appropriate secondary antibody (goat anti-mouse IgG-HRP) was then applied at 1:3000 dilution before blots were washed and developed. Ovary 1 and Ovary 2 represent two separate protein extracts prepared from different pools of whole ovaries. The silver stain demonstrates that equal quantities of protein were loaded for each protein extract. Mouse anti-HYAL2 detected protein bands within both ovary 1 and ovary 2 protein extracts at the expected molecular weight for HYAL2 of 54 kDa. A protein band with an approximate molecular weight of 54 kDa was also detected within the mouse brain protein extract.

Immunoblot analysis of protein extracts from whole ovaries therefore provided initial evidence supporting the possible expression of HYAL2 within mouse ovaries, which may have been attributable to HYAL2 expression by oocytes.
5.3.1.4 Characterisation of LPL Expression

The gene Lipoprotein Lipase, also known as Lipoprotein Lipase precursor, encodes a 53.12 kDa protein responsible for the hydrolysis of triglycerides (Beigneux et al., 2007). Similar to HYAL2 expression, LPL expression was detected across a broad range of tissues, with a maximum of 4746 TPM detected in the bone marrow. Reviewing the developmental breakdown, LPL expression was observed in the both the oocyte and unfertilised ovum. The transition from unfertilised ovum to zygote was associated with a rapid decline in LPL expression, and by post-implantation stage LPL transcript was no longer detectable.

**Breakdown by Tissue**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Pool Name</th>
<th>Transcripts per Million (TPM)</th>
<th>Spot Intensity based on TPM</th>
<th>Gene EST/Total EST in Pool</th>
</tr>
</thead>
<tbody>
<tr>
<td>adipose tissue</td>
<td>prostate</td>
<td>64</td>
<td>2/30947</td>
<td></td>
</tr>
<tr>
<td>blood</td>
<td>skin</td>
<td>117</td>
<td>10/85203</td>
<td></td>
</tr>
<tr>
<td>bone</td>
<td>spinal cord</td>
<td>40</td>
<td>1/24406</td>
<td></td>
</tr>
<tr>
<td>bone marrow</td>
<td>spleen</td>
<td>241</td>
<td>24/99272</td>
<td></td>
</tr>
<tr>
<td>brain</td>
<td>sympathetic ganglion</td>
<td>0</td>
<td>0/10208</td>
<td></td>
</tr>
<tr>
<td>connective tissue</td>
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<td></td>
</tr>
<tr>
<td>dorsal root ganglion</td>
<td>thymus</td>
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<td>10/126584</td>
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<tr>
<td>embryonic tissue</td>
<td>urinary</td>
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<td>83/136152</td>
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<td>vascular</td>
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<td>1/11132</td>
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<td>0/2182</td>
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<tr>
<td>eye</td>
<td>female genital</td>
<td>193</td>
<td>7/36152</td>
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<tr>
<td>gastrointestinal tract</td>
<td>head and neck</td>
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<td>14/114900</td>
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<tr>
<td>heart</td>
<td>heart</td>
<td>56</td>
<td>7/123955</td>
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<td>inner ear</td>
<td>359</td>
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<td>limb</td>
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<td>2/29784</td>
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<td>lung</td>
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<td>mammary gland</td>
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<td>muscle</td>
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<tr>
<td>pancreas</td>
<td>676</td>
<td>18/26598</td>
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</tr>
<tr>
<td></td>
<td>148</td>
<td>16/107785</td>
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**Breakdown Developmental Stage**

<table>
<thead>
<tr>
<th>Stage</th>
<th>Transcripts per Million (TPM)</th>
<th>Spot Intensity based on TPM</th>
<th>Gene EST/Total EST in Pool</th>
</tr>
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<tbody>
<tr>
<td>oocyte</td>
<td>99</td>
<td>2/20028</td>
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</tr>
<tr>
<td>unfertilized ovum</td>
<td>338</td>
<td>7/20660</td>
<td></td>
</tr>
<tr>
<td>zygote</td>
<td>35</td>
<td>1/27827</td>
<td></td>
</tr>
<tr>
<td>pre-implantation embryo</td>
<td>6</td>
<td>1/150475</td>
<td></td>
</tr>
<tr>
<td>post implantation embryo</td>
<td>0</td>
<td>0/43247</td>
<td></td>
</tr>
<tr>
<td>mid-gestation embryo</td>
<td>39</td>
<td>7/176010</td>
<td></td>
</tr>
<tr>
<td>late gestation embryo</td>
<td>33</td>
<td>3/88290</td>
<td></td>
</tr>
<tr>
<td>fetus</td>
<td>131</td>
<td>80/608632</td>
<td></td>
</tr>
<tr>
<td>neonate</td>
<td>102</td>
<td>11/106970</td>
<td></td>
</tr>
<tr>
<td>juvenile</td>
<td>303</td>
<td>91/299187</td>
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</tr>
<tr>
<td>adult</td>
<td>253</td>
<td>260/1025000</td>
<td></td>
</tr>
</tbody>
</table>

* Taken from the online UniGene EST Profile viewer (May, 2007)*

Figure 5.5 Distribution of LPL transcript within different tissues and developmental stages

Expression data for the gene LPL was collected by means of EST analysis across a broad range of murine tissues and developmental stages. LPL exhibited expression within 29 of the 33 tissue types examined. In addition, LPL transcript was detected at 10 of the 11 developmental stages investigated. LPL expression was particularly high within the unfertilised ovum, with 338 TPM detected, however expression levels declined following fertilisation.
Based on the results of the EST analysis, LPL was anticipated to be present within the mouse ovary at the protein level. Immunoblot analysis using mouse anti-human LPL revealed antibody labelling at the expected molecular weight for LPL of approximately 53.1 kDa. Although labelling was observed at the expected molecular weight, the bands identified were not clearly defined. Within the brain protein extract, anti-LPL failed to recognise a protein band of appropriate molecular weight for LPL. This observation is consistent with results of the EST analysis, which revealed that only 13 TPM were detected within the brain tissue assayed.

**Figure 5.6 Immunoblot detection of LPL within ovarian protein extracts**

SDS protein extracts were prepared from decapsulated whole ovaries and mouse brain tissue. The protein content for each extract was quantified before equal quantities of each sample (4µg) were separated by SDS-PAGE (10% gel). Proteins were either silver stained for visualisation, or electrobotted to nitrocellulose membrane. Nitrocellulose membranes were blocked (3% BSA) and then probed with the mouse anti-human LPL at 1:2500 dilution in TBS-T. The appropriate secondary antibody (goat anti-mouse IgG-HRP) was then applied at 1:3000 dilution before blots were washed and developed. Ovary 1 and Ovary 2 represent two separate protein extracts prepared from different pools of whole ovaries. Mouse anti-human LPL appeared to detect a protein band of the appropriate molecular weight for LPL of 53.1 kDa within both ovary protein extracts. The observed labelling was however undefined. Within the brain protein extract, anti-LPL did not detect a protein band of appropriate molecular weight for LPL.
Following the results of the anti-LPL immunoblot analysis, the presence of LPL at the protein level within the ovary remained unconfirmed. Antibody labelling at the expected molecular weight was observed within the ovarian protein extracts; however, discrete bands were not detected.

5.3.1.5 Characterisation of GPC2 Expression
Glypican 2, also known as cerebroglycan, is one of four members of the heparin sulfate proteoglycan family. This 64.6 kDa protein is believed to be expressed exclusively within the developing nervous system where it has been implicated in neurite outgrowth (Wang and Denburg, 1992), cell-cell adhesion (Reyes et al., 1990; Stanley et al., 1995), cell-substratum adhesion (LeBaron et al., 1988) and growth factor signalling (Zioncheck et al., 1995). During EST analysis, maximal expression of GPC2 was observed within the dorsal root ganglion (658 TPM). GPC2 transcript was however also detected within oocytes at a level of 49 TPM. Despite reports that GPC2 is expressed exclusively within neural tissue, GPC2 may therefore also be expressed within oocytes where it may potentially be fulfilling a role in murine fertilisation, similar to its previously documented role in cell-cell adhesion (Reyes et al., 1990; Stanley et al., 1995).
**Characterisation of Candidate Proteins**

**Figure 5.7 Distribution of GPC2 transcript within different tissues and developmental stages**

Expression data for the gene GPC2 was collected by means of EST analysis across a broad range of murine tissues and developmental stages. GPC2 exhibited expression within 16 of the 33 tissue type examined. In addition, GPC2 transcript was detected at 7 of the 11 developmental stages investigated, including the oocytes where detection was relatively low, at just 49 TPM.

Based upon the transcript expression levels reported from the EST analysis, it was not anticipated that GPC2 would be detected within ovarian protein extracts. Attempts to detect GPC2 within ovarian protein extracts utilised the monoclonal antibody rat anti-mouse GPC2. Results of the immunoblot analyses appear in Figure 5.8.

![Table of EST counts and developmental stage breakdown](image)

*Taken from the online UniGene EST Profile viewer (May, 2007)*
SDS protein extracts were prepared from decapsulated whole ovaries and mouse brain tissue. The protein content for each extract was quantified before equal quantities of each sample (4µg) were separated by SDS-PAGE (10% gel). Proteins were either silver stained for visualisation, or electroblotted to nitrocellulose membrane. Nitrocellulose membranes were blocked (3% BSA) and then probed with rat anti-mouse GPC2 at 1:2500 dilution in TBS-T. The appropriate secondary antibody (goat anti-rat IgG-HRP) was then applied at 1:3000 dilution before blots were washed and developed. Ovary 1 and Ovary 2 represent two separate protein extracts prepared from different pools of whole ovaries. The silver stain demonstrates that equal quantities of protein were loaded for each protein extract. Immunoblot labelling with rat anti-mouse GPC2 resulted labelling of an extensive number of protein bands within the ovarian extracts. Discrete bands at the appropriate molecular weight for GPC2 of 64.6 kDa were unable to be observed.

It was observed that rat anti-mouse GPC2 labelling of ovarian protein extracts via immunoblot resulted in detection of a large number of protein bands. Very dense labelling was observed within the vicinity of the expected molecular weight for GPC2 of 64.6 kDa. Discrete bands that appeared to be attributed to the expression of GPC2 were therefore unable to be observed within ovarian proteins extracts.
5.3.1.6 Characterisation of LYPD3 Expression
LYPD3 possesses a variety of alternative names, including C4.4A, GPI-anchored metastasis-associated protein C4.4A homolog, Matrigel-induced gene C4 protein and MIG-C4. The LYPD3 gene encodes a 363 amino acid GPI-anchored protein with a predicted molecular weight of 37.5 kDa. LYPD3 was first identified within a highly metastasising rat pancreatic adenocarcinoma cell line, and as such was proposed to be involved in metastasis and wound healing (Rosel et al., 1998). At that point in time LYPD3 was regarded as an orphan receptor, more recently however LYPD3 has been shown to interact with laminin proteins, in particular laminins 1 and 5 (Paret et al., 2005). The protein possesses repetitive cysteine residues, which through the formation of disulphide bonds, result in a ‘3 finger’ folding of the protein structure (Tsetlin, 1999).

Expression of the LYPD3 transcript has been documented within 8 murine tissues, these include brain, eye, female genital, gastrointestinal tract, head and neck, mammary gland, urinary tissue and embryonic tissue (see Figure 5.9). Analysis of LYPD3 expression by developmental stages reveals strong expression of the LYPD3 transcript within the oocyte (204 TPM) and unfertilised ovum (94 TPM).
Figure 5.9 Distribution of LYPD3 transcript within different tissues and developmental stages

Expression data for the gene LYPD3 was collected by means of EST analysis across a broad range of murine tissues and developmental stages. LYPD3 exhibited a restricted expression pattern in that transcript was detected in only 8 of 33 tissues examined. Similar to CD160, LYPD3 was expression was strong within the oocyte and unfertilised ovary, with 209 and 99 TPM detected respectively. Post-fertilisation, LYPD3 transcript was not detected within the zygote or early stages of embryo development.

Results for the EST analyses therefore indicated that LYPD3 was an excellent candidate as a potential mediator of sperm-egg binding. Antibody characterisation studies were however unable to be performed due to the inability to source an appropriate antibody.
5.3.1.7 Characterisation of RAET1C Expression

The gene RAET1C, also known as RAE-1gamma and Retinoic acid early inducible protein 1 gamma precursor, encodes a 253 amino acid protein with a predicted molecular weight of 28.5 kDa. The retinoic acid early inducible-1 (Rae-1) family of proteins, to which RAET1C belongs to, are a class of proteins implicated in host immunity. Upon bacterial, viral or fungal-mediated activation of macrophages, the cells signal their detection of the infection by means of expressing ligands on their surface. These ligands, which are encoded by the macrophages themselves, are then recognised by other cells of the immune system. Evidence suggests that RAET1C is the ligand expressed by activated macrophages that is detected by NK cells, activated CD8\(^+\) T cells and other activated macrophages. Among these cell types, the receptor for the RAET1C ligand is the membrane protein NKG2D, which is implicated in both adaptive and innate immunity (Hamerman et al., 2004).

Unfortunately, at the time of compiling this research the EST profile for RAET1C was yet to be made available through UniGene. As a result the virtual expression profile of RAET1C is unable to be presented. Additionally, antibodies against RAET1C were unable to be obtained. Questions as to whether RAET1C is expressed on the surface of eggs and whether this GPI-anchored protein fulfils a role in mediating sperm-egg binding therefore remain to be answered.

5.3.2 Characterisation of GPI-Anchored Protein Candidates in Ovarian Sections

Following efforts to detect the GPI-anchored protein candidates within ovarian lysates, expression of the GPI-anchored protein candidates was investigated by means of immunohistochemical analysis of ovarian sections.

The CD160 antibody clones 7H1 and 2C7 were raised in rat against murine CD160. Since these antibodies were biotin labelled, streptavidin-HRP was utilised as the secondary antibody for detecting these clones. The primary antibody control pictured in Figure 5.10A revealed no non-specific labelling of ovarian cell types by the streptavidin-HRP. Probing sections with either the 7H1 or 2C7 clones of anti-CD160 failed to label any cells in the ovarian sections. The CL1-R2 clone of anti-CD160 was raised in mouse against human CD160. For analysis of CL1-R2 mediated detection of CD160, a HRP-conjugated goat anti-mouse IgG was utilised as the secondary antibody.
As evidenced in the primary control of Figure 5.10B, this goat anti-mouse IgG-HRP revealed non-specific labelling of the peripheral region of oocytes as well as stromal cells. Relative to this non-specific labelling however, the CL1-R2 clone of anti-CD160 exhibited cytoplasmic labelling of oocytes.

Antibodies that were sourced to characterise LPL and HYAL2 expression were also raised in mouse. Anti-LPL was raised in mouse against human LPL protein whilst anti-HYAL2 was raised in mouse against partial recombinant HYAL2 (species not disclosed). Since these antibodies were raised in mouse, goat anti-mouse IgG-HRP was once again utilised as the secondary antibody. The labelling pattern for anti-LPL did not deviate from that observed for the secondary antibody control (see Figure 5.10B). HYAL2 however, exhibited very strong labelling of the oocyte cytoplasm as well as granulosa cells, thecal and stromal cells.

For the purpose of investigating GPC2 expression a monoclonal rat anti-mouse GPC2 antibody was successfully sourced. To detect rat anti-mouse GPC2, a goat anti-rat IgG-HRP was utilised as the secondary antibody. Goat anti-rat IgG-HRP revealed no non-specific labelling of ovarian cells (see Figure 5.10C). Anti-GPC2 exhibited labelling of the nuclear region within oocytes. In addition, anti-GPC2 also labelled some, but not all, granulosa cells within close proximity of the oocytes. For more information on the antibodies utilised refer to Appendix D, Table D1.
Characterisation of Candidate Proteins

Figure 5.10 Detection of candidate GPI-anchored proteins in ovarian sections
Sections cut from superovulated Swiss ovaries were de-waxed and subjected to sodium citrate-mediated antigen retrieval. Sections were incubated in anti-CD160 (7H1, 2C7 and CL1-R2 clones), anti-GPC2, anti-HYAL2 or anti-LPL at 1:50 dilutions overnight at 4°C. After washing, sections were incubated in the appropriate HRP-conjugated secondary antibodies (either streptavidin-HRP, goat anti-mouse IgG-HRP or goat anti-rat IgG-HRP as labelled) at 1:50 dilutions for 2 hrs at 37°C. Sections were developed using DAB, counter-stained with haematoxylin and visualised. The 7H1 and 2C7 clones of anti-CD160 did not label ovarian sections. CL1-R2 produced weak nuclear labelling relative to the anti-mouse IgG control, however this anti-mouse secondary exhibited non-specific labelling of the periphery of oocytes. LPL did not label ovarian sections relative to the anti-mouse secondary control, however HYAL2 strongly labelled oocytes, granulosa cells and stromal cells. GPC2 labelled the nucleus of oocytes as well as some granulosa cells.
5.3.3 Effect of Antibodies against GPI-Anchored Protein Candidates on IVF

In an attempt to determine whether the GPI-anchored proteins of interest were implicated in sperm-egg binding and fusion, antibodies against the GPI-anchored proteins were assayed for their capacity to competitively inhibit sperm-oocyte binding and fusion. During these bioassays, ZP-free oocytes were pre-incubated in the antibodies raised against the GPI-anchored proteins for a period of 60 min. The oocytes were then washed and transferred into droplets of capacitated spermatozoa under standard IVF conditions.

Pre-incubation of oocytes in the CL1-R2 clone of anti-CD160 resulted in a mild reduction in the rates of sperm-egg binding and fusion when compared to sperm-binding rates of the control. Control oocytes bound an average of 13.3 ± 1.1 sperm per egg, whilst anti-CD160 treated oocytes bound an average of 10.7 ± 1.3 sperm per egg. This reduction in rates of sperm binding was however not statistically significant (P = 0.085). Similarly, no significant difference was observed for sperm-egg fusion rates between anti-CD160 treated oocytes and oocytes of the control (P = 0.155).

The observed rates of sperm-egg binding varied significantly between untreated oocytes and oocytes pre-incubated in anti-GPC2 (P = 0.003). In this instance, incubating oocytes in anti-GPC2 actually increased the observed rates of sperm-egg binding. Anti-GPC2 treated oocytes exhibited an average of 20.4 ± 4.3 sperm bound per egg, compared to the 13.3 ± 1.1 sperm bound per egg for the control oocytes. Despite the increased rate of sperm-egg binding, pre-incubation of oocytes in anti-GPC2 had no significant effect on rates of sperm-egg fusion compared to control oocytes (P = 0.291).

Similar to the pre-incubation of oocytes in anti-GPC2, incubating oocytes in anti-HYAL2 resulted a significant increase in observed rates of sperm-egg binding compared to control oocytes (P = 0.003). However once again this increased rate of sperm-egg binding had no significant effect on rates of sperm-egg fusion upon comparison of control and anti-HYAL2 treated oocytes (P = 0.473).
Pre-incubating oocytes in anti-LPL produced no significant differences in the rates of sperm-egg binding (P = 0.095) or sperm-egg fusion (P = 0.209) upon comparison with control oocytes.

![Figure 5.11 Effect of GPI-anchored protein antibodies on sperm-egg interaction](image)

**Figure 5.11 Effect of GPI-anchored protein antibodies on sperm-egg interaction**

ZP-free oocytes were pre-incubated in antibodies against CD160 (CL1-R2 clone), GPC2, HYAL2, or LPL at 1:50 dilutions for 60 min at 37°C. The oocytes were then washed and used to perform the sperm-egg binding and fusion bioassay. Incubating oocytes in anti-CD160 resulted in a mild reduction in sperm-egg binding rates however the reduction was not statistically significant. Anti-CD160 had no affect on rates of sperm-egg fusion. Pre-treating oocytes in anti-GPC2 or anti-HYAL2 resulted significant increases in rates of sperm-egg binding. However antibodies against GPC2 or HYAL2 had no affect on rates of sperm-egg fusion. Treatment of oocytes with anti-LPL had significant affect on rates of sperm binding or sperm fusion when compared to oocytes of the untreated control. (Data are mean ± sem, n = number of oocytes. *P < 0.05; unpaired Student T-test).
5.4 Discussion

A total of 11 GPI-anchored protein identifications were obtained from the results of the previous chapter. One or more of these GPI-anchored proteins potentially represented the egg surface protein(s) implicated in sperm-egg binding. The experiments presented in this chapter aimed to investigate these identified candidate proteins in the interest of characterising their expression within ovaries and oocytes as well as their potential roles as mediators of sperm-egg interaction. As previously stated, gene deletion studies are a desirable means of investigating protein function. For this reason, the known literature was consulted to determine whether any of the 11 identified GPI-anchored proteins had been the subject of previous gene deletion studies, and if so, to determine whether the resultant phenotypes exhibited impaired fertility.

Previously published findings immediately eliminated 3 of the 11 candidates as mediators of fertilisation. Sun et al., 1999 published results of a study in which the role of CD55 was investigated by means of gene deletion (Sun et al., 1999). Also known as decay accelerating factor, the study revealed that CD55 functions to regulate homologous and heterologous complement activation, and that CD55 is capable of fulfilling this role via either the alternative or classical pathway for complement activation (Sun et al., 1999). Whilst complement activation is of little relevance to this project, the authors also report that CD55 null mice ‘were able to develop, grow and reproduce, showing no overt abnormal phenotypes’ when housed under non-specific pathogen-free conditions (Sun et al., 1999). The fact that CD55 null mice exhibited normal reproduction precludes CD55 as a key GPI-anchored protein mediating sperm-egg binding. Similarly, gene deletion studies performed for CNTN2 and CNTN6 revealed that fertility remained unaffected following deletion of either of these two genes (Fukamauchi et al., 2001; Takeda et al., 2003). The contactin proteins, which are members of the immunoglobulin superfamily, are a class of neural cell adhesion molecules involved in axon growth, guidance and fasciculation in the central nervous system (Fernandez et al., 2004). Although GPI-anchored, deletion of CNTN2 or CNTN6 had no apparent affect on fertility, and as such were unlikely to represent the pivotal GPI-anchored protein implicated in sperm-egg interaction.

In addition, although gene deletion studies had not been performed for CNTN4, it was deemed highly unlikely that CNTN4 was implicated in sperm-egg interaction due to evidence linking CNTN4 disruption in humans to the 3p Deletion Syndrome in which
neural activity is affected (Fernandez et al., 2004). CNTN4 was therefore anticipated to fulfil very similar roles to CNTN2 and CNTN6, both of which had no affect on fertility.

Although knockout studies had been performed for GFRα1, the resultant phenotype was neonatally lethal with mice dying within 24 hours of birth due to uremia (Tomac et al., 2000). Nonetheless, GFRA1 has a very well documented role within nervous tissue development and as such was also deemed unlikely to be implicated in sperm-egg interaction. In addition, deletion of the closely related protein GFRA3 has no effect on fertility (Nishino et al., 1999), further suggesting that proteins of this family are not implicated in sperm-egg interaction.

CD55, CNTN2, CNTN4, CNTN6 and GFRA1 were therefore all eliminated as potential mediators of sperm-egg interaction. Reports of gene deletion studies were however unable to be found for the remainder of the GPI-anchored proteins. These proteins, which included CD160, LYPD3, LPL, RAET1C, GPC2 and HYAL2, therefore represented potential mediators of sperm-egg interaction and were selected for further characterisation studies.

Characterisation studies began by interrogating the virtual expression profile database of each of the proteins via the UniGene website. Inspection of the UniGene profiles provided an indication of whether the gene for each GPI-anchored protein of interest was actively transcribed in a variety of different murine tissues or whether expression of the gene was restricted to specific cells types and tissue. It was hoped that one or more of the GPI-anchored protein candidates would reveal high levels of expression within oocytes and the unfertilised ovum whilst remaining tissues of the body exhibited little or no expression.

One such candidate for which gene expression was highly restricted was CD160. This 20.55 kDa protein exists as a multimeric GPI-anchored surface protein on circulating cytotoxic lymphocytes (Bensussan et al., 1993). Despite its documented presence on the surface of cytotoxic lymphocytes, detection of the CD160 transcript was actually greatest within oocytes and unfertilised ovum (see Figure 5.1). Other tissues in which the CD160 transcript was detected include the spleen (50 TPM), thymus (31 TPM), liver (27 TPM) and mammary gland (6 TPM). Detection of CD160 transcript within the
spleen was to be expected given that CD160 is expressed on the surface of white blood cells, many of which are contained within the spleen. Yet despite the accumulation of white blood cells within the spleen, the level of CD160 transcript within oocytes (199 TPM) and unfertilised ovum (193 TPM) was almost 4 times that detected within the spleen. The high level of CD160 transcript detected within oocytes in conjunction with the highly restricted expression of the gene therefore identified CD160 as strong candidate for the GPI-anchored protein mediating sperm-egg binding. In addition, the level CD160 transcript within oocytes rapidly declined following fertilisation to the point that it was undetectable by the stage of implantation. This switching off of CD160 expression suggests that the protein is no longer required following successful fertilisation, and may therefore be indicative of a role for CD160 in the events of fertilisation.

The antibodies sourced for CD160 were utilised to examine expression of the protein within ovarian lysates. The monoclonal antibodies 7H1 and 2C7 were raised in rat against mouse CD160. During Western blot analysis, both antibodies successfully detected a discrete protein band of the expected molecular weight for CD160 of 20 kDa. For both these antibody clones, this 20 kDa band was observed within ovarian lysates but absent from lanes of the brain extract, which was included as a negative control (see Figure 5.2).

The third antibody clone sourced for the purpose of CD160 characterisation, CL1-R2, was raised in mouse against human CD160. Unlike the 7H1 and 2C7 clones however, CL1-R2 failed to detect a protein band of the appropriate molecular weight for CD160 within either the ovarian lysate or brain lysate. Failure of CL1-R2 to detect a band of the appropriate molecular weight for CD160 may be due to the fact that CL1-R2 was raised against human CD160, which exhibits just 66.85% sequence homology with murine CD160.

In addition to the CD160 transcript exhibiting restricted expression that was greatest in the oocyte, immunoblot analysis therefore provided initial evidence for the translation of this transcript into detectable levels of protein. Results obtained for the 7H1 and 2C7 clones provided the first indication that CD160 may indeed be expressed at the protein level within ovaries where it may be facilitating sperm-egg binding.
As previously mentioned, the GPI-anchored protein mediating sperm-egg interaction was expected to exhibit a highly restricted expression profile. The virtual expression profile for HYAL2 however deviated from this expectation considerably in that HYAL2 expression was detected within 24 of the 33 tissue types examined (see Figure 5.3). Among these tissues, HYAL2 detection was greatest within the endocrine system where over 250 TPM were detected. Looking at the developmental time course however, a mere 49 TPM were detected within the oocyte. Although GPI-anchored, based on these reports, HYAL2 was therefore not expected to represent the GPI-anchored protein mediating sperm-egg interaction.

For the purposes of characterising expression of HYAL2 at the protein level however, a polyclonal antibody raised in mouse against recombinant HYAL2 was sourced. Although this antibody was not ideal, anti-HYAL2 successfully labelled a strong protein band of the correct molecular weight for HYAL2 (53 kDa) when used to probe proteins present in ovarian lysates (see Figure 5.4). In humans, HYAL2 is reportedly expressed in almost all tissues analysed, including the brain (Danilkovitch-Miagkova et al., 2003). In mouse, the UniGene expression profile for HYAL2 (see Figure 5.3) exhibited detection of the HYAL2 gene within brain also. As such, for the purpose of HYAL2 characterisation brain lysate was utilised as a positive control. It can be seen in Figure 5.4 that in addition to detection of HYAL2 within the two ovarian lysates, HYAL2 also appeared to be identified within mouse brain protein extracts.

Discrete bands correlating to the correct molecular weight for both CD160 and HYAL2 were therefore visualised within ovarian protein extracts. The next protein candidate whose expression was characterised was LPL. Similar to HYAL2 expression, the virtual expression profile for LPL revealed detection of the transcript for this gene within almost all of the tissues analysed. Of the 33 tissues examined, LPL transcript was detected all but 4. Of the 29 tissues found to express LPL, the level of transcript exhibited by bone marrow was by far the greatest with 4746 TPM detected. Bone and heart also exhibited very high levels of transcript detection with 448 and 659 TPM detected respectively. Interestingly, unfertilised ova were also found to express high levels of LPL, with 338 TPM detected. Although LPL detection was widespread, the high abundance of this transcript within ovum was a positive sign that LPL may in fact be the GPI-anchored protein mediating sperm-egg interaction. As
was the case with CD160, within the developmental time course the level of LPL transcript decreased significantly following successful fertilisation. The fact that the level of LPL transcript plummets from 338 TPM detected in ovum to just 35 TPM in zygotes may again be an indication that within oocytes, LPL performs a role specifically related to sperm-egg interaction.

Antibodies against LPL were obtained and utilised to characterise the expression of LPL within ovaries. The only antibody able to be obtained was raised in mouse against human LPL. Fortunately murine LPL exhibits 92.63% sequence homology with human LPL, and thus use of this monoclonal mouse anti-human LPL antibody to detect murine LPL was a strong possibility.

The expected molecular weight for LPL was 53 kDa. During immunoblot analysis it was anticipated that LPL detection within ovarian lysates would be strong. Within brain protein extracts however, LPL detection was expected to be weak or non-existent due to reports that only 13 TPM were detected within mouse brain (see Figure 5.5). Looking at the results of the immunoblot analysis presented in Figure 5.6, it can be seen that antibody detection was indeed observed at the correct molecular weight for LPL within the two ovarian extracts. However, although labelling is evident at the 53 kDa range for the ovarian lysates, discrete bands were not detected. It remained plausible however that the diffuse labelling observed in this region of the blot may in fact have constituted LPL, given that the brain lysate, which was anticipated to exhibit little or no labelling, revealed no protein detection at this molecular weight range.

GPC2 did not reveal a highly restricted transcript expression like CD160, however widespread detection of the transcript was not detected as with LPL and HYAL2. Of the 16 tissues in which GPC2 transcript was detected, dorsal root ganglion exhibited the greatest level of GPC2 expression with 658 TPM detected. Despite consensus that GPC2 is expressed exclusively within the developing nervous system where it has been implicated in neurite outgrowth (Wang and Denburg, 1992), cell-cell adhesion (Reyes et al., 1990; Stanley et al., 1995), cell-substratum adhesion (LeBaron et al., 1988) and growth factor signalling (Zioncheck et al., 1995), GPC2 transcript was detected within oocytes at a level of 49 TPM. Whilst 49 TPM is not regarded as a high level of
detection, it remained possible therefore that GPC2 was expressed at the protein level within murine ovaries and oocytes.

To ascertain whether GPC2 was expressed at the protein level within ovaries, a monoclonal antibody raised in rat against mouse GPC2 was utilised to probe ovarian protein lysates. The results of this immunoblot analysis, which appear in Figure 5.8, were inconclusive in that discrete bands attributable to GPC2 expression were unable to be visualised due to high levels of background labelling. The inability to visualise discrete bands persisted despite efforts to optimise the labelling. GPC2 expression was however able to be visualised by means immunohistochemistry, as will be discussed.

As was the case for CD160, retrieval of the UniGene virtual expression profile for LYPD3 revealed a highly restricted expression profile. Of the 33 tissues analysed, LYPD3 transcript was detected in just 8, most notably these tissues included the female genital (77 TPM), the head and neck (55 TPM), and the gastrointestinal tract (33). Looking at developmental time course however, the LYPD3 gene was expressed at its highest level within oocytes, where over 200 TPM were detected. This restricted expression pattern and high level of transcript detection within oocytes therefore identified LYPD3 as a strong candidate for the GPI-anchored protein responsible for mediating sperm-egg interaction.

Due to the fact that LYPD3 presented as a strong candidate for the GPI-anchored protein mediating sperm-egg interaction, it was therefore desirable to perform antibody based characterisation studies in an attempt to confirm the presence of this protein within ovarian lysates as well as visualise its expression profile on ovarian sections. Unfortunately however, an antibody against LYPD3 was unable to be obtained from either commercial or non-commercial sources. Characterisation studies for this particular GPI-anchored protein therefore remain outstanding.

Of the 6 identified GPI-anchored proteins that warranted further characterisation, RAET1C remains the final protein to be discussed. As was the case for LYPD3 however, antibodies against RAET1C were unable to obtained, so characterisation studies for this protein once again remain outstanding. In addition, although RAET1C has been identified as a ligand for NKG2D and implicated in immunity (Hamerman et
al., 2004), information surrounding this protein is scarce to the point that a virtual expression profile for RAET1C is not yet available on the UniGene website. Although RAET1C is a GPI-anchored protein potentially expressed within oocytes, the role of this protein in sperm-egg interaction was therefore unable to be characterised.

The previously outlined immunoblotting analyses were performed as preliminary investigations into the expression of the GPI-anchored candidates at the protein level. Although it was preferential to perform these investigations on protein extracts prepared from isolated oocytes, the large number of oocytes required to generate a protein pool of sufficient quantity meant that this approach was not feasible. It was however possible to visualise expression patterns for the GPI-anchored proteins of interest through the use of immunohistochemistry analyses. The results of the immunohistochemistry analyses appear in Figure 5.10 and demonstrate which cell types of the murine ovary expressed each of the GPI-anchored proteins of interest.

As was the case with the immunoblotting analyses, all three antibody clones raised against CD160 were utilised to visualise CD160 expression within ovaries. Since antibody clones 7H1 and 2C7 were biotin labelled, streptavidin-HRP was utilised to achieve primary antibody detection. Relative to the streptavidin-HRP only control, incubation of ovarian sections in either the 7H1 or 2C7 antibody clones resulted in no apparent labelling of ovarian cell types as evidenced by the lack of brown staining (see Figure 5.10A). Failure of these antibody clones to label ovarian sections, in particular the oocytes which are known to express 199 TPM for CD160, was disappointing in that both of these clones had successfully labelled a protein band of the appropriate molecular weight for CD160 during the previous immunoblot analyses. During immunoblot analyses proteins are subjected to denaturing conditions such as boiling in the presence of SDS and the reducing agent 2-mercaptoethanol. Immunohistochemistry however, does not include a denaturation step and may account for observed differences in the ability of these antibodies clones to label proteins of the murine ovary during either immunoblot or immunohistochemistry analyses.

Whilst 7H1 and 2C7 failed to label ovarian sections, the CD160 antibody clone CL1-R2 revealed apparent labelling of the cytoplasm of oocytes (see Figure 5.10B). This labelling was evident as a brown staining of the oocytes cytoplasm that was clearly
intensified relative to the oocytes visualised in the secondary antibody only control. Although unexpected, this cytoplasmic labelling may be attributable to the immature oocytes synthesising CD160 in anticipation of ovulation and fertilisation.

In addition, it is important to note that although efforts were made to eliminate non-specific binding, the HRP-conjugated secondary antibody utilised in the detection of CL1-R2 revealed its own inherent labelling of ovarian fractions. Unfortunately the goat anti-mouse IgG-HRP in question labelled the periphery of oocytes, which is exactly where CL1-R2 mediated labelling of CD160 was anticipated. It remained possible therefore that CL1-R2 did indeed label the surface of oocytes as expected, in addition to the oocyte cytoplasm, but that this surface labelling was unable to be observed relative to the control due the non-specific labelling of the secondary antibody. Whilst 7H1 and 2C7 revealed no labelling of non-denatured oocyte proteins, CL1-R2 therefore revealed cytoplasmic labelling of oocytes, with labelling of the oocyte surface remaining a possibility also.

Although non-specific labelling observed for the goat anti-mouse IgG-HRP interfered with interpretation of CL1-R2 results, no such concerns were evident in the visualisation of anti-HYAL2 labelling. Relative to the secondary antibody only control, incubation of ovarian sections in anti-HYAL2 revealed exceptionally strong labelling of multiple ovarian cell types including granulosa cells and stromal layer cells as well as the oocytes themselves (see Figure 5.10B). This observation was consistent with results of the immunoblot analysis in which anti-HYAL2 was observed to detect a protein band of the correct molecular weight for HYAL2 within ovarian protein extracts. Although it would have been ideal for ovaries to exhibit HYAL2 expression exclusively within oocytes, HYAL2 therefore remained a strong candidate protein as the GPI-anchored molecule responsible for mediating sperm-egg interaction.

Such positive results were not however able to be visualised upon analysis of LPL expression within ovarian sections. Incubation of ovarian sections in mouse anti-human LPL revealed no apparent labelling of any ovarian cell types. This was evident in the fact that the labelling pattern observed for sections incubated in the anti-LPL exactly reflected that observed for the secondary antibody control. It remained possible that anti-LPL had indeed labelled the surface of oocytes and that this labelling was simply
unable to be observed due to non-specific labelling of the secondary antibody, as was the case for CD160. Therefore, despite this negative result, anti-LPL was still deemed worthwhile for analysis during subsequent sperm-egg binding and fusion assays to ascertain whether the antibody does indeed have the capacity to bind to oocytes and in doing so potentially influence rates of sperm-egg binding and/or fusion.

Fortunately, during the analysis of anti-GPC 2 labelling of ovarian sections, the secondary antibody utilised exhibited no non-specific labelling of ovarian cell types. As such, when strong staining of oocyte nuclei as well as some granulosa cells was observed following incubation of sections in anti-GPC2, the results was directly attributable to labelling of these cell types with anti-GPC2. Labelling observed for anti-GPC2 was interesting with respect to both cell types in which staining occurred. Firstly, as a GPI-anchored protein GPC2 was expected to be localised to the surface of oocytes. HRP-mediated staining however was clearly localised to the oocyte nuclei. Secondly, although it was not deemed unusual for anti-GPC2 to have labelled granulosa cells, it was deemed unusual for GPC2 labelling to have been observed in only some granulosa cells, and that the granulosa cells in which labelling was observed were all localised within close proximity to the oocyte. This selective labelling or expression of GPC2 on granulosa cells close to the oocytes remains to be understood. The nuclear labelling observed for GPC2 however may simply have been the result of the rat anti-mouse GPC2 non-specifically labelling a protein or proteins present in the oocyte nucleus. Alternatively, a non-GPI-anchored isoform of GPC2 may very well have been expressed within the nuclei of oocytes from pre-ovulatory follicles, although this was deemed unlikely.

Although anti-GPC2 labelling revealed nuclear staining of oocytes that may or may not have been attributable to GPC2, the uncertainty surrounding GPC2 expression meant that it remained necessary to ascertain the affect of anti-GPC2 on rates of sperm-egg binding fusion by means of antibody inhibition studies. In addition, although the antibody clones 7H1 and 2C7 clones had failed to label oocytes in ovarian sections, the CL1-R2 clone of anti-CD160 revealed cytoplasmic labelling, as well as potential surface labelling of oocytes. CL1-R2 was therefore also chosen for further investigation to ascertain its capacity to inhibit or block sperm-egg binding and/or fusion. HYAL2 revealed high levels of expression within oocytes and was therefore deemed important.
Characterisation of Candidate Proteins

for further characterisation by means of antibody inhibition studies. Finally, LPL did not appear to be expressed in oocytes however the uncertainty of surface labelling for anti-LPL, as a result of the non-specific labelling of the secondary antibody, meant that this protein also needed to be investigated by means of the sperm-egg binding and fusion bioassay.

As previously mentioned, the use of antibodies has already proved valuable in the identification of proteins implicated in sperm-egg interaction. In particular the antibody JF9 binds to the tetraspanin CD9 and effectively inhibits sperm-egg binding and fusion in a dose-dependent manner (Chen et al., 1999). It was hoped therefore that at least some of the antibodies being utilised to characterise the GPI-anchored proteins of interest would exhibit a capacity to significantly impede rates of sperm-egg binding and/or fusion in vitro. The results of these competitive inhibition studies appear in Figure 5.11. During these studies, oocytes were pre-incubated in anti-CD160 (CL1-R2 clone), anti-HYAL2, anti-GPC2 or anti-LPL at 1:50 dilutions for 60 min prior to being utilised in sperm-egg binding and fusion bioassays. Any observed reductions in the number of sperm bound or fused to these oocytes would therefore be interpreted as antibody binding having successfully inhibited the interaction of the egg membrane proteins with complementary sperm surface proteins.

Of the GPI-anchored proteins analysed, CD160 was believed to represent the most likely candidate as an egg surface protein responsible for mediating sperm-egg interaction. Following incubation of oocytes in anti-CD160, it was observed that a mild decrease in rate of sperm-egg binding was observed. Although this reduction in sperm binding rate was exactly what was hoped for, the reduction was found not to be statistically upon analysis of the data (P = 0.085). Additionally, incubation of oocytes in CD160 had no affect on rates of sperm-egg fusion (P = 0.155). A greater number of replicates with increased numbers of oocytes will be necessary to confirm reduced sperm-oocyte binding which time constraint prevented.

Similar to pre-incubation of oocytes in anti-CD160, pre-incubation of oocytes in anti-LPL revealed no statistical significance in rates of sperm binding or fusion when compared to untreated control oocytes. This observation was expected given that anti-
LPL failed to exhibit labelling oocytes during previous ovarian immunohistochemistry analyses (see Figure 5.10B).

Statistically significant affects on rates of sperm-egg binding were however observed upon pre-incubating oocytes in anti-HYAL2 or anti-GPC2. In both cases however, pre-incubation of the oocytes in the respective antibodies resulted in increases in the observed rates of sperm-egg binding. Following completion of the IVF incubation, an average of 13.28 ± 1.14 sperm/egg bound to control oocytes. In comparison, oocytes pre-incubated anti-HYAL2 bound and average of 19.25 ± 1.03 sperm/egg, whilst oocytes pre-incubated in anti-GPC2 bound an average of 20.4 ± 1.96 sperm/egg. In both cases however, this increased rate of sperm binding did not translate into increased rates of sperm-egg fusion. The precise reason why binding rates were increased as opposed to decreased remains to be determined. The fact that increased binding did not translate into increased rates of membrane fusion however suggests that the additional sperm were not bound in a manner consistent with enhanced fertilisation.

Despite resulting in a statistically significant increase in sperm-egg binding rates HYAL2 and GPC2 are therefore not expected to be implicated in sperm-egg interaction based on these results. However although none of the antibodies utilised exhibited a capacity to impede sperm-egg binding or fusion in a manner similar to JF9, the results should not be taken as conclusive evidence that one or more of the GPI-anchored proteins analysed are not responsible for mediating sperm-egg interaction. As previously outlined, antibody based characterisation studies are a relatively quick and cost effective means of obtaining valuable information regarding a proteins distribution and function. There is no guarantee with this approach however that a particular antibody will impede a target protein’s function upon binding to the epitope recognised within the protein’s secondary structure. For this reason, antibodies may well have bound to CD160 on the surface of mouse oocytes, and yet may not have produced enough steric hindrance to preclude the interaction of CD160 with its complementary ligand on the sperm surface. As such, although an antibody based characterisation approach had the potential to identify GPI-anchored proteins implicated in sperm-egg interaction, the fact that the approach has not succeeded simply means that alternative strategies will need to be adopted to obtain conclusive answers. These additional
measures along with other future experiments will be discussed in detail in the following chapter.
CHAPTER 6:

Final Discussion
CHAPTER 6: FINAL DISCUSSION

6.1 Discussion
Investigations presented in previous chapters are wide ranging in their strategies and approaches to elucidate the various components of sperm-egg interaction. The aim of this final chapter is to provide an integrated and concise overview of the findings and how the data may be viewed collectively to provide greater insight into the oocyte’s mechanisms for mediating sperm-egg interaction.

During the early stages of this project, investigations designed to elucidate and characterise factors that affect murine sperm-egg interaction were presented. These included previously known treatments, such as release of GPI-anchored proteins to reduce sperm-egg binding, as well as exploratory studies to identify novel means of impacting sperm-egg interaction, such as xenobiotic modification of the oolemma.

Studies in which PI-PLC treatment of oocytes was utilised to release egg surface GPI-anchored proteins clearly demonstrated the importance of this class of protein in mediating sperm-egg interaction. Effective release of GPI-anchored proteins by PI-PLC was demonstrated using confocal microscopy to monitor the release of the known GPI-anchored protein CD55. When coupled with the sperm-egg binding and fusion bioassay, it was observed that this release of GPI-anchored proteins was associated with significant reductions in the rates of sperm-egg binding in vitro. This result was consistent with the in vitro studies performed by Coonrod and colleagues in 1999 (Coonrod et al., 1999b), and is supported by the pig-a knockout studies performed by Alfieri and colleagues in 2003, in which infertility in null female mice was observed upon the oocyte specific deletion of a subunit of an enzyme involved in GPI-anchor biosynthesis (Alfieri et al., 2003). The findings therefore supported a role for one or more GPI-anchored proteins in mediating fertilisation at the level of sperm-egg binding, and highlighted the need to identify the oocytes complement of GPI-anchored proteins in order to advance the current state of knowledge in this field.
The fact that release of oolemmal GPI-anchored proteins resulted in inhibition at the level of sperm-egg interaction is important when viewed in context with the results of the exploratory xenobiotic studies. Initial studies were performed in an attempt to expand upon the known series of factors found to impact upon sperm-egg interaction. Major factors identified in the literature were the release of egg surface GPI-anchored proteins, which affected sperm-egg binding (Coonrod et al., 1999a; Coonrod et al., 1999b), as well as the deletion of the tetraspanin CD9, which affected sperm-egg fusion (Kaji et al., 2000; Le Naour et al., 2000; Miyado et al., 2000). Following the series of xenobiotic experiments outlined in Section 3.3.3, it is now apparent that treatment of oocytes with specific xenobiotics capable of arylation and alkylation of cell surface components may be added to this list of factors capable of impacting upon sperm-egg interaction.

In what was a novel finding, it was discovered that incubating oocytes in the presence of 10 µM benzoquinone or bis maleimide modified oocytes in such a manner as to significantly reduce the ability of sperm to bind to the oolemma. Whilst the mechanism of bis maleimide action is yet to be understood, confocal microscopy revealed that benzoquinone treatment resulted in alkylation of free thiols on the egg surface. This alkylation of surface thiols is believed to underpin the inhibitory affects of benzoquinone on sperm-egg interaction, and is supported by the findings of Ellerman et al., 2006. DTNB is an agent that covalently binds to free thiols effectively alkylating these functional groups. During IVF studies, Ellerman and colleagues observed that incubating spermatozoa in the presence of DTNB resulted in significant reductions in the fertilising capacity of sperm following thiol alkylation (Ellerman et al., 2006). Alkylation of oocyte surface thiols by means of specific xenobiotics may therefore be anticipated to exert a similar effect fertilising capacity.

An explanation as to importance of surface thiols may be obtained from the results of the proteomic analyses of oocytes. The proteomic analysis of oocytes by 2D LC-MS/MS constituted a major component of this research. During this analysis, 5 different isoforms of PDI were detected in oocytes (P < 0.05), these included PDI-A1 (ERp59), PDI-A3 (ERp57), PDI-A4 (ERp72), PDI-A5 and PDI-A6 (see Appendix B, Table B1). The different isoforms of PDI function to catalyse disulphide bond formation, reduction and isomerization (Turano et al., 2002). Given reports that at least one member of the
PDI family are expressed on the surface of oocytes (Calvert et al., 2003), one may anticipate egg surface PDIs are continually reducing, rearranging and maintaining thiol bonds within proteins on the oocyte surface to ensure correct protein structure (Turano et al., 2002). It is expected therefore, that benzoquinone was able to alkylate free surface thiols while these functional groups were being maintained by egg surface PDIs. Once covalently alkylated by benzoquinone, these thiols would no longer be available for disulphide bond formation, and would thus impact upon protein structure and function. In support of this, it has been demonstrated that inhibition of ERp57 on the sperm surface results in an inhibition of murine fertilisation (Ellerman et al., 2006). This finding suggests that PDIs are indeed active in modifying disulphide bonds within proteins expressed on the surface of cells, and such a mechanism may account for the inhibitory action of benzoquinone on fertilisation upon treating oocytes.

The remodelling of disulphides has been demonstrated to play an important role in membrane fusion during envelope virus infection of host cells (Barbouche et al., 2003; Gallina et al., 2002; Matthias et al., 2002). Moreover, it has been hypothesised throughout this body of research that the mechanisms fundamental to sperm-egg interaction may parallel those of envelope virus – host cell fusion. As such, the proteomic data presented in Section 4.3.6.4 documenting the presence of 5 PDI isoforms in oocytes, together with the findings of Ellerman et al., 2006 suggests that this may very well be the case. In the most thoroughly studied system of membrane fusion that is HIV infection, data supports a situation whereby PDI is associated with CD4 on the surface of T cells. Upon binding of viral gp120 to CD4 and a co-receptor (CCR5 or CXCR4), PDI functions to reduce two disulphide bonds within gp120 (Barbouche et al., 2003; Gallina et al., 2002). The reduction of these disulphide bonds triggers conformational changes that lead to the exposure of a fusion peptide hidden within gp41 and insertion of this peptide into the host cell membrane to facilitate fusion (Barbouche et al., 2003; Gallina et al., 2002).

It could be anticipated therefore, that when sperm and oocyte membranes come into close proximity, one or more of the PDI isoforms expressed in the oocyte, possibly in concert with ERp57 on the sperm surface, may serve to remodel thiol-containing proteins on the oocyte and/or sperm surface to initiate protein conformational changes that promote membrane fusion. Paralleling HIV - T cell membrane fusion with that of
sperm – egg fusion, a GPI-anchored protein(s) could replace CD4, as strong evidence suggests that GPI-anchored proteins are implicated in the initial binding of spermatozoa to oocytes. PDI may therefore be associated with GPI-anchored proteins on the egg surface in anticipation of catalysing thiol-disulphide exchange reactions upon sperm binding. Applying the HIV – T cell paradigm to the spermatozoa, the protein Izumo may represent viral Env, which is composed of the gp41 and gp120 subunits. However as is the case for all retroviruses, HIV has one envelope protein that is responsible for both binding and fusion. The function of Izumo however appears to be specifically limited to promoting membrane fusion possibly, through mediating the correct alignment of the gamete membranes, in that while sperm from Izumo knockout males cannot fuse with oocytes, they remain capable of binding to the egg surface (Inoue et al., 2005). Another class of protein expressed on the head of acrosome reacted sperm therefore appears to be responsible for mediating the binding of sperm to the oolemma.

GPI-anchored proteins and Izumo may therefore be the functional counterparts of CD4 and gp120/gp41 respectively in the mediation of gamete recognition and fusion. However, proteins on the egg surface are yet to be identified that correspond to the co-receptors on the T-cell surface, CCR5 and CXCR4, which are essential for achieving fusion. Continuing the analogy, a different class of protein(s) expressed on the egg surface would therefore need to be responsible for interacting with Izumo to induce membrane fusion, subsequent to sperm binding. Interestingly however, CXCR4 is actually expressed on the surface of oocytes within developing antral follicles (Holt et al., 2006). Rather then finding proteins on the egg surface to parallel the function of CXCR4 during viral fusion, it is possible therefore that the same protein responsible for mediating HIV – T cell membrane fusion may also be responsible for mediating membrane fusion during fertilisation. The above analogy between HIV – T cell membrane fusion and sperm – egg fusion is clarified in Figure 6.1.
Figure 6.1 Possible functional counterparts in the mediation of HIV – T cell membrane fusion and sperm – egg membrane fusion.

CD4 on the T cell surface is implicated in HIV binding whilst the co-receptors CCR5 and CXCR4 are implicated in subsequent membrane fusion events. Counterparts for these proteins on the oocyte surface may be GPI-anchored proteins and CXCR4 respectively. Viral gp120 mediates binding to T cells whilst gp41 facilitates membrane merger. On the sperm surface Izumo is essential for membrane fusion but not binding. Izumo may therefore be the sperm surface counterpart for gp41, however additional proteins on the sperm surface are anticipated to be mediators of sperm binding.

During HIV infection, it has been shown that the spatial orientation of PDI in relation to gp120, CD4 and CXCR4 is critical for PDIs ability to facilitate membrane fusion (Markovic et al., 2004). Observations by Calvert et al., 2003 localising oocyte PDI to the oolemma, in conjunction with the proteomic identification of 5 different oocyte isoforms of PDI reported herein (Chapter 4, Section 3.3.6.4), therefore provides a premise for a possible interaction between egg surface PDI and CXCR4 in the mediation of sperm-egg membrane fusion.

Whilst an association of egg surface CXCR4 with PDI during gamete fusion is an attractive extrapolation of viral-host cell fusion events, evidence is yet to be obtained in support of this. However, much evidence has already been obtained in support of other proteins involvement as discrete mediators of membrane fusion. The fact that membrane fusion occurs downstream of binding suggests that it should be possible to
delete the egg proteins implicated in membrane fusion whilst having no apparent effect on sperm-egg binding. This is very much the case considering results of tetraspanin studies. As previously discussed, deletion of the murine CD9 was simultaneously reported by three independent laboratories to result in severely reduced fertility in females (Kaji et al., 2000; Le Naour et al., 2000; Miyado et al., 2000). Despite normal health and vitality, females in all cases exhibited severely reduced fertility attributable to a lack of fusibility of their CD9-deficient oocytes. While no significant differences were observed for sperm binding capacity between control and CD9-deficient eggs, CD9-deficient eggs rarely fused with spermatozoa during either in vivo or in vitro fertilisation studies (Kaji et al., 2000; Le Naour et al., 2000; Miyado et al., 2000). Furthermore, deletion of murine CD81 resulted in a similar phenotype, in that females exhibited a 40% reduction in fertility attributable to reduced oocyte fusibility (Rubinstein et al., 2006a).

Each of these tetraspanins therefore conform to the virus – host cell analogy, in that they represent proteins expressed on the surface of oocytes responsible for mediating the membrane fusion events of fertilisation. In addition to these findings however, reported herein is the novel finding that deletion of the tetraspanin CD151 also affects sperm-egg interaction at the level of membrane fusion. It was seen in Section 3.3.4 that deletion of murine CD151 resulted in an abnormal ovarian phenotype (see Figure 3.10), with ovaries exhibiting an increased prevalence of atretic bodies, smaller corpora lutea, basement membrane detachment and friable tissue. Subjecting ovulated oocytes to the sperm-egg binding and fusion bioassay revealed that fusion rates were reduced by >75% in oocytes obtained from CD151 null females. Within oocytes, successful membrane fusion may therefore be impeded by disrupting expression of CD9 or CD151. Furthermore, it has also been shown that exogenous expression of the tetraspanin CD81 can partially rescue fertility in CD9 deficient oocytes (Kaji et al., 2002).

Within the literature, evidence for tetraspanin function has led to the consensus that these molecules operate as membrane organisers (Rubinstein et al., 1996). By establishing cis interactions with adjacent proteins, tetraspanins are believed to interact with a web of binding partners, and in doing so facilitate the organisation of components within a receptor complex or other membrane complexes. Binding partners
for CD9 include CD9P-1 and EWI-2 (Charrin et al., 2003; Charrin et al., 2001; Clark et al., 2001; Stipp et al., 2001a; Stipp et al., 2001b). In the case of murine fertilisation, it therefore remains unclear as to whether CD9 and CD151 facilitate fusion by interacting directly with a protein(s) expressed on the sperm surface, such as Izumo, or whether these tetraspanins function in a more traditional role as membrane organisers and interact with a host of lateral binding partners. Experimental evidence has been obtained suggesting that it may be possible for tetraspanins to interact directly with binding ligands. PSG17 is a ligand found to interact directly with CD9 (Ellerman et al., 2003). Although PSG17 is not expressed on the surface of sperm, this example of a trans interaction between a tetraspanin and ligand raises the possibility that CD9 and CD151 may interact directly with the proteins on the sperm itself.

A more likely scenario however, is that on the surface of oocytes CD9, CD81 and CD151 again serve as membrane organisers and are implicated in membrane fusion through facilitating the organization of a receptor complex. If this were the case then the receptor complex would be anticipated to contain an as yet unidentified component capable of interacting with sperm proteins directly, and in doing so serve as a co-receptor for membrane fusion. This latter scenario is supported by the fact that deletion of CD9, CD81 or CD151 by themselves do not result in complete abolition of oocyte fusibility. Double knockout of CD9 and CD81 however achieves complete abolition of oocyte fusibility (Rubinstein et al., 2006a). This finding suggests that oocyte tetraspanins perform complementary roles, with each specific tetraspanin responsible for recruiting one or more binding partners to the receptor complex. These binding partners may overlap between different tetraspanins, such as CD9P-1, which can interact with both CD9 and CD81, or may be specifically recruited by a particular tetraspanin, such as integrins, which interact preferentially with CD151 (Sterk et al., 2000). It would therefore be anticipated that as more oolemmal tetraspanins are deleted, the oocytes sperm receptor complex would become increasingly disordered and may lack individual components. Such a notion is supported by observations that deletion of different tetraspanins results in the loss of different levels of fertility. Deletion of CD81 results in a 40% reduction in female fertility (Rubinstein et al., 2006a), CD9 deletion however results in a 94% reduction in fertility (Miyado et al., 2000) while CD151 deletion resulted in a 75% reduction in oocyte fusibility (see Section 3.3.4). In this regard, the binding partners recruited to the complex by CD9 may play a more
important role in mediating fusion then those recruited by CD81 for example. The loss of any one component is therefore unlikely to completely abolish function of the receptor complex, and this is evidenced in the fact that many different classes of proteins may be deleted or inhibited to achieve reductions in oocyte fertilising capability, however none completely abolishes fertility; for review see (Rubinstein et al., 2006b).

Discussed previously was the finding that CXCR4 is expressed on the surface of antral follicle oocytes (Holt et al., 2006) and the plausibility that interaction of CXCR4 with PDI may be important in mediating the events of sperm-egg interaction, for which thiol/disulphide exchange appears to be important (Ellerman et al., 2006). In light of the view that tetraspanins facilitate membrane fusion by forming cis interactions with binding partners, tetraspanins may represent the molecular organisers responsible for maintaining a close association between egg surface PDI and CXCR4. Such a notion is consistent with reports that PDI does not possess a transmembrane domain, and its association with the plasma membrane is maintained by way of non-covalent interactions with integral membrane proteins, lipids and glycans (Terada et al., 1995). Under such circumstances, deletion of the tetraspanins (CD9, CD81 and CD151) would be expected to disrupt the close association between PDI and CXCR4 and impair the process of sperm-oocyte fusion. This is because PDI would no longer be maintained in close proximity to CXCR4, and as such would not be able to catalyse the fusion-promoting thiol/disulphide exchange events, which during HIV infection occur downstream of binding but upstream of fusion (Markovic et al., 2004).

For reasons previously discussed, the identification of the GPI-anchored protein(s) implicated in sperm-egg interaction was of high priority. In an attempt to address this, a proteomics approach was adopted in order to identify the oolemma’s complement of GPI-anchored proteins. The characterisation studies presented in Section 3.3.2 revealed that PI-PLC was effective in releasing GPI-anchored proteins from oocyte membranes. As such, initial attempts to sequence egg GPI-anchored proteins focused on collecting proteins liberated into the egg culture medium supernatant by PI-PLC. This approach also enabled the concurrent collection of proteins from the remainder of the oocytes, which were fractionated into oocyte surface proteins and oocyte lysate. Proteomic identifications were successfully obtained for the oocyte lysate and surface protein
fractions (see Tables 4.2 and 4.3), however it soon became apparent that the abundance of oocyte GPI-anchored proteins was very low (see Figure 4.2), and even though the professional LC-MS/MS services of APAF were eventually utilised, difficulties in obtaining sufficient protein quantities for effective LC-MS/MS analysis persisted. In an attempt to enhance proteomic identifications, it was decided to perform fractionation at the peptide level, as opposed to fractionation at the protein level. For this purpose, protein extracts prepared from readily available whole ovaries were utilised to develop the appropriate protocols. In addition, whole ovary extracts were utilised to compare the number of proteomic identifications obtained from non-fractionated peptides extracts with that of peptides fractionated according to pI.

In-house LC-MS/MS analysis of non-fractionated peptides yielded a total of 722 successful identifications from 30 µg of ovarian protein extract (see Appendix A, Table A1). However by using IPG strips to separate peptides into 8 discrete fractions, a total of 1162 protein identifications were obtained from 500 µg of ovarian protein extract (see Appendix A, Table A2). Fractionation of peptides prior to LC-MS/MS analysis therefore yielded a greater number of protein identifications. In addition, upon interpreting the data it became apparent that a large percentage of peptides had actually been lost throughout the fractionation and recovery process. Peptide fractionation therefore provided enhanced levels of protein identification despite the loss of substantial amounts of material. A means of fractionating peptides whilst sustaining minimal loss of sample was therefore envisaged as the most appropriate means of obtaining GPI-anchored protein identifications from the an oocyte extract.

For this purpose, the protein extract from 2346 oocytes was sent to APAF for 2D LC-MS/MS analysis. Where IPG strips had been used to separate ovarian peptides into 8 fractions based on pI prior to LC-MS/MS, a size exclusion column was utilised to separate oocyte peptides into 12 fractions according to size, prior to LC-MS/MS analysis. This column-based approach meant that peptides were not lost through failure to elute from IPG strip segments or during the recovery of peptides from the StrataX clean up columns. LC-MS/MS analysis performed on the 12 individual fractions yielded the successful identification of 337 oocyte proteins (see Appendix B, Table B1). This protein database potentially represents the most comprehensive list of oocyte proteins.
identified to date in that the 337 successful identifications exceeds previously published results.

Although 2D LC-MS/MS was successful in identifying 337 oocyte proteins, including the oocyte specific proteins ZP1, ZP2 and ZP3, the list did not include any known GPI-anchored proteins. This is believed to be due to the fact that proteins such as ePAD and L-lactate dehydrogenase B chain represent relatively large percentages of the oocyte’s total protein pool. For every MS scan performed during the analysis, the 3 most intense peaks (detected peptides) were selected for acquisition of MS/MS data. The fact that peptides from ePAD and L-lactate dehydrogenase B chain were present in such high abundance meant that lower abundance peptides, which may have been only the 4th or 5th most intense, were not selected for acquisition of MS/MS data. Since mass to charge ratio data was not obtained for these lower abundance peptides, identification of the proteins they were generated from was not possible. 2D fractionation of peptides was implemented in an attempt to reduce the silencing affect of these high abundance proteins, however it is now apparent that even 2D fractionation of peptides was not sufficient to allow identification of the lower abundance proteins. It is anticipated however that additional processing of protein samples could be implemented to overcome this problem, however time constraints did not permit the regeneration of another pool of oocyte proteins on which to perform the analyses, as will be discussed in the future directions.

Although proteomic analysis of oocytes did not yield the identification of egg surface GPI-anchored proteins, concurrent studies undertaken to identify these proteins at the gene level proved highly successful. Through accessing the MGI website it was possible to view a list of genes known to encode GPI-anchored proteins that were found to be expressed in one or more murine tissues. By itself this list of known murine GPI-anchored proteins afforded little insight into the identity of the GPI-anchored protein(s) implicated in sperm-egg interaction. Cross-referencing this list against the results of a study performed by Evsikov et al., 2006 however enabled significant progress to be made. Given that Eviskov and colleagues had identified and published a list of 4790 gene transcripts detected within mouse oocytes, it was possible to screen the list of 116 GPI-anchored proteins listed on the MGI website against the list of genes detected in oocytes. This is the first reported instance of the application of such an approach to the
identification of egg surface GPI-anchored proteins, and the result was the identification of 11 GPI-anchored protein encoding genes expressed within murine oocytes.

Of the 11 genes identified, 5 were precluded as potential mediators of sperm-egg interaction (for reason previously outlined) upon consulting the relevant published literature (see Section 5.3.1.1). Genes that passed the filtering process included CD160, LYPD3, LPL, RAET1C, GPC2 and HYAL2. Of these proteins, CD160 and HYAL2 were deemed the most probable candidates as potential mediators of sperm-egg interaction. Previously published findings had already identified CD160 as a receptor for T cell activation where it serves as ligand for MHC class 1a and 1b binding (Agrawal et al., 1999; Maeda et al., 2005). The protein therefore had a documented role in receptor activity, which was consistent with its possible function during sperm binding. In addition to this however, the amino acid sequence for CD160 indicated that the protein contains 2 cysteine residues indicating the possible presence of a disulphide bond. This presence of a putative disulphide bond was consistent with evidence suggesting that PDI-mediated disulphide exchange may be implicated in sperm-egg interaction (Ellerman et al., 2006). Finally, the UniGene expression profile for CD160 revealed that high-level expression of this gene was restricted to oocytes (see Figure 5.1), indicating that the protein may play an important role in processes occurring at this stage of development, such as fertilisation. Conversely, the UniGene profile for HYAL2 revealed broad range expression across 24 different tissues. However HYAL2 was deemed a high probability candidate given that the protein is known to serve as a receptor for the jaagsiekte sheep retrovirus (Dirks et al., 2002; Rai et al., 2001). HYAL2 therefore conformed to the ongoing paradigm that the events mediating sperm-egg fusion parallel those mediating virus-host cell fusion. Despite the fact that CD160 and HYAL2 appeared to be stronger candidates then the remaining GPI-anchored proteins, it remained necessary to characterise the expression profile of each and ascertain their potential role in mediating sperm-egg interaction.

During expression studies HYAL2 appeared to be the only candidate for which strong detection was observed within oocytes in a manner consistent with a role in mediating sperm-egg interaction. A positive result was observed for GPC2, however the observed nuclear labelling was not consistent with a role in mediating receptor binding events taking place on the surface of oocytes. CD160 appeared to be detected during
immunoblot analysis with antibody clones 7H1 and 2C7, however each of these antibodies failed to label oocytes present in ovarian sections. CD160 may however have been detected within oocytes during immunohistochemistry analysis with the CL1-R2 antibody clone in that incubation of slides with CL1-R2 led to cytoplasmic labelling of oocytes. In contrast, attempts to detect LPL expression did not support a role for this particular GPI-anchored protein in mediating sperm-egg interaction in that immunoblot detection was questionable while immunohistochemistry analysis failed to detect LPL in ovarian sections. Thus investigations into the expression of GPI-anchored proteins once again highlighted HYAL2 and CD160 as the strongest candidates for potential mediators of sperm-egg interaction. It remains possible that RAE1T1C and LYPD3 may have exhibited strong oocyte expression, however the inability to source antibodies against these candidates meant that their expression could not be investigated. This of course also meant that antibody inhibition studies were unable to performed for these particular proteins using the sperm-egg binding and fusion bioassay.

The sperm-egg binding and fusion bioassay was utilised in conjunction with antibody labelling in an attempt to explore the role of the GPI-anchored protein candidates in mediating sperm-egg interaction (with the exception of RAET1C and LYPD3 for which antibodies were unable to be sourced). Antibody labelling has the potential to disrupt receptor-ligand interaction, and in doing so provide great insight into role of a particular protein within a given cell type. However effective disruption of protein function upon antibody labelling is not guaranteed, and during IVF studies this limitation was kept in mind.

Of the 6 oocyte GPI-anchored proteins identified, CD160 and HYAL2 had emerged as the highest probable candidates as mediators of sperm-egg interaction. Incubation of oocytes with the anti-CD160 resulted in a mild reduction in the average rate of sperm-egg binding. However this reduction did not attain statistical significance ($P = 0.08$) when compared to binding rates of the untreated control oocytes (see Figure 5.11). Nonetheless, the result was exciting in that pre-incubating oocytes in the presence of CD160 resulted in inhibition of IVF at the level of sperm-egg binding, which was precisely in accordance with strong evidence suggesting that GPI-anchored proteins are involved in mediating this process. Failure to attain statistical significance may have been attributable to factors such as oocyte sample size, the affinity of the antibody or
the nature of the epitope targeted. With increased numbers of oocyte replicates it is anticipated statistically significant levels of inhibition of sperm-egg binding would have been achieved. No such expectations are retained for LPL however in that even with the limited sample size analysed, anti-LPL labelling of oocytes revealed no apparent effects on sperm-egg interaction.

Although the effects of anti-CD160 were not statistically significant, they were at least consistent with expectations that a reduction in binding rates would be observed. Pre-incubating oocytes in anti-HYAL2 however, actually resulted in a statistically significant increase in the observed rates of sperm-egg binding ($P = 0.003$). This phenomenon was also observed upon anti-GPC2 treatment of oocytes. As previously discussed however, these increased rates of sperm-egg binding did not translate into increased rates of sperm-egg fusion. This important observation therefore suggests that the additional sperm bound to anti-HYAL2 or anti-GPC2 treated oocytes were bound non-specifically in a manner inconsistent with successful fertilisation. In what was a disappointing outcome, anti-HYAL2 therefore failed to competitively inhibit murine sperm-egg binding \textit{in vitro}.

When faced with a disappointing outcome, such as results of antibody inhibition studies outlined above, it is possible to speculate as to the reasons why the failure occurred and how the desired result may possibly be achieved. Alternatively, the results may simply indicate, in this particular case, that GPI-anchored proteins are not directly implicated in sperm-egg interaction. Within membranes GPI-anchored proteins partition into lipid rafts, which are plasma membrane microdomains enriched in glycosphingolipids and cholesterol. These finite membrane regions are postulated to function in signalling events as well as membrane trafficking (Brown and London, 1998; Horejsi et al., 1999; Simons and Toomre, 2000). The attachment of saturated alkyl groups to proteins, such as the attachment of a GPI anchor, can result in the localisation of proteins within lipid rafts (Simons and Toomre, 2000). Given the specialised nature of lipid rafts, it remains possible that the enzyme-mediated release of GPI-anchored proteins from the oocyte surface (Coonrod et al., 1999a; Coonrod et al., 1999b), or deletion of the GPI anchor itself (Alfieri et al., 2003) may have disrupted these lipid rafts potentially altering the surface architecture of the oocytes. Studies into the effects of CD9 deletion have already demonstrated that deletion of this gene product is associated in ultrastructural changes.
in oocyte membrane topography, including altered length, thickness and density of the microvilli (Runge et al., 2007). Whilst tetraspanins partition into their own discrete lipid microdomains, as opposed to traditional lipid rafts (Wright et al., 2004b), the finding is an example of how the loss of one particular protein can have drastic ramifications on the structure and function of cellular membranes. The fact that PI-PLC may very well indiscriminately cleave the oocytes entire complement of GPI-anchored proteins may therefore affect lipid raft structure and function, and in doing so, indirectly impact upon sperm-egg interaction. Under such circumstances, antibodies against GPI-anchored proteins would therefore not be anticipated to inhibit sperm-egg interaction. In this regard, analysis of the membrane topography of GPI-anchor deficient oocytes would be an exciting avenue to explore.

Laboratories in possession of mice with the oocyte-specific GPI knockout are however yet to perform such ultrastructural experiments, and until such time as GPI anchor cleavage or deletion is associated with detrimental changes in oocyte membrane structure, it remains possible that a GPI-anchored protein(s) is directly implicated in mediating sperm-egg interaction.

Although none of the antibodies utilised were effective in producing statistically significant levels of IVF inhibition, the results do not eliminate the analysed candidates as potential mediators of sperm-egg interaction given the limitations of antibody-based functional characterisation (see Chapter 5, Section 5.1). That said, in light of these findings and other published information, LPL and GPC2 are at this stage not anticipated to be mediators of this process. Optimism does however remain high that HYAL2 or CD160 in particular, are mediators of murine sperm-egg interaction. To cite two final publications in support for CD160, tetraspanins, like those mediating sperm-egg membrane fusion, have been shown to form complexes containing MHC antigens, reviewed in (Hemler, 2003). As an MHC antigen (Agrawal et al., 1999), CD160 may therefore be held in close proximity to fusion machinery, such as CXCR4 and PDI, by means of associations with one or more of the egg surface tetraspanins CD9, CD81 and CD151.

The aim of this final chapter was to provide an integrated and concise overview of the research conducted within the scope of this project so that the data may be viewed
collectively to provide greater insight into the mechanisms mediating murine sperm-egg interaction. In the following section these data have been integrated with the existing relevant literature in order to postulate an updated model of the mechanisms that may be mediating murine sperm-egg interaction. In this model, GPI-anchored proteins and tetraspanins once again play important roles in mediating sperm-egg interaction.

As spermatozoa enter the perivitelline space following a successful acrosome reaction and penetration of the ZP, a complement of newly exposed proteins are now presented to the oocyte surface. Mouse SLLP1, which has been implicated in sperm-binding (Herrero et al., 2005), interacts with a GPI-anchored protein on the oocyte surface. As a result of this research, it is known that possible identities for these GPI-anchored proteins now include CD160, HYAL2, RAET1C and LYPD3. Following this initial binding, proteins such as ADAMs on the sperm surface and integrins on the oocyte surface may serve to stabilise and reinforce the interaction between sperm and egg to assist in holding the membranes juxtaposed. With sperm and oocyte membranes now held in close proximity, proteins involved in membrane fusion are then able to interact. During this interaction, Izumo on the sperm surface may bind to oolemmal CXCR4 or another tetraspanin binding partner such as CD9P-1. A PDI held in close proximity to CXCR4 or CD9P-1 by means of cis interactions with tetraspanins may then reduce the putative disulphide linkage of Izumo on the sperm surface. Due to the fact that disulphide bonds play a role in maintaining protein structure, Izumo would then be anticipated to undergo a series of conformational changes as the protein shifts to adopt its native, lower energy folding state. This may result in the insertion of a segment of Izumo into the oocyte membrane anchoring it to the egg surface, similar to viral fusogen function. ERp57 on the sperm surface may concurrently serve to catalyse disulphide bond rearrangement in key oocyte proteins, such as a yet to be identified thiol containing tetraspanin binding partner. As the proteins shift and rearrange following thiol/disulphide exchange, sperm and oocyte membranes may be anchored then warped leading to stalk formation between the two membranes, which serves as a prelude to complete bilayer fusion (Chernomordik et al., 1995; Leikina and Chernomordik, 2000; Yang and Huang, 2002). Also included in this model is thioredoxin, which would be anticipated to perform a complementary role to cell surface PDIs in catalysing oxidation/reduction of thiols/disulphides. Thioredoxin is included in this model as although strong evidence suggests that PDIs catalyse disulphide bond reduction during
HIV entry, evidence also suggests that thioredoxin actually catalyses the reduction of at least one disulphide bond in T-cell CD4 in addition to the PDI activity.

**Figure 6.2 Proposed model for membrane components mediating murine sperm-egg interaction**

Sperm surface mSLLP1 and a GPI-anchored protein on the oocyte surface (CD160, HYAL2) serve as initial proteins involved in sperm-egg binding. ADAMs and integrins then stabilise the interaction between sperm and egg assisting in holding membranes in close proximity. A PDI on the oocyte surface along with ERp57 on the sperm surface then catalyse thiol/disulphide exchange between Izumo and tetraspanin binding partners, which possibly include CXCR4 and CD9P-1. Rearrangement of disulphide bonds leads to conformational changes in protein structures. These changes result in insertion of protein domains into opposing lipid bilayers and the generation of torsional strain on the membranes. Outer leaflets of the bilayer fuse leading to membrane stalk formation prior to complete bilayer fusion.

As can be seen, the postulated model uses findings inferred from the results of these series of investigations to build on existing knowledge in the literature. Importantly however, although attempts have not been made to directly implicate proteins involved in virus-host cell membrane fusion with sperm-egg membrane fusion, except for CXCR4 which is conveniently expressed on oocytes (Holt et al., 2006), the model is
based on the concept that these two processes parallel one another. In this context, efforts have been made to highlight the importance of disulphide switching as the means by which the necessary protein conformational changes can occur to bring about the energetically costly event that is membrane fusion.

Finally, at the initiation of this project, the idea that the events of sperm-egg fusion may parallel those of virus-host cell fusion seemed a remote ideal around which to lay a foundation for progress. In what may perhaps be impeccable timing, a recent study by Shanmukhappa et al., 2007 has just reported that the tetraspanin CD151 serves at a receptor for the porcine reproductive and respiratory syndrome virus (PRRSV) ((Shanmukhappa et al., 2007). CD151 was identified as the membrane protein utilised by PRRSV to gain entry to porcine host cells. Such a finding is important in that it reinforces the concept that sperm-egg fusion has parallels with virus-host cell fusion. The observation that PRRSV mediates membrane entry into porcine alveolar macrophages through none other then the protein identified by this research as a mediator of membrane fusion during sperm-egg interaction, CD151, certainly provides reassurance that researchers in this field are on the right track.

6.2 Future Directions
Although this research has contributed to a greater understanding of the events mediating sperm-egg interaction, there remain numerous areas of research where additional experimentation could be applied to further knowledge in this field. Many of these future directions, along with a host of other important experiments, were actually planned for completion within the scope of this project. The limited supply of mice and oocytes however meant that these investigations were unable to be performed within the project timeframe.

Firstly, xenobiotic modification of the oolemma revealed that benzoquinone and bis maleimide each have the capacity to modify oocyte proteins in such a manner as to inhibit sperm-egg binding. Inhibitory effects were believed to be attributable to the alkylation of thiols in cell surface proteins. Further experimentation is therefore required to identify those proteins modified upon treatment of oocytes with benzoquinone or bis maleimide. For this purpose, oocyte proteins could be extracted, separated by 2D PAGE and electroblotted to nitrocellulose membranes. Nitrocellulose
membranes could then be labelled with a thiol reactive probe to highlight thiol-containing proteins. Additional membranes could be treated with benzoquinone prior to incubation with the thiol-detecting probe and the loss of positive signals documented. Proteins that were no longer labelled following benzoquinone treatment would therefore have been alkylated as a result of benzoquinone action. These proteins could be cored from 2D gels and identified by MS/MS analysis. This particular series of experiments was planned for inclusion in the present body of research, limitations on the collection of oocyte proteins however meant that the oocytes needed to be utilised for higher priority research areas such as 2D LC-MS/MS analysis.

2D LC-MS/MS analysis was performed in the hope of obtaining protein identifications for oocyte GPI-anchored proteins. Although 11 candidates were identified by an alternative gene-based approach, identification of the oocytes GPI-anchored proteome would contribute greatly to understanding the processes mediating sperm-egg interaction. For this purpose, a 2D LC-MS/MS analysis remains the best approach, however measures would be required to enhance detection of the lower abundance proteins. It is anticipated that this enhanced detection of lower abundance oocyte proteins could be achieved by removing high abundance proteins such as ePAD and L-lactate dehydrogenase B chain from protein lysate prior to 2D LC-MS/MS analysis. Removal of these proteins could be accomplished through simply incubating the lysate in the presence of magnetic Dynabeads coupled to antibodies against the respective proteins. The supernatant could be collected following magnetic sequestration of the beads to yield an oocyte protein extract deficient in these overwhelming proteins. Unfortunately this revised proteomic analysis of murine oocytes could not to be performed due to limited sample material.

Limited sample material also prevented the identification of tetraspanin binding partners within murine oocytes. Future directions for this project therefore also include co-immunoprecipitation studies with antibodies against CD9, CD81 and CD151. Magnetic Dynabeads would be coupled to antibodies against the respective tetraspanins and incubated with oocyte lysates. Proteins would then be eluted off the beads and sequenced to identify any proteins that immunoprecipitated with the tetraspanins due to a stable molecular interaction. Such proteins would be anticipated to be mediators of membrane fusion events during sperm-egg interaction.
Among the tetraspanin binding partners one would hope to find CXCR4 given the association of this protein with HIV – T cell membrane fusion. However evidence is yet to be obtained implicating CXCR4 in the events of fertilisation. In addition to identifying CXCR4 during tetraspanin pull downs, experiments are therefore also necessary to investigate the role of this protein by means of IVF inhibition studies. In this regard, oocytes could be pre-incubated in AMD3100 prior to exposure to sperm during IVF. AMD3100 binds to the region of CXCR4 that serves as the binding site for the ligand SDF-1. This SDF-1 binding site may also be important for forming associations with proteins involved in membrane fusion, such as the tetraspanins.

Finally, although 6 GPI-anchored proteins were identified as potential mediators of sperm-egg interaction, antibody based studies were unable to fully characterise the expression of these proteins within ovaries and oocytes. This is particularly the case for proteins such as LYPD3 and RAET1C for which antibodies could not even be sourced. LYPD3 remains a protein of particular interest based on the repetitive cysteine sequence within the protein’s structure, which through the formation of disulphide bonds results in a 3-finger folding structure (Tsetlin, 1999). Where antibodies were available, competitive inhibition studies during IVF gave little insight into the involvement of these proteins in the events of fertilisation. Future directions for this project therefore include the generation of transgenic mice to study the consequences of deleting these genes on fertility as well as the deletion of the genes encoding any additional GPI-anchored proteins that may be identified through the optimised 2D LC-MS/MS analysis approach.
References


References


References


## Appendix A: Whole Ovary Proteomic Identifications

### A.1 LC-MS/MS analysis of Non-fractionated Whole Ovaries

Table A1. Sequest Protein identities obtained for non-fractionated Whole Ovaries (Tables spans pages 1-16 of Appendix A)

<table>
<thead>
<tr>
<th>No.</th>
<th>Protein Identification</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0 DAY NEONATE LUNG CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:E030024J03 PRODUCT:ISOCITRATE DEHYDROGENASE 1 (NADP⁺), SOLUBLE, FULL INSERT SEQUENCE.</td>
</tr>
<tr>
<td>2</td>
<td>10 DAY OLD MALE PANCREAS CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:1810009J06 PRODUCT:TRYPSIN IV (EC &lt;A HREF=&quot;wgetz?id+16pH91V5hRl+[enzyme-ECNumber:3.4.21.4]+&quot; e&amp;quot;&gt;3.4.21.4&amp;lt;/A&amp;gt;) (PRETRYPSINOGEN IV) HOMOLOG.</td>
</tr>
<tr>
<td>3</td>
<td>10 DAYS EMBRYO WHOLE BODY CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:2610027B07 PRODUCT:HISTONE 4 PROTEIN, FULL INSERT SEQUENCE.</td>
</tr>
<tr>
<td>4</td>
<td>10 DAYS NEONATE CEREBELLUM CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:B930041E13 PRODUCT:HYPOTHETICAL PROTEIN, FULL INSERT SEQUENCE.</td>
</tr>
<tr>
<td>5</td>
<td>10 DAYS NEONATE CORTEX CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:A830011C08 PRODUCT:HYPOTHETICAL RNI-LIKE STRUCTURE CONTAINING PROTEIN, FULL INSERT SEQUENCE.</td>
</tr>
<tr>
<td>6</td>
<td>10 DAYS NEONATE CORTEX CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:A830026L17 PRODUCT:HYPOTHETICAL PROTEIN, FULL INSERT SEQUENCE.</td>
</tr>
<tr>
<td>7</td>
<td>10 DAYS NEONATE SKIN CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:4732455O04 PRODUCT:INFERRED: POTENTIAL PHOSPHOLIPID-TRANSPORTING ATPASE IH.</td>
</tr>
<tr>
<td>8</td>
<td>10 DAYS NEONATE SKIN CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:4732496B02 PRODUCT:UBIQUITIN PROTEIN LIGASE E3A, FULL INSERT SEQUENCE.</td>
</tr>
<tr>
<td>9</td>
<td>10 DAYS PREGNANT ADULT FEMALE OVARY AND UTERUS CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:G630042H19 PRODUCT:IMPLANTATION SERINE PROTEASE 2, FULL INSERT SEQUENCE.</td>
</tr>
<tr>
<td>10</td>
<td>11 DAYS EMBRYO HEAD CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:6230411L24 PRODUCT:APOPTOSIS INHIBITOR 5, FULL INSERT SEQUENCE.</td>
</tr>
<tr>
<td>11</td>
<td>11 DAYS EMBRYO WHOLE BODY CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:2700086E01 PRODUCT:RIBOSOMAL PROTEIN S23, FULL INSERT SEQUENCE.</td>
</tr>
<tr>
<td>12</td>
<td>11 KDA PROTEIN.</td>
</tr>
<tr>
<td>13</td>
<td>116 KDA PROTEIN.</td>
</tr>
<tr>
<td>14</td>
<td>12 DAYS EMBRYO EMBRYONIC BODY BETWEEN DIAPHRAGM REGION AND NECK CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:9430025F20 PRODUCT:PROSTAGLANDIN F SYNTHASE HOMOLOG.</td>
</tr>
<tr>
<td>15</td>
<td>12 DAYS EMBRYO EYEBALL CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:D230010H24 PRODUCT:HYPOTHETICAL PROTEIN, FULL INSERT SEQUENCE.</td>
</tr>
<tr>
<td>16</td>
<td>12 DAYS EMBRYO FEMALE MULLERIAN DUCT INCLUDES SURROUNDING REGION CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:6820433C17 PRODUCT:PROSTAGLANDIN F SYNTHASE HOMOLOG.</td>
</tr>
<tr>
<td>17</td>
<td>12 DAYS EMBRYO SPINAL CORD CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:6820433C17 PRODUCT:HOMOLOC13, FULL INSERT SEQUENCE.</td>
</tr>
<tr>
<td>18</td>
<td>12 DAYS EMBRYO SPINAL GANGLION CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:D130030I02 PRODUCT:HYPOTHETICAL PROTEIN, FULL INSERT SEQUENCE (FRAGMENT).</td>
</tr>
</tbody>
</table>
19  13 DAYS EMBRYO HEAD CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:3110025H23 PRODUCT:HYPOTHETICAL PROTEIN, FULL INSERT SEQUENCE.
20  13 DAYS EMBRYO HEAD CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:3110047H05 PRODUCT:HYPOTHETICAL PROTEIN, FULL INSERT SEQUENCE (FRAGMENT).
21  13 DAYS EMBRYO LIVER CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:2510040B16 PRODUCT:HEMOGLOBIN, BETA ADULT MAJOR CHAIN, FULL INSERT SEQUENCE.
22  14-3-3 PROTEIN EPSILON.
23  14-3-3 PROTEIN ZETA/DELTA.
24  15 DAYS EMBRYO HEAD CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:D930044C15 PRODUCT:HYPOTHETICAL PROTEIN, FULL INSERT SEQUENCE (FRAGMENT).
25  15 KDA PROTEIN.
26  16 DAYS EMBRYO HEAD CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:C130023M09 PRODUCT:RAB33B, MEMBER OF RAS ONCOGENE FAMILY, FULL INSERT SEQUENCE.
27  17 DAYS EMBRYO HEART CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:I920064F16 PRODUCT:HYPOTHETICAL PROTEIN, FULL INSERT SEQUENCE.
28  17 KDA PROTEIN.
29  18 DAYS PREGNANT ADULT FEMALE PLACENTA AND EXTRA EMBRYONIC TISSUE CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:3830423B07 PRODUCT:PEROXIREDOXIN 5, FULL INSERT SEQUENCE.
30  18 KDA PROTEIN.
31  19 KDA PROTEIN.
32  2 DAYS NEONATE THYMUS THYMIC CELLS CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:E430016E04 PRODUCT:HETEROCHROMATIN PROTEIN 1, BINDING PROTEIN 3, FULL INSERT SEQUENCE.
33  2 DAYS NEONATE THYMUS THYMIC CELLS CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:E430030L01 PRODUCT:HYPOTHETICAL PROTEIN, FULL INSERT SEQUENCE.
34  2' - 5' OLIGOADENYLATE SYNTHETASE 2.
35  26 KDA PROTEIN.
36  28 KDA HEAT- AND ACID-STABLE PHOSPHOPROTEIN.
37  3 BETA-HYDROXYSTEROID DEHYDROGENASE/DELTA 5--4-ISOMERASE TYPE I.
38  3 BETA-HYDROXYSTEROID DEHYDROGENASE/DELTA 5--4-ISOMERASE TYPE III.
39  3 BETA-HYDROXYSTEROID DEHYDROGENASE/DELTA 5--4-ISOMERASE TYPE VI.
40  3 BETA-HYDROXYSTEROID DEHYDROGENASE/DELTA 5--4-ISOMERASE TYPE VI.
41  3 DAYS NEONATE THYMUS CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:A630024M13 PRODUCT:ISCHEMIA/REPERFUSION INDUCIBLE PROTEIN, FULL INSERT SEQUENCE.
42  3,2-TRANS-ENOYL-COA ISOMERASE, MITOCHONDRIAL PRECURSOR.
43  33 KDA PROTEIN.
44  34 KDA PROTEIN.
45  39 KDA PROTEIN.
46  3-KETOACYL-COA THIOLASE, MITOCHONDRIAL.
47  3-KETO-STEROID REDUCTASE.
48  40S RIBOSOMAL PROTEIN S13.
49  40S RIBOSOMAL PROTEIN S20.
50  40S RIBOSOMAL PROTEIN S21.
51  40S RIBOSOMAL PROTEIN S21.
52  40S RIBOSOMAL PROTEIN S28.
53  40S RIBOSOMAL PROTEIN S3.
Appendix A

54 40S RIBOSOMAL PROTEIN S4, X ISOFORM.
55 40S RIBOSOMAL PROTEIN S8.
56 40S RIBOSOMAL PROTEIN S9.
57 4930506M07RIK PROTEIN.
58 4933409K07Rik PROTEIN.
59 6 DAYS NEONATE SPLEEN CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:F420002G12 PRODUCT:CCAAT/ENHANCER BINDING PROTEIN (C/EBP), EPSILON HOMOLOG.
60 6 KDA PROTEIN.
61 60S ACIDIC RIBOSOMAL PROTEIN P1.
62 60S ACIDIC RIBOSOMAL PROTEIN P2.
63 60S RIBOSOMAL PROTEIN L10.
64 60S RIBOSOMAL PROTEIN L10A.
65 60S RIBOSOMAL PROTEIN L13.
66 60S RIBOSOMAL PROTEIN L13A.
67 60S RIBOSOMAL PROTEIN L14.
68 60S RIBOSOMAL PROTEIN L15.
69 60S RIBOSOMAL PROTEIN L17.
70 60S RIBOSOMAL PROTEIN L18.
71 60S RIBOSOMAL PROTEIN L19.
72 60S RIBOSOMAL PROTEIN L22.
73 60S RIBOSOMAL PROTEIN L23.
74 60S RIBOSOMAL PROTEIN L27.
75 60S RIBOSOMAL PROTEIN L28.
76 60S RIBOSOMAL PROTEIN L29.
77 60S RIBOSOMAL PROTEIN L3.
78 60S RIBOSOMAL PROTEIN L31.
79 60S RIBOSOMAL PROTEIN L4.
80 60S RIBOSOMAL PROTEIN L5.
81 60S RIBOSOMAL PROTEIN L7.
82 60S RIBOSOMAL PROTEIN L8.
83 66 KDA PROTEIN.
84 6-PHOSPHOFRUCTO-2-KINASE/FRUCTOSE-2,6-BIPHOSPHATASE 2.
85 7 DAYS NEONATE CEREBELLUM CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:A730022A14 PRODUCT:HYPOTHEtical PROTEIN, FULL INSERT SEQUENCE.
86 78 KDA GLUCOSE-REGULATED PROTEIN PRECURSOR.
87 7-DEHYDROCHOLESTEROL REDUCTASE.
88 8 DAYS EMBRYO WHOLE BODY CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:5730433G16 PRODUCT:CHROMOBOX HOMOLOG 1 (DROSOPHILA HP1 BETA), FULL INSERT SEQUENCE.
89 8 DAYS EMBRYO WHOLE BODY CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:5730511L01 PRODUCT:HYPOTHEtical SIX-HAIRPIN GLYCOSYLTRANSFERASES STRUCTURE CONTAINING PROTEIN, FULL INSERT SEQUENCE.
90 9 DAYS EMBRYO WHOLE BODY CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:D030036J20 PRODUCT:WEAKLY SIMILAR TO UBIQUITIN-CONJUGATING ENZYME E2.
91 9 DAYS EMBRYO WHOLE BODY CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:D030044A21 PRODUCT:HYPOTHEtical CYTOCHROME C HEME-BINDING SITE CONTAINING PROTEIN, FULL INSERT SEQUENCE.
92 A KINASE (PRKA) ANCHOR PROTEIN 2.
93 ABHYDROLASE DOMAIN-CONTAINING PROTEIN 11.
94 ACETYL-COA CARBOXYLASE 1.
95 ACONITATE HYDRATASE, MITOCHONDRIAL PRECURSOR.
96 ACTIN, CYTOPLASMIC 1.
Appendix A

97 ACTIVATED SPLEEN CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:F830014P17 PRODUCT:P300/CBP-ASSOCIATED FACTOR, FULL INSERT SEQUENCE.

98 ADENOMATOSIS POLYPOSIS COLI 2.

99 ADIPOCYTE PLASMA MEMBRANE-ASSOCIATED PROTEIN.

100 ADP/ATP TRANSLOCASE 1.

101 ADP/ATP TRANSLOCASE 2.

102 ADP-RIBOSYLATION FACTOR GTPASE-ACTIVATING PROTEIN 3.

103 ADP-RIBOSYLATION FACTOR GUANINE NUCLEOTIDE-EXCHANGE FACTOR 2.

104 ADULT INNER EAR CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:F930017M11 PRODUCT:HYPOTHETICAL PROTEIN, FULL INSERT SEQUENCE.

105 ADULT INNER EAR CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:F930044B09 PRODUCT:MATRICE EXTRACELLULAR PHOSPHOGLYCOPROTEIN WITH ASARM MOTIF (BONE), FULL INSERT SEQUENCE.

106 ADULT INNER EAR CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:F930104E18 PRODUCT:HYPOTHETICAL PROTEIN, FULL INSERT SEQUENCE.

107 ADULT MALE BRAIN UNDEFINED_CELL_LINE CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:M5C1068G17 PRODUCT:DELETED IN LIVER CANCER 1, FULL INSERT SEQUENCE.

108 ADULT MALE BRAIN UNDEFINED_CELL_LINE CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:M5C1105K07 PRODUCT:PROTEIN TYROSINE PHOSPHATASE, RECEPTOR TYPE, B. FULL INSERT SEQUENCE.

109 ADULT MALE CORPORA QUADRIGEMINA CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:B230039D16 PRODUCT:HYPOTHETICAL PROTEIN, FULL INSERT SEQUENCE.

110 ADULT MALE EPIDIDYMIS CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:9230002K05 PRODUCT:NIBAN PROTEIN, FULL INSERT SEQUENCE.

111 ADULT MALE HIPPOCAMPUS CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:2900074C18 PRODUCT:SIMILAR TO CDNA FLJ30373 FIS, CLONE BRACE2007882, WEAKLY SIMILAR TO ACTIN-DEPOLYMERIZING PROTEIN N-WASP.

112 ADULT MALE HYPOTHALAMUS CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:A230035L05 PRODUCT:HYPOTHETICAL DIVALENT CATION TRANSPORTER CONTAINING PROTEIN, FULL INSERT SEQUENCE.

113 ADULT MALE PITUITARY GLAND CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:5330413N23 PRODUCT:INHIBIN BINDING PROTEIN SHORT ISOFORM HOMOLOG.

114 ADULT MALE SMALL INTESTINE CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:2010309E21 PRODUCT:HYPOTHETICAL 15.6 KDA PROTEIN HOMOLOG.

115 ADULT MALE TESTIS CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:1700016B04 PRODUCT:4933431D05RIK PROTEIN HOMOLOG (FRAGMENT).

116 ADULT MALE TESTIS CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:1700058G18 PRODUCT:HYPOTHETICAL PROTEIN, FULL INSERT SEQUENCE.

117 ADULT MALE TESTIS CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:1700104B16 PRODUCT:HYPOTHETICAL PROTEIN, FULL INSERT SEQUENCE.

118 ADULT MALE TESTIS CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:4922505F07 PRODUCT:ATP CITRATE LYASE, FULL INSERT SEQUENCE.

119 ADULT MALE TESTIS CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:4930415O10 PRODUCT:RIKEN CDNA 4930415O10 (FRAGMENT).

120 ADULT MALE TESTIS CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:4930467L20 PRODUCT:HYPOTHETICAL PROTEIN, FULL INSERT SEQUENCE.
Appendix A

121 ADULT MALE TESTIS CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:4930483I10 PRODUCT: WEAKLY SIMILAR TO TESTIS SPECIFIC ANKYRIN-LIKE PROTEIN 1.

122 ADULT MALE TESTIS CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:4930548L11 PRODUCT: SAM DOMAIN, SH3 DOMAIN AND NUCLEAR LOCALISATION SIGNALS, 1, FULL INSERT SEQUENCE.

123 ADULT MALE TESTIS CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:4932418E24 PRODUCT: HYPOTHETICAL PROTEIN, FULL INSERT SEQUENCE.

124 ADULT MALE TESTIS CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:4932438M10 PRODUCT: WEAKLY SIMILAR TO HYPOTHETICAL 66.8 KDA PROTEIN.

125 ADULT MALE TESTIS CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:4933412G19 PRODUCT: REGULATORY FACTOR X, 4 (INFLUENCES HLA CLASS II EXPRESSION), FULL INSERT SEQUENCE.

126 ADULT MALE THYMUS CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:5830428C01 PRODUCT: FERREDOXIN REDUCTASE, FULL INSERT SEQUENCE.

127 AGRIN.

128 AHNAK NUCLEOPROTEIN ISOFORM 1.

129 ALANINE AMINOTRANSFERASE 2.

130 ALDEHYDE DEHYDROGENASE AHD-2-LIKE.

131 ALDEHYDE DEHYDROGENASE, MITOCHONDRIAL PRECURSOR.

132 ALDO-KETO REDUCTASE FAMILY 1, MEMBER C19.

133 ALDOSE REDUCTASE.

134 ALPHA-1-ANTITRYSIN 1-6 PRECURSOR.

135 ALPHA-2-HS-GLYCOPROTEIN PRECURSOR.

136 AMINE OXIDASE [FLAVIN-CONTAINING] A.

137 ANKYRIN REPEAT, SAM AND BASIC LEUCINE ZIPPER DOMAIN-CONTAINING PROTEIN 1.

138 ANNEXIN A2.

139 ANNEXIN A2.

140 AP-2 COMPLEX SUBUNIT ALPHA-2.

141 APOLIPOPROTEIN A-I PRECURSOR.

142 APOLIPOPROTEIN C-III PRECURSOR.

143 ARF GTPASE-ACTIVATING PROTEIN GIT1.

144 ARGININOSUCCINATE LYASE.

145 ARP3 ACTIN-RELATED PROTEIN 3 HOMOLOG B.

146 ASPH PROTEIN.

147 ATP SYNTHASE O SUBUNIT, MITOCHONDRIAL PRECURSOR.

148 ATP SYNTHASE SUBUNIT ALPHA, MITOCHONDRIAL PRECURSOR.

149 ATP SYNTHASE SUBUNIT BETA, MITOCHONDRIAL PRECURSOR.

150 ATP-BINDING CASSETTE (ABC) TRANSPORTER ABCA14.

151 B6-DERIVED CD11 +VE DENDRITIC CELLS CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:F730209H22 PRODUCT: INTEGRIN ALPHA M, FULL INSERT SEQUENCE.

152 B6-DERIVED CD11 +VE DENDRITIC CELLS CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:F730311N10 PRODUCT: TAX1-BINDING PROTEIN TRX HOMOLOG, FULL INSERT SEQUENCE.


154 BLASTOCYST BLASTOCYST CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:11C0031B10 PRODUCT: HYPOTHETICAL RIBOSOMAL PROTEIN L1 CONTAINING PROTEIN, FULL INSERT SEQUENCE.

155 BLASTOCYST BLASTOCYST CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:11C0031M24 PRODUCT: HYPOTHETICAL RNI-LIKE STRUCTURE CONTAINING PROTEIN HOMOLOG.
Appendix A

156 BONE MARROW MACROPHAGE CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:G530009C13 PRODUCT:X-PROLYL AMINOPEPTIDASE (AMINOPEPTIDASE P) 1, SOLUBLE, FULL INSERT SEQUENCE.

157 BONE MARROW MACROPHAGE CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:I830029L12 PRODUCT:SIMILAR TO BRAIN MITOCHONDRIAL CARRIER PROTEIN-1.

158 BONE MARROW MACROPHAGE CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:I830041H02 PRODUCT:SULFATASE MODIFYING FACTOR 1 (C-ALPHA- FORMGYLCINE- GENERATING ENZYME 1), FULL INSERT SEQUENCE.

159 BONE MARROW MACROPHAGE CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:I830077J02 PRODUCT:WEAKLY SIMILAR TO BA552M11.4.1.

160 BONE MARROW STROMA CELL CRL-2028 SR-4987 CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:G430067B09 PRODUCT:HISTONE H1.0, FULL INSERT SEQUENCE.

161 BRAIN-SPECIFIC ANKYRIN-G.

162 BREAST CANCER TYPE 1 SUSCEPTIBILITY PROTEIN HOMOLOG.

163 BULLOUS PEMPHIGOID ANTIGEN 1, ISOFORM 5.

164 CALSEQUESTRIN-1 PRECURSOR.

165 CASPASE-4 PRECURSOR.

166 CD180 ANTIGEN.

167 CDC2-RELATED KINASE, ARGinine/SERINE-RICH.

168 CDNA SEQUENCE BC021614.

169 CDNA SEQUENCE BC043118.

170 CDNA SEQUENCE BC051070.

171 CELL ADHESION MOLECULE-RELATED/DOWN-REGULATED BY ONCOGENES PRECURSOR.

172 CELL DIVISION CYCLE 5-RELATED PROTEIN.

173 CELL DIVISION CYCLE 6 HOMOLOG ISOFORM A.

174 CENTROsomal PROTEIN 250.

175 CHLORIDE ANION EXCHANGER.

176 CHLORIDE CHANNEL 1 ISOFORM (FRAGMENT).

177 CHLORIDE CHANNEL CALCIUM ACTIVATED 3.

178 CHROMAFFIN GRANULE AMINE TRANSPORTER.

179 CLASS IB MAJOR HISTOCOMPATIBILITY COMPLEX.

180 CLEAVAGE AND POLYADENYLATION SPECIFICITY FACTOR 6.

181 COFACTOR REQUIRED FOR SP1 TRANSCRIPTIONAL ACTIVATION, SUBUNIT 7.

182 COHESIN SUBUNIT SA-3.

183 COILED-COIL DOMAIN-CONTAINING PROTEIN 73.

184 COILED-COIL DOMAIN-CONTAINING PROTEIN C1ORF110 HOMOLOG.

185 COL4A5 PROTEIN.

186 COLLAGEN ALPHA-1(X) CHAIN PRECURSOR.

187 COLLAGEN ALPHA-2(I) CHAIN PRECURSOR.

188 COLLAGEN ALPHA-2(IV) CHAIN PRECURSOR.

189 COLLAGEN TYPE VI ALpha 4.

190 COLLAGENASE 3 PRECURSOR.

191 COPPER HOMEOSTASIS PROTEIN CUTC HOMOLOG.

192 CORTACTIN BINDING PROTEIN 2 ISOFORM 1.

193 CRL-1722 L5178Y-R CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:I730059D17 PRODUCT:CHRONIC MYELOGENOUS LEUKEMIA TUMOR ANTIGEN 66 HOMOLOG, FULL INSERT SEQUENCE.

194 C-TYPE LECTIN DOMAIN FAMILY 4 MEMBER D.

195 CYTOCHROME P450 11A1, MITOCHONDRIAL PRECURSOR.

196 CYTOCHROME P450 11A1, MITOCHONDRIAL PRECURSOR.

197 CYTOSKELETON ASSOCIATED PROTEIN 2-LIKE.

198 CYTOSKELETON-DIMENSION-ASSOCIATED PROTEIN 4.

199 CYTOSOLIC PHOSPHOLIPASE A2 ZETA.

200 D930015E06RIK PROTEIN.

201 DEDICATOR OF CYTOKINESIS 5.
DEDICATOR OF CYTOKINESIS PROTEIN 4.
DEHYDROGENASE/REDUCTASE SDR FAMILY MEMBER 1.
DESMIN.
DESTRIN.
DIAZEPAM BINDING INHIBITOR ISOFORM 1.
DIPHTHERIA TOXIN RESISTANCE PROTEIN REQUIRED FOR DIPHTHAMIDE BIOSYNTHESIS (SACCHAROMYCES)-LIKE 1.
DISHEVELED-ASSOCIATED ACTIVATOR OF MORPHOGENESIS 1.
DISPATCHED HOMOLOG 2.
DNA POLYMERASE EPSILON, CATALYTIC SUBUNIT A.
DNA TOPOISOMERASE 1.
DNA-DIRECTED RNA POLYMERASE III SUBUNIT D.
DOWN SYNDROME CELL ADHESION MOLECULE.
DYSTROPHIN.
E130112L23RIK PROTEIN.
E3 UBQUITIN-PROTEIN LIGASE UBR1.
ECTONUCLEOTIDE PYROPHOSPHATASE/PHOSPHODIESTERASE FAMILY MEMBER 2 PRECURSOR.
EF-HAND DOMAIN-CONTAINING FAMILY MEMBER B.
ELONGATION FACTOR 1-ALPHA 1.
ELONGATION FACTOR 1-ALPHA 2.
EMILIN-2 PRECURSOR.
ENDOPLASMIN PRECURSOR. (HSP90beta)
ENDOTHELIAL LIPASE PRECURSOR.
ENHANCER OF POLYCOMB HOMOLOG 2.
ENKURIN.
ENO2 PROTEIN.
EPIPLAKIN.
ES CELL ASSOCIATED TRANSCRIPT 11.
ES CELLS CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:2410042M16 PRODUCT:DAZ ASSOCIATED PROTEIN 1, FULL INSERT SEQUENCE (FRAGMENT).
ES CELLS CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:C330011M18 PRODUCT:HYPOTHETICAL PROTEIN, FULL INSERT SEQUENCE.
ESTRADIOL 17-BETA-DEHYDROGENASE 2.
EUCHAROMATIC HISTONE METHYLTRANSFERASE 1.
EUKARYOTIC INITIATION FACTOR 4A-I.
EXPERIMENTAL AUTOIMMUNE PROSTATITIS ANTIGEN 2.
EXPRESSED SEQUENCE AA407659.
FANCONI ANEMIA GROUP A PROTEIN HOMOLOG.
FARNESYL PYROPHOSPHATE SYNTHETASE.
F-BOX AND WD-40 DOMAIN PROTEIN 11.
F-BOX PROTEIN 10.
F-BOX/WD REPEAT PROTEIN 7.
FIBRINOGEN BETA CHAIN PRECURSOR.
FIBROBLAST GROWTH FACTOR RECEPTOR 2 ISOFORM IIIC.
FILAMIN-BINDING LIM PROTEIN 1.
FRIZZLED-1 PRECURSOR.
FRIZZLED-8 PRECURSOR.
FRUCTOSE-BISPHOSPHATE ALDOLASE A.
FURIN PRECURSOR.
FUSED TOES PROTEIN.
G PROTEIN PATHWAY SUPPRESSOR 1.
GAP JUNCTION ALPHA-6 PROTEIN.
GASTRICSIN PRECURSOR.
GEMININ.
GENE MODEL 323.
254 GLUTAMATE--CYSTEINE LIGASE CATALYTIC SUBUNIT.
255 GLUTATHIONE S-TRANSFERASE MU 1.
256 GLYCOGEN [STARCH] SYNTHASE, LIVER.
257 GLYPICAN-4 PRECURSOR.
258 GOLGIN SUBFAMILY A MEMBER 4.
259 GRANULE CELL ANTISERUM POSITIVE 14 ISOFORM 2.
260 GRANZYME E PRECURSOR.
261 GROWTH FACTOR RECEPTOR-BOUND PROTEIN 7.
262 GTP-AMP PHOSPHOTRANSFERASE MITOCHONDRIAL.
263 GTPASE, IMAP FAMILY MEMBER 1.
264 H-2 CLASS I HISTOCOMPATIBILITY ANTIGEN, L-D ALPHA CHAIN PRECURSOR.
265 H2AFJ PROTEIN.
266 HEAT SHOCK 70 KDA PROTEIN 1L.
267 HEAT SHOCK COGNATE 71 KDA PROTEIN.
268 HEAT SHOCK PROTEIN 84B.
269 HEAT SHOCK PROTEIN HSP 90-ALPHA.
270 HEMATOPOIETIC CELL TRANSCRIPT 1.
271 HEMATOPOIETIC LINEAGE CELL-SPECIFIC PROTEIN.
272 HEMOGLOBIN SUBUNIT BETA-1.
273 HEMOGLOBIN SUBUNIT BETA-2.
274 HERMANSKY-PUDLAK SYNDROME 3 PROTEIN HOMOLOG.
275 HIGH MOBILITY GROUP PROTEIN B1.
276 HISTONE H1.1.
277 HISTONE H1.2.
278 HISTONE H1.4.
279 HISTONE H1.5.
280 HISTONE H2B TYPE 1-F/J/L.
281 HISTONE H3.2.
282 HISTONE H4.
283 HMG-L6.
284 HOMEOBOX PROTEIN CUT-LIKE 2.
285 HOMEOBOX PROTEIN EMX1.
286 HOMEOBOX PROTEIN HMX2.
287 HYDROXYACYL-COENZYME A DEHYDROGENASE/3-KETOACYL-COENZYME A THIOLASE/ENOYL-COENZYME A HYDRATASE (TRIFUNCTIONAL PROTEIN), ALPHA SUBUNIT.
288 HYPOTHETICAL PROTEIN ISOFORM 1.
289 HYPOTHETICAL PROTEIN LOC218333 ISOFORM 1.
290 HYPOTHETICAL PROTEIN LOC230991.
291 HYPOTHETICAL PROTEIN LOC240185.
292 HYPOTHETICAL PROTEIN LOC241134.
293 HYPOTHETICAL PROTEIN LOC28135 ISOFORM 11.
294 HYPOTHETICAL PROTEIN LOC319807.
295 HYPOTHETICAL PROTEIN LOC330788.
296 HYPOTHETICAL PROTEIN LOC399568.
297 HYPOTHETICAL PROTEIN LOC67555.
298 HYPOTHETICAL PROTEIN LOC67655.
299 HYPOTHETICAL PROTEIN LOC70005.
300 HYPOTHETICAL PROTEIN P4(21)N (FRAGMENT).
301 HYPOTHETICAL PROTEIN.
302 IGH PROTEIN.
303 IL-27 P28 SUBUNIT.
304 IN VITRO FERTILIZED EGGS CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:7420493B02 PRODUCT:SIMILAR TO PAN2 PROTEIN.
305 INOSITOL 1,4,5-TRISPHOSPHATE RECEPTOR TYPE 3.
306 INSULIN RECEPTOR SUBSTRATE 4.
307 INTEGRIN ALPHA-V PRECURSOR.
308 INTERLEUKIN-5 RECEPTOR ALPHA CHAIN PRECURSOR.
Appendix A

309  INTERMEDIATE CONDUCTANCE CALCIUM-ACTIVATED POTASSIUM CHANNEL PROTEIN 4.
310  IQ MOTIF CONTAINING GTPASE ACTIVATING PROTEIN 2.
311  IROQUOIS-CLASS HOMEODOMAIN PROTEIN IRX-4.
312  ISLR2 PROTEIN.
313  ISOFORM 1 OF 14-3-3 PROTEIN THETA.
314  ISOFORM 1 OF 1-PHOSPHATIDYLINOSITOL-4,5-BISPHOSPHATE PHOSPHODIESTERASE EPSILON 1.
315  ISOFORM 1 OF 28S RIBOSOMAL PROTEIN S18B, MITOCHONDRIAL PRECURSOR.
316  ISOFORM 1 OF 60 KDA HEAT SHOCK PROTEIN, MITOCHONDRIAL PRECURSOR.
317  ISOFORM 1 OF A-KINETASE ANCHOR PROTEIN 4 PRECURSOR.
318  ISOFORM 1 OF ALDO-KETO REDUCTASE FAMILY 1 MEMBER C18.
319  ISOFORM 1 OF ANKYRIN REPEAT AND SOCS BOX PROTEIN 2.
320  ISOFORM 1 OF ATP-BINDING CASSETTE SUB-FAMILY B MEMBER 9 PRECURSOR.
321  ISOFORM 1 OF BCL-6 COREPRESSOR.
322  ISOFORM 1 OF BRAIN-SPECIFIC ANGIOGENESIS INHIBITOR 2 PRECURSOR.
323  ISOFORM 1 OF CENTROMERE PROTEIN O.
324  ISOFORM 1 OF COLLAGEN ALPHA-1(I) CHAIN PRECURSOR.
325  ISOFORM 1 OF COLLAGEN ALPHA-4(IV) CHAIN PRECURSOR.
326  ISOFORM 1 OF CONTACTIN-6 PRECURSOR.
327  ISOFORM 1 OF COP9 SIGNALOSOME COMPLEX SUBUNIT 7A.
328  ISOFORM 1 OF CORE HISTONE MACRO-H2A.1.
329  ISOFORM 1 OF DEDICATOR OF CYTOKINESIS PROTEIN 1.
330  ISOFORM 1 OF DEVELOPMENT AND DIFFERENTIATION-ENHANCING FACTOR 2.
331  ISOFORM 1 OF DOUBLE-STRANDED RNA-SPECIFIC ADENOSINE DEAMINASE.
332  ISOFORM 1 OF EH DOMAIN-BINDING PROTEIN 1-LIKE PROTEIN 1.
333  ISOFORM 1 OF ENDONUCLEASE VIII-LIKE 3.
334  ISOFORM 1 OF EPOXIDE HYDRASE 2.
335  ISOFORM 1 OF EWING’S TUMOR-ASSOCIATED ANTIGEN 1 HOMOLOG.
336  ISOFORM 1 OF FATTY ACYL-COA REDUCTASE 1.
337  ISOFORM 1 OF FILAMIN-C.
338  ISOFORM 1 OF FOCAL ADHESION KINASE 1.
339  ISOFORM 1 OF GLUTAMINYL-PEPTIDE CYCLOTRANSFERASE PRECURSOR.
340  ISOFORM 1 OF HETEROGENEOUS NUCLEAR RIBONUCLEOPROTEIN A3.
341  ISOFORM 1 OF HIV TAT-SPECIFIC FACTOR 1 HOMOLOG.
342  ISOFORM 1 OF HOMEBOX PROTEIN NANOG.
343  ISOFORM 1 OF HOMEBOX PROTEIN SIX4.
344  ISOFORM 1 OF IMPLANTATION-ASSOCIATED PROTEIN PRECURSOR.
345  ISOFORM 1 OF INAD-LIKE PROTEIN.
346  ISOFORM 1 OF KIT LIGAND PRECURSOR.
347  ISOFORM 1 OF LOW-DENSITY LIPOPROTEIN RECEPTOR-RELATED PROTEIN 4 PRECURSOR.
348  ISOFORM 1 OF LYSOPHOSPHATIDIC ACID PHOSPHATASE TYPE 6 PRECURSOR.
349  ISOFORM 1 OF MITOFUSIN-2.
350  ISOFORM 1 OF M-PHASE PHOSPHOPROTEIN 1.
351  ISOFORM 1 OF MYOSIN-11.
352  ISOFORM 1 OF MYOSIN-IXB.
353  ISOFORM 1 OF NMRA-LIKE FAMILY DOMAIN-CONTAINING PROTEIN 1.
354  ISOFORM 1 OF NON-RECEPTOR TYROSINE-PROTEIN KINASE TNK1.
355  ISOFORM 1 OF ORPHAN NUCLEAR RECEPTOR NR4A3.
356  ISOFORM 1 OF PAB-DEPENDENT POLY(A)-SPECIFIC RIBONUCLEASE SUBUNIT 3.
357  ISOFORM 1 OF PHOSPHATIDYLINOSITOL-4-PHOSPHATE 5-KINASE-LIKE PROTEIN 1.
ISOFORM 1 OF PROBABLE PROTEIN-CYSTEINE N-PALMITOYLTRANSFERASE PORCUPINE.
ISOFORM 1 OF PROLINE-RICH PROTEIN 6.
ISOFORM 1 OF PROTEIN FAM40B.
ISOFORM 1 OF PROTEIN JADE-1.
ISOFORM 1 OF PROTEIN KIAA0284.
ISOFORM 1 OF PROTEIN SET.
ISOFORM 1 OF PUTATIVE NEURONAL CELL ADHESION MOLECULE PRECURSOR.
ISOFORM 1 OF RETROTRANSPOSON GAG DOMAIN-CONTAINING PROTEIN 1.
ISOFORM 1 OF RHOMBOID DOMAIN-CONTAINING PROTEIN 3.
ISOFORM 1 OF ROOTLETIN.
ISOFORM 1 OF SERINE/THREONINE-PROTEIN KINASE DCAMKL3.
ISOFORM 1 OF SH2 DOMAIN-CONTAINING PROTEIN 2A.
ISOFORM 1 OF SID1 TRANSMEMBRANE FAMILY MEMBER 2 PRECURSOR.
ISOFORM 1 OF SIGNAL-INDUCED PROLIFERATION-ASSOCIATED 1-LIKE PROTEIN 1.
ISOFORM 1 OF SMALL GLUTAMINE-RICH TETRATRICOPEPTIDE REPEAT-CONTAINING PROTEIN A.
ISOFORM 1 OF SMEK HOMOLOG 1.
ISOFORM 1 OF SODIUM BICARBONATE COTRANSPORTER 3.
ISOFORM 1 OF SPlicing FACTOR, ARGININE/SERINE-RICH 1.
ISOFORM 1 OF TRANSCRIPTION FACTOR SOX-5.
ISOFORM 1 OF TROPOMYOSIN-1 ALPHA CHAIN.
ISOFORM 1 OF TUBBY-RELATED PROTEIN 2.
ISOFORM 1 OF UNCG5 HOMOLOG B.
ISOFORM 1 OF UNCHARGERIZED PROTEIN C9ORF126 HOMOLOG.
ISOFORM 1 OF WD REPEAT AND FYVE DOMAIN-CONTAINING PROTEIN 3.
ISOFORM 1 OF Y-BOX-BINDING PROTEIN 2.
ISOFORM 2 OF 40S RIBOSOMAL PROTEIN S24.
ISOFORM 2 OF BCL-2-ASSOCIATED TRANSCRIPTION FACTOR 1.
ISOFORM 2 OF DEDICATOR OF CYTOKINESIS PROTEIN 10.
ISOFORM 2 OF DI-N-ACETYLCHITOBIASE PRECURSOR.
ISOFORM 2 OF E1B-BINDING PROTEIN P400.
ISOFORM 2 OF HETEROGENEOUS NUCLEAR RIBONUCLEOPROTEIN D0.
ISOFORM 2 OF KINASE SUPPRESSOR OF RAS-1.
ISOFORM 2 OF MYELIN TRANSCRIPTION FACTOR 1-LIKE PROTEIN.
ISOFORM 2 OF POLIOVIRUS RECEPTOR-RELATED PROTEIN 3 PRECURSOR.
ISOFORM 2 OF RETICULON-4.
ISOFORM 2 OF TRIPARTITE MOTIF-CONTAINING PROTEIN 45.
ISOFORM 2 OF TYROSINE-PROTEIN KINASE RECEPTOR TYRO3 PRECURSOR.
ISOFORM 3 OF DYNAMIN-1.
ISOFORM 3 OF ECHINOderm MICROTUBULE-ASSOCIATED PROTEIN-LIKE 4.
ISOFORM 3 OF HEPARAN-SULFATE 6-O-SULFOTRANSPORTER 2.
ISOFORM 3 OF HETEROGENEOUS NUCLEAR RIBONUCLEOPROTEINS A2/B1.
ISOFORM 3 OF LEUCINE-RICH REPEAT-CONTAINING PROTEIN 33 PRECURSOR.
ISOFORM 3 OF NASAL EMBRYONIC LUTEINIZING HORMONE-RELEASEING HORMONE FACTOR.
ISOFORM 3 OF PROTEIN PELLINO HOMOLOG 2.
ISOFORM 3 OF SERINE/ARGININE REPETITIVE MATRIX PROTEIN 2.
ISOFORM 3 OF SIALOADHESIN PRECURSOR.
ISOFORM 4 OF LIMKAIN-B1.
ISOFORM 4 OF SH3 DOMAIN-CONTAINING KINASE-BINDING PROTEIN 1.
ISOFORM 5 OF SH3-CONTAINING GRB2-LIKE PROTEIN 3-INTERACTING PROTEIN 1.
ISOFORM 5 OF ZINC FINGER PROTEIN 638.
ISOFORM A OF AP-2 COMPLEX SUBUNIT ALPHA-1.
ISOFORM A OF GLYCINE RECEPTOR SUBUNIT ALPHA-1 PRECURSOR.
ISOFORM A OF METHYL-CPG-BINDING PROTEIN 2.
ISOFORM C2 OF LAMIN-A/C.
ISOFORM LONG OF HEMATOPOIETIC PROGENITOR CELL ANTIGEN CD34 PRECURSOR.
ISOFORM M2 OF PYRUVATE KINASE ISOZYMES M1/M2.
ISOFORM PL-VDAC1 OF VOLTAGE-DEPENDENT ANION-SELECTIVE CHANNEL PROTEIN 1.
ISOFORM SHORT OF RETINOIC ACID RECEPTOR RXR-BETA.
ISOFORM SHORT OF TRANSCRIPTIONAL ENHANCER FACTOR TEF-3.
ISOLEUCYL-TRNA SYNTHETASE, MITOCHONDRIAL PRECURSOR.
KERATIN, TYPE I CYTOSKELETAL 23.
KIAA1447.
KIDNEY-SPECIFIC TRANSPORT PROTEIN.
KINESIN FAMILY MEMBER 26B.
KINESIN-LIKE PROTEIN KIF1A.
KINESIN-RELATED PROTEIN KIF27A.
LAMININ SUBUNIT BETA-3 PRECURSOR.
LAMIN, GAMMA 2.
LATENT TRANSFORMING GROWTH FACTOR BETA BINDING PROTEIN 1 ISOFORM LTPB- 1S.
KINESIN-LIKE PROTEIN KIF27A.
LAMININ SUBUNIT BETA-3 PRECURSOR.
LAMININ, GAMMA 2.
LATS2C.
LEUCINE ZIPPER PROTEIN 1.
LEUCINE ZIPPER PUTATIVE TUMOR SUPPRESSOR 2.
LEUCINE-RICH REPEAT AND CALPONIN HOMOLOGY DOMAIN-CONTAINING PROTEIN 4.
L-LACTATE DEHYDROGENASE B CHAIN.
LON PROTEASE HOMOLOG, MITOCHONDRIAL PRECURSOR.
LOW-DENSITY LIPOPROTEIN RECEPTOR-RELATED PROTEIN 1 PRECURSOR.
MACROPHAGE MANNOSE RECEPTOR 1 PRECURSOR.
MACROPHAGE MIGRATION INHIBITORY FACTOR.
MACROPHAGE RECEPTOR MARCO.
MAMMARY DEHYDROGENASE, MITOCHONDRIAL PRECURSOR.
MAMMARY GLAND RCB-0527 JYG-MC(B) CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:G930022G24 PRODUCT:HYPOTHETICAL FORMIN HOMOLOGY 2 DOMAIN CONTAINING PROTEIN, FULL INSERT SEQUENCE.
MATRIX METALLOPROTEINASE 27.
MAX-INTERACTING PROTEIN.
MEMBRANE-ASSOCIATED PROGESTERONE RECEPTOR COMPONENT 1.
MITOGEN-ACTIVATED PROTEIN KINASE 1.
MITOGEN-ACTIVATED PROTEIN KINASE KINASE 11.
MKIAA1030 PROTEIN.
MOESIN.
MORC FAMILY CW-TYPE ZINC FINGER PROTEIN 2A.
MTPRD.
MUCIN 5, SUBTYPES A AND C, TRACHEOBRONCHIAL/GASTRIC.
MUCIN-6 PRECURSOR.
MULTIDRUG RESISTANCE-ASSOCIATED PROTEIN 5.
MURINOglobulin-2 PRECURSOR.
MYELOID/LYMPHOID OR MIXED-LINEAGE LEUKEMIA 3 ISOFORM 4.
MYOSIN LIGHT CHAIN 2.
MYOSIN, LIGHT POLYPEPTIDE KINASE.
MYOSIN-9.
MYRISTOYLATED ALANINE-RICH C-KINASE SUBSTRATE.
N-ACETYLYTRANSFERASE 9.
NACHT, LRR AND PYD DOMAINS-CONTAINING PROTEIN 4A.
NACHT-, LRR-, AND PYD-CONTAINING PROTEIN 1 PARALOG B.
NADP-DEPENDENT LEUKOTRIENE B4 12-HYDROXYDEHYDROGENASE.
NADP-DEPENDENT MALIC ENZYME.
NEBULIN.
NEXT TO BRCA1 GENE 1 PROTEIN.
NITRIC OXIDE SYNTHASE 3, ENDOTHELIAL CELL.
NODAL MODULATOR 1.
NOD-DERIVED CD11C +VE DENDRITIC CELLS CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:F630022E15 PRODUCT:HYPOTHETICAL PROLINE-RICH REGION PROFILE CONTAINING PROTEIN, FULL INSERT SEQUENCE.
NOD-DERIVED CD11C +VE DENDRITIC CELLS CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:F630222A05 PRODUCT:5'-METHYLTHETRAHYDROFOLATE- HOMOCYSTEINE METHYLTRANSFERASE REDUCTASE, FULL INSERT SEQUENCE.
NOD- DERIVED CD11C +VE DENDRITIC CELLS CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:F630328I21 PRODUCT:SIMILAR TO MYELOBLAST KIAA0223 (HYPOTHETICAL PROTEIN) HOMOLOG.
NOVEL HISTONE H2A FAMILY MEMBER.
NOVEL PROTEIN CONTAINING A RHOGEF DOMAIN.
NOVEL PROTEIN SIMILAR TO HUMAN HEMICENTIN.
NOVEL PROTEIN.
NOVEL PROTEIN.
NOVEL PROTEIN.
NUCLEAR MITOTIC APPARATUS PROTEIN 1.
NUCLEASE SENSITIVE ELEMENT-BINDING PROTEIN 1.
NUCLEOLIN.
NUCLEOPORIN 153.
NUCLEOSIDE DIPHOSPHATE KINASE B.
OLFACTORY RECEPTOR 127.
ORF 1 PROTEIN.
OSTEOCLAST- LIKE CELL CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:I420039N16 PRODUCT:HETEROGENEOUS NUCLEAR RIBONUCLEOPROTEIN U, FULL INSERT SEQUENCE.
PAD-1-LIKE ISOFORM 1.
PALMITOYLTRANSFERASE ZDHHC18.
PALMITOYLTRANSFERASE ZDHHC9.
PEPTIDYL-PROLYL CIS-TRANS ISOMERASE A.
PEPTIDYLPROLYL ISOMERASE B.
PERFORIN-1 PRECURSOR.
PEROXIREDOXIN-1.
PEROXIREDOXIN-2.
PEROXIREDOXIN-4.
PHOSPHOGLYCERATE MUTASE 1.
PHOSPHORIBOSYLGLYCINAMIDE FORMYLTRANSFERASE.
PHOSPHOTRIESTERASE-RELATED PROTEIN.
PICCOLO.
PIWI-LIKE PROTEIN 2.
PKP3 PROTEIN.
PLASMA MEMBRANE CALCIUM-TRANSPORTING ATPASE 2.
PLEXIN-A1 PRECURSOR.
POLY (A) POLYMERASE BETA.
POLYDUCTIN.
POLYPEPTIDE N-ACETYLGLACTOSAMINYLTANSFERASE 6.
PFIBP2 PROTEIN.
PRE-MRNA-PROCESSING-SPlicing FACTOR 8.
PROBABLE CATION-TRANSPORTING ATPASE 13A1.
PROBABLE G-PROTEIN COUPLED RECEPTOR 146.
PROBABLE NUCLEOLAR COMPLEX PROTEIN 14.
PRO-EPI-DEMAL GROWTH FACTOR PRECURSOR.
PROFILIN-1.
PROHIBITIN-2.
PROTEASOME ACTIVATOR COMPLEX SUBUNIT 1.
PROTEASOME ACTIVATOR COMPLEX SUBUNIT 2.
PROTEASOME SUBUNIT ALPHA TYPE 7-LIKE.
PROTEIN DISULFIDE-ISOMERASE A3 PRECURSOR.
PROTEIN JUMONJI.
PROTEIN KINASE LYSINE DEFICIENT 1.
PROTEIN S100-A11.
PROTEIN S100-A4.
PROTEIN TYROSINE PHOSPHATASE-LIKE A DOMAIN CONTAINING 1.
PROTHYMOSIN ALPHA.
PROTACADHERIN 8 ISOFORM 2.
PROTACADHERIN ALPHA 9.
PUTATIVE ATP-DEPENDENT RNA HELICASE PL10.
PUTATIVE SPLIChING FACTOR, ARGININE/SERINE-RICH 14.
PWWP DOMAIN CONTAINING 2.
QY1.5 VH (FRAGMENT).
R3H DOMAIN.
RAC GTPASE-ACTIVATING PROTEIN 1.
RASGRP3.
RAS-INTERACTING PROTEIN 1.
RAS-RELATED PROTEIN RAB-24.
REARRANGED L-MYC FUSION SEQUENCE.
RECEPTOR TYROSINE-PROTEIN KINASE ERBB-2 PRECURSOR.
RETAIL DEHYDROGENASE 1.
RETNOL DEHYDROGENASE 10.
RHO GTPASE-ACTIVATING PROTEIN 11A PRECURSOR.
RIKEN CDNA 1500011H22 GENE.
RIKEN CDNA 1810015C04 GENE.
RIKEN CDNA 2010001M09 GENE.
RIKEN CDNA 2310022A10 GENE.
RIKEN CDNA 3732412D22 GENE.
RIKEN CDNA A530050D06 GENE.
RING FINGER PROTEIN 157.
RPS16 PROTEIN.
SERINE DEHYDRATASE-LIKE.
SERINE PROTEASE INHIBITOR A3K PRECURSOR.
SERINE/THREONINE-PROTEIN KINASE 25.
SERINE/THREONINE-PROTEIN KINASE 38.
SERPIN H1 PRECURSOR.
SERUM ALBUMIN PRECURSOR.
SET DOMAIN CONTAINING 1B.
SH2 DOMAIN-CONTAINING ADAPTER PROTEIN E.
SH2/SH3 ADAPTOR PROTEIN.
SIALIC ACID-BINDING IG-LIKE LECTIN 5 PRECURSOR.
SIMILAR TO 40S RIBOSOMAL PROTEIN S6.
Appendix A

SIMILAR TO 40S RIBOSOMAL PROTEIN SA.
SIMILAR TO 60S RIBOSOMAL PROTEIN L12.
SIMILAR TO 60S RIBOSOMAL PROTEIN L13.
SIMILAR TO 60S RIBOSOMAL PROTEIN L18.
SIMILAR TO 60S RIBOSOMAL PROTEIN L21.
SIMILAR TO 60S RIBOSOMAL PROTEIN L23A.
SIMILAR TO 60S RIBOSOMAL PROTEIN L29.
SIMILAR TO 60S RIBOSOMAL PROTEIN L3.
SIMILAR TO 60S RIBOSOMAL PROTEIN L6.
SIMILAR TO 60S RIBOSOMAL PROTEIN L7A (SURFEIT LOCUS PROTEIN 3) ISOFORM 1.
SIMILAR TO 60S RIBOSOMAL PROTEIN L7A.
SIMILAR TO AFADIN (AF-6 PROTEIN) ISOFORM 4.
SIMILAR TO CARBONYL REDUCTASE [NADPH] 1.
SIMILAR TO CG18437-PA.
SIMILAR TO CG5514-PB. ISOFORM B ISOFORM 4.
SIMILAR TO COFILIN-1 (COFILIN, NON-MUSCLE ISOFORM) ISOFORM 1.
SIMILAR TO CYTOCHROME P450 2J3.
SIMILAR TO DYNEN HEAVY CHAIN FAMILY MEMBER.
SIMILAR TO EARLY ESTROGEN-INDUCED GENE 1 PROTEIN.
SIMILAR TO ENOLASE 1, ALPHA NON-NEURON.
SIMILAR TO FERM AND PDZ DOMAIN CONTAINING 2 ISOFORM 1.
SIMILAR TO FRAS1-RELATED EXTRACELLULAR MATRIX PROTEIN 3 PRECURSOR.
SIMILAR TO GERM CELL-LESS.
SIMILAR TO GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE.
SIMILAR TO H-2 CLASS I HISTOCOMPATIBILITY ANTIGEN, TLA(C) ALPHA CHAIN PRECURSOR.
SIMILAR TO HB1-LIKE PROTEIN.
SIMILAR TO HEAT SHOCK PROTEIN HSP 90-BETA.
SIMILAR TO HEMOGLOBIN, BETA ADULT MAJOR CHAIN.
SIMILAR TO HIGH MOBILITY GROUP PROTEIN 1.
SIMILAR TO IMPORTIN-8 (IMP8) (RAN-BINDING PROTEIN 8) (RANBP8) ISOFORM 5.
SIMILAR TO INHIBITOR OF GROWTH FAMILY, MEMBER 1.
SIMILAR TO INTERFERON-INDUCED GUANYLATE-BINDING PROTEIN 1.
SIMILAR TO LAMININ GAMMA-3 CHAIN PRECURSOR.
SIMILAR TO LAS1-LIKE ISOFORM 1 ISOFORM 2.
SIMILAR TO LEIOMODIN 3.
SIMILAR TO MAP/MICROTUBULE AFFINITY-REGULATING KINASE 4.
SIMILAR TO MELANOMA-ASSOCIATED ANTIGEN B3.
SIMILAR TO MIDASIN (MIDAS-CONTAINING PROTEIN) ISOFORM 1.
SIMILAR TO MYOSIN HEAVY CHAIN, CARDIAC MUSCLE ALPHA ISOFORM.
SIMILAR TO MYOSIN LIGHT POLYPEPTIDE 6.
SIMILAR TO NEURON NAVIGATOR 3 ISOFORM 5.
SIMILAR TO PDZ DOMAIN-CONTAINING RING FINGER PROTEIN 4.
SIMILAR TO PHD FINGER PROTEIN 20.
SIMILAR TO PROTEIN KINASE BSK146.
SIMILAR TO PUTATIVE RETROVIRUS-RELATED GAG PROTEIN.
SIMILAR TO RIBOSOMAL PROTEIN L10.
SIMILAR TO RIBOSOMAL PROTEIN L21.
SIMILAR TO RIBOSOMAL PROTEIN L24.
SIMILAR TO RIBOSOMAL PROTEIN L24.
SIMILAR TO RIBOSOMAL PROTEIN S14.
SIMILAR TO RIBOSOMAL PROTEIN S18.
SIMILAR TO RIBOSOMAL PROTEIN S27A.
SIMILAR TO RIBOSOMAL PROTEIN S8 ISOFORM 1.
SIMILAR TO SODIUM- AND CHLORIDE-ACTIVATED ATP-SENSITIVE POTASSIUM CHANNEL.
SIMILAR TO SPETEX-2C PROTEIN.
SIMILAR TO SPLICING FACTOR 3A, SUBUNIT 3.
SIMILAR TO SPOC DOMAIN CONTAINING 1.
SIMILAR TO SUMO/SENTRIN SPECIFIC PROTEASE 6 ISOFORM 1 ISOFORM 2.
SIMILAR TO SYNAPE DEFECTIVE 1, RHO GTPASE, HOMOLOG 1.
SIMILAR TO TISSUE-TYPE VOMERONASAL NEURONS PUTATIVE PHEROMONE RECEPTOR V2R2 ISOFORM 1.
SIMILAR TO TRANSCRIPTIONAL REGULATING FACTOR 1.
SIMILAR TO UBIQUITIN A-52 RESIDUE RIBOSOMAL PROTEIN FUSION PRODUCT 1.
SIMILAR TO UBIQUITIN-CONJUGATING ENZYME E2E 1 ISOFORM 2.
SIMILAR TO VACUOLAR PROTEIN SORTING 13D ISOFORM 2 ISOFORM 5.
SIMILAR TO VOMERONASAL 2, RECEPTOR, 14 ISOFORM 2.
SIMILAR TO VOMERONASAL 2, RECEPTOR, 1B.
SIMILAR TO Y51B11A.1.
SMALL NUCLEAR RIBONUCLEOPROTEIN SM D1.
SMALL NUCLEAR RIBONUCLEOPROTEIN-ASSOCIATED PROTEIN B.
SOC5 BOX PROTEIN ASB-18.
SODIUM- AND CHLORIDE-DEPENDENT GABA TRANSPORTER 4.
SOLUTE CARRIER FAMILY 12, MEMBER 2.
SOX-12 PROTEIN.
SP140 NUCLEAR BODY PROTEIN FAMILY MEMBER.
SPERM TAIL ASSOCIATED PROTEIN.
SPERMATOGENIC LEUCINE ZIPPER PROTEIN 1.
SPERM-SPECIFIC SODIUM PROTON EXCHANGER.
SPHINGOSINE-1-PHOSPHATE LYASE 1.
SPLICING FACTOR 4.
SPLICING FACTOR, ARGININE/SERINE-RICH 15.
START DOMAIN CONTAINING 9.
STEROL O-ACYLTRANSFERASE 1.
STRESS-70 PROTEIN, MITOCHONDRIAL PRECURSOR.
STRUCTURAL MAINTENANCE OF CHROMOSOMES PROTEIN 3.
STRUCTURAL MAINTENANCE OF CHROMOSOMES PROTEIN 4.
SUCCINATE DEHYDROGENASE [UBIQUINONE] FLAVOPROTEIN SUBUNIT, MITOCHONDRIAL PRECURSOR.
SYMPELIN.
SYNAPTIC GLYCOPROTEIN SC2.
SYNAPTIC NUCLEAR ENVELOPE 2.
SYNTROPHIN ACIDIC 1.
TCDD-INDUCIBLE POLY [ADP-RIBOSE] POLYMERASE.
T-COMPLEX EXPRESSED 7.
T-COMPLEX PROTEIN 1 SUBUNIT ETA.
TENASCIN X.
TENSIN 3.
TENSIN 3.
TETRATRICOPEPTIDE REPEAT PROTEIN 28.
THIOREDOKIN.
THYROID HORMONE RECEPTOR INTERACTOR 12.
TIB-55 BB88 CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:I730069E17 PRODUCT:HPAI1 TINYS LOCUS 9C, FULL INSERT SEQUENCE.
TOLL-LIKE RECEPTOR 12 PRECURSOR.
TORSIN-3A PRECURSOR.
TRANSCRIPTION ELONGATION FACTOR B POLYPEPTIDE 1.
TRANSCRIPTION FACTOR SOX-8.
TRANSCRIPTION INITIATION FACTOR IIB.
TRANS-GOLGI NETWORK INTEGRAL MEMBRANE PROTEIN 2 PRECURSOR.
TRANS-GOLGI NETWORK INTEGRAL MEMBRANE PROTEIN 2 PRECURSOR.
TRANSIENT RECEPTOR POTENTIAL CATION CHANNEL, SUBFAMILY C, MEMBER 3.
TRANSKETOLASE.
TRANSLATION INITIATION FACTOR EIF-2B SUBUNIT BETA.
TRANSLOCATION-ASSOCIATED MEMBRANE PROTEIN 2.
TRIOSEPHOSPHATE ISOMERASE.
TRIOSEPHOSPHATE ISOMERASE.
TRIPARTITE MOTIF-CONTAINING PROTEIN 10.
TRIPARTITE MOTIF-CONTAINING PROTEIN 17.
TRIPARTITE MOTIF-CONTAINING PROTEIN 2.
TRIPARTITE MOTIF-CONTAINING PROTEIN 72.
TRANSIENT RECEPTOR POTENTIAL CATION CHANNEL, SUBFAMILY C, MEMBER 3.
TUBULIN ALPHA-4 CHAIN.
TUBULIN ALPHA-1 CHAIN.
TUBULIN BETA-2B CHAIN.
TUBULIN BETA-4 CHAIN.
TUBULIN BETA-5 CHAIN.
TUMOR NECROSIS FACTOR RECEPTOR SUPERFAMILY MEMBER 1A PRECURSOR.
UBIQUITIN CARBOXYL-TERMINAL HYDROLASE 10.
UBIQUITIN CARBOXYL-TERMINAL HYDROLASE 5.
UBIQUITIN CARBOXYL-TERMINAL HYDROLASE ISOZYME L4.
UBIQUITIN-CONJUGATING ENZYME E2 N.
UBIQUITIN-PROTEIN LIGASE E3B.
UDP-GLCNAC:BETAGAL BETA-1,3-N-ACETYLGLUCOSAMINYLTANSFERASE 6.
UNC-119 PROTEIN HOMOLOG.
UNCHARACTERIZED PROTEIN C12ORF34 HOMOLOG.
UNCHARACTERIZED PROTEIN C17ORF47 HOMOLOG.
UNCHARACTERIZED PROTEIN C1ORF190 HOMOLOG.
UNCHARACTERIZED PROTEIN C2ORF29 HOMOLOG.
UNCHARACTERIZED PROTEIN C3ORF33 HOMOLOG.
UNCHARACTERIZED PROTEIN C6ORF206 HOMOLOG.
UNCHARACTERIZED PROTEIN KIAA0157.
UPF0369 PROTEIN C6ORF57 HOMOLOG PRECURSOR.
VACUOLAR PROTEIN SORTING-ASSOCIATING PROTEIN 4A.
VIMENTIN.
VOLTAGE-DEPENDENT ANION-SELECTIVE CHANNEL PROTEIN 2.
VOMERONASAL RECEPTOR V1RH18.
VOMERONASAL RECEPTOR V1R19.
WD REPEAT DOMAIN 40A.
WD REPEAT PROTEIN 61.
WISKOTT-ALDRICH SYNDROME PROTEIN HOMOLOG.
XIN2.
ZFP473 PROTEIN.
ZINC FINGER PROTEIN 536.
ZINC FINGER PROTEIN OF THE CEREBELLUM 4.
ZINC FINGERS AND HOMEBOXES PROTEIN 2.
ZYXIN.
## A.2 LC-MS/MS analysis of IPG Strip Fractionated Whole Ovaries

### Table A2. Sequest Protein identities obtained for fractionated Whole Ovaries

<table>
<thead>
<tr>
<th>No.</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0 DAY NEONATE CEREBELLUM CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:C230015M18 PRODUCT: HYPOTHETICAL PROTEIN, FULL INSERT SEQUENCE (FRAGMENT).</td>
</tr>
<tr>
<td>2</td>
<td>0 DAY NEONATE CEREBELLUM CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:C230073G03 PRODUCT: HYPOTHETICAL PROTEIN, FULL INSERT SEQUENCE.</td>
</tr>
<tr>
<td>3</td>
<td>10 DAYS LACTATION, ADULT FEMALE MAMMARY GLAND CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:D730001C10 PRODUCT: HYPOTHETICAL PROTEIN, FULL INSERT SEQUENCE.</td>
</tr>
<tr>
<td>4</td>
<td>12 DAYS EMBRYO SPINAL CORD CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:C530023H18 PRODUCT: GANGLIOSIDE-INDUCED DIFFERENTIATION-ASSOCIATED-PROTEIN 2, FULL INSERT SEQUENCE.</td>
</tr>
<tr>
<td>5</td>
<td>13 DAYS EMBRYO LIVER CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:2500002L14 PRODUCT: HYPOTHETICAL RING FINGER CONTAINING PROTEIN, FULL INSERT SEQUENCE.</td>
</tr>
<tr>
<td>6</td>
<td>17 KDA PROTEIN.</td>
</tr>
<tr>
<td>7</td>
<td>337 KDA PROTEIN.</td>
</tr>
<tr>
<td>8</td>
<td>3-KETOACYL-COA THIOLASE B, PEROXISOMAL PRECURSOR.</td>
</tr>
<tr>
<td>9</td>
<td>6 KDA PROTEIN.</td>
</tr>
<tr>
<td>10</td>
<td>60S RIBOSOMAL PROTEIN L8.</td>
</tr>
<tr>
<td>11</td>
<td>ALDO-KETO REDUCTASE FAMILY 1, MEMBER C19.</td>
</tr>
<tr>
<td>12</td>
<td>AMYLOID BETA A4 PRECURSOR PROTEIN-BINDING FAMILY B MEMBER 3.</td>
</tr>
<tr>
<td>13</td>
<td>BC040756 PROTEIN (FRAGMENT).</td>
</tr>
<tr>
<td>14</td>
<td>BROMODOMAIN AND WD REPEAT DOMAIN CONTAINING 2.</td>
</tr>
<tr>
<td>15</td>
<td>ELONGATION FACTOR RNA POLYMERASE II 2.</td>
</tr>
<tr>
<td>16</td>
<td>H2AFJ PROTEIN.</td>
</tr>
<tr>
<td>17</td>
<td>HISTONE H3.2.</td>
</tr>
<tr>
<td>18</td>
<td>HYPOTHETICAL PROTEIN LOC77011.</td>
</tr>
<tr>
<td>19</td>
<td>IN VITRO FERTILIZED EGGS CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:7420437O10 PRODUCT: PLECKSTRIN HOMOLOGY DOMAIN CONTAINING, FAMILY G (WITH RHOGEF DOMAIN) MEMBER 1, FULL INSERT SEQUENCE.</td>
</tr>
<tr>
<td>20</td>
<td>ISOFORM 1 OF APRATAxin AND PNK-LIKE FACTOR.</td>
</tr>
<tr>
<td>21</td>
<td>ISOFORM 1 OF LYSOSOMAL-TRAFFICKING REGULATOR.</td>
</tr>
<tr>
<td>22</td>
<td>ISOFORM 1 OF NEUROGENIC LOCUS NOTCH HOMOLOG PROTEIN 1 PRECURSOR.</td>
</tr>
<tr>
<td>23</td>
<td>ISOFORM 1 OF PROTEIN SIDEKICK-1 PRECURSOR.</td>
</tr>
<tr>
<td>24</td>
<td>ISOFORM 1 OF TIMELESS HOMOLOG.</td>
</tr>
<tr>
<td>25</td>
<td>ISOFORM 1 OF UNC45 HOMOLOG B.</td>
</tr>
<tr>
<td>26</td>
<td>ISOFORM 2 OF DIACYLGLYCEROL KINASE BETA.</td>
</tr>
<tr>
<td>27</td>
<td>ISOFORM 2 OF KERATIN, TYPE I CYTOSKELETAL 12.</td>
</tr>
<tr>
<td>28</td>
<td>ISOFORM 2 OF NIPPED-B-LIKE PROTEIN.</td>
</tr>
</tbody>
</table>
ISOFORM 2 OF ZINC FINGER CCCH DOMAIN-CONTAINING PROTEIN C190R7 HOMOLOG.

NICOTINAMIDE MONONUCLEOTIDE ADENYLTRANSFERASE 1.

NUCLEAR RECEPTOR COACTIVATOR 3.

RIKEN CDNA 2410075B13 GENE.

SECRETED AND TRANSMEMBRANE 1B.

SIMILAR TO CHROMOSOME 9 OPEN READING FRAME 61.

SIMILAR TO DYNEIN, AXONEMAL, HEAVY POLYPEPTIDE 9 ISOFORM 2 ISOFORM 5.

SIMILAR TO PRAF3 FAMILY MEMBER 3.

SIMILAR TO RASD FAMILY, MEMBER 2.

TOLL-LIKE RECEPTOR 1 PRECURSOR.

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0 DAY NEONATE CEREBELLUM CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:C230073G03 PRODUCT:HYPOTHETICAL PROTEIN, FULL INSERT SEQUENCE.

0 DAY NEONATE LUNG CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:E030024J03 PRODUCT:ISOCITRATE DEHYDROGENASE 1 (NADP+), SOLUBLE, FULL INSERT SEQUENCE.

10 DAYS EMBRYO WHOLE BODY CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:2610027B07 PRODUCT:HISTONE 4 PROTEIN, FULL INSERT SEQUENCE.

10 DAYS NEONATE SKIN CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:4732474G14 PRODUCT:WEAKLY SIMILAR TO HYPOTHETICAL 64.0 KDA PROTEIN.

12 DAYS EMBRYO EMBRYONIC BODY BETWEEN DIAPHRAGM REGION AND NECK CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:9430025F20 PRODUCT:PROSTAGLANDIN F SYNTHASE HOMOLOG.

12 DAYS EMBRYO FEMALE MULLERIAN DUCT INCLUDES SURROUNDING REGION CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:6820433C17 PRODUCT:PROSTAGLANDIN F SYNTHASE HOMOLOG.

13 DAYS EMBRYO LIVER CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:2510040B16 PRODUCT:HEMOGLOBIN, BETA ADULT MAJOR CHAIN, FULL INSERT SEQUENCE.

13 DAYS EMBRYO MALE TESTIS CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:6030457M03 PRODUCT:C321D2.2 (NOVEL PROTEIN SIMILAR TO REPLICATION FACTORS) HOMOLOG.

14-3-3 PROTEIN ZETA/Delta.

15 KDA PROTEIN.

16 DAYS EMBRYO HEART CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:1920080J03 PRODUCT:HYPOTHETICAL PROTEIN (SWI/SNF RELATED, MATRIX ASSOCIATED, ACTIN DEPENDENT REGULATOR OF CHROMATIN, SUBFAMILY C, MEMBER 2) HOMOLOG.
18 DAYS PREGNANT ADULT FEMALE PLACENTA AND EXTRA EMBRYONIC TISSUE CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:3830403N18 PRODUCT:X- LINKED LYMPHOCYTE-REGULATED PROTEIN PM1 HOMOLOG.

2 DAYS NEONATE THYMUS THYMIC CELLS CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:E430016E04 PRODUCT:HETEROCHROMATIN PROTEIN 1, BINDING PROTEIN 3, FULL INSERT SEQUENCE.

2,4-DIENOYL-COA REDUCTASE, MITOCHONDRIAL PRECURSOR.

26S PROTEASE REGULATORY SUBUNIT 6A.

3 BETA-HYDROXYSTEROID DEHYDROGENASE/Delta 5--4-ISOMERASE TYPE I.

3-KETOACYL-COA THIOLASE, MITOCHONDRIAL.

3-OXO-5-BETA-STEROID 4-DEHYDROGENASE.

40S RIBOSOMAL PROTEIN S13.

43 KDA PROTEIN.

6 KDA PROTEIN.

60S RIBOSOMAL PROTEIN L13A.

60S RIBOSOMAL PROTEIN L14.

60S RIBOSOMAL PROTEIN L18A.

60S RIBOSOMAL PROTEIN L23.

60S RIBOSOMAL PROTEIN L4.

80 KDA MCM3-ASSOCIATED PROTEIN.

9.5 DAYS EMBRYO PARTHENOGENOTE CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:B130008G09 PRODUCT:ATP-BINDING CASSETTE, SUB-FAMILY C (CFTR/MRP), MEMBER 4, FULL INSERT SEQUENCE.

ACTIN, ALPHA SKELETAL MUSCLE.

ACTIVATED SPLEEN CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:F830207J03 PRODUCT:YES-ASSOCIATED PROTEIN, FULL INSERT SEQUENCE (FRAGMENT).

ACTIVATED SPLEEN CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:F830217J08 PRODUCT:HYPOTHETICAL ZN-FINGER, C2H2 TYPE CONTAINING PROTEIN, FULL INSERT SEQUENCE.

ADP/ATP TRANSLOCASE 1.

ADULT MALE BRAIN UNDEFINED_CELL_LINE CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:M5C1068G17 PRODUCT:DELETED IN LIVER CANCER 1, FULL INSERT SEQUENCE.

ADULT MALE CORPORA QUADRIGEMINA CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:B230349L23 PRODUCT:POTASSIUM VOLTAGE-GATED CHANNEL, SHAKER-RELATED SUBFAMILY, BETA MEMBER 3, FULL INSERT SEQUENCE.

ADULT MALE HYPOTHALAMUS CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:A230026I24 PRODUCT:HYPOTHETICAL PROTEIN, FULL INSERT SEQUENCE.

ADULT MALE HYPOTHALAMUS CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:A230065N10 PRODUCT:HYPOTHETICAL PROTEIN, FULL INSERT SEQUENCE.
ADULT MALE OLFATORY BRAIN CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:6430517J16 PRODUCT:HYPOTHETICAL METALLO-HYDROLASE/OXIDOREDUCTASE STRUCTURE CONTAINING PROTEIN, FULL INSERT SEQUENCE.

ADULT MALE SMALL INTESTINE CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:2010300P09 PRODUCT:1810004I06RIK PROTEIN (SIMILAR TO NADH DEHYDROGENASE (UBIQUINONE) 1, SUBCOMPLEX UNKNOWN, 2) (14.5KD, B14.5B) HOMOLOG.

ADULT MALE TESTIS CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:4930503E14 PRODUCT:HYPOTHETICAL PROTEIN, FULL INSERT SEQUENCE.

ADULT MALE TESTIS CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:4931412F17 PRODUCT:SOLUBLE ADENYLYL CYCLASE HOMOLOG.

ADULT MALE TESTIS CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:4932412N08 PRODUCT:HYPOTHETICAL GLUTAMIC ACID-RICH REGION CONTAINING PROTEIN, FULL INSERT SEQUENCE.

ADULT MALE TESTIS CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:4932443F20 PRODUCT:ZINC FINGER PROTEIN (C2H2 TYPE) 276, FULL INSERT SEQUENCE.

ADULT MALE TESTIS CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:4933400B06 PRODUCT:HYPOTHETICAL SERINE-RICH REGION CONTAINING PROTEIN, FULL INSERT SEQUENCE (FRAGMENT).

ADULT MALE TESTIS CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:4933413C22 PRODUCT:HYPOTHETICAL 52.3 KDA PROTEIN HOMOLOG.

ADULT PANCREAS ISLET CELLS CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:C820003N21 PRODUCT:SIMILAR TO DJ506.2.

ANKYRIN REPEAT DOMAIN-CONTAINING PROTEIN 5.

ARISTALESS 3.

BONE MARROW MACROPHAGE CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:I830042F18 PRODUCT:ASPARTYL-TRNA SYNTHETASE, FULL INSERT SEQUENCE.

CARBOXYPEPTIDASE N CATALYTIC CHAIN PRECURSOR.

CCAAT/ENHANCER-BINDING PROTEIN BETA.

CDNA SEQUENCE AK129341.

CGG TRIPLET REPEAT-BINDING PROTEIN 1.

COHESIN SUBUNIT SA-3.

COILED-COIL DOMAIN-CONTAINING PROTEIN 110.

COMPLEMENT C1Q TUMOR NECROSIS FACTOR-RELATED PROTEIN 3 PRECURSOR.

CONSERVED OLIGOMERIC GOLGI COMPLEX COMPONENT 1.

CORTACTIN BINDING PROTEIN 2 ISOFORM 1.

CYTOCHROME P450 11A1, MITOCHONDRIAL PRECURSOR.

D-AMINO-ACID OXIDASE.

DEAD (ASP-GLU-ALA-ASP) BOX POLYPEPTIDE 23.

DIACYLGLYCEROL KINASE ZETA.

DOWN-REGULATED IN METASTASIS ISOFORM 1.
101 DUAL SPECIFICITY PROTEIN PHOSPHATASE 1.
102 ELONGATION FACTOR 2.
103 EOSINOPHIL PEROXIDASE PRECURSOR.
104 EPIDERMAL GROWTH FACTOR RECEPTOR PRECURSOR.
105 EXTRACELLULAR MATRIX PROTEIN FRAS1 PRECURSOR.
106 F-BOX AND WD-40 DOMAIN PROTEIN 9.
107 FILAMIN BINDING LIM PROTEIN 1.
108 FORKHEAD-RELATED TRANSCRIPTION FACTOR 10.
109 GALECTIN-1.
110 GENERAL CONTROL OF AMINO ACID SYNTHESIS PROTEIN 5-LIKE 2.
111 GLUTATHIONE S-TRANSFERASE MU 1.
112 G-RICH SEQUENCE FACTOR 1.
113 H2AFJ PROTEIN.
114 HEMOGLOBIN SUBUNIT BETA-1.
115 HEMOGLOBIN SUBUNIT BETA-2.
116 HEPATOCELLULAR GROWTH FACTOR-LIKE PROTEIN PRECURSOR.
117 HISTIDINE ACID PHOSPHATASE DOMAIN CONTAINING 1.
118 HISTONE H1.5.
119 HISTONE H3.2.
120 HYDROXYACYL-COA DEHYDROGENASE/3-KETOACYL
COENZYME A COENZYME A A THIOLASE/ENOLASE
HYDROLASE (TRIFUNCTIONAL PROTEIN), ALPHA SUBUNIT.
121 HYPOTHETICAL PROTEIN LOC212448.
122 HYPOTHETICAL PROTEIN P4(21)N (FRAGMENT).
123 HYPOTHETICAL PROTEIN.
124 ISOFORM 1 OF ATAXIN-2.
125 ISOFORM 1 OF CELL DIVISION PROTEIN KINASE 3.
126 ISOFORM 1 OF COMPLEMENT FACTOR D PRECURSOR.
127 ISOFORM 1 OF CYCLIN-L2.
128 ISOFORM 1 OF E3 UBIQUITIN-PROTEIN LIGASE NRDPI.
129 ISOFORM 1 OF EPITHELIAL DISCOIDIN DOMAIN-CONTAINING
RECEPTOR 1 PRECURSOR.
130 ISOFORM 1 OF INOSITOL 1,4,5-TRISPHOSPHATE RECEPTOR TYPE 1.
131 ISOFORM 1 OF JOUBERIN.
132 ISOFORM 1 OF NUCLEOLAR PROTEIN 11.
133 ISOFORM 1 OF PROBABLE ATP-DEPENDENT RNA HELICASE DDX46.
134 ISOFORM 1 OF PROTEIN KIAA0284.
135 ISOFORM 1 OF RHOMBOID DOMAIN-CONTAINING PROTEIN 2.
136 ISOFORM 1 OF SEPTIN-9.
137 ISOFORM 1 OF SPERMATAGENESIS-ASSOCIATED PROTEIN 17.
138 ISOFORM 1 OF STRIATED MUSCLE-SPECIFIC SERINE/THREONINE
PROTEIN KINASE.
139 isoform 1 of tyrosine-protein kinase tec.
140 ISOFORM 2 OF CHROMOMADOMAIN-HELICASE-DNA-BINDING PROTEIN
9.
141 ISOFORM 2 OF KERATIN, TYPE I CYTOSKELETAL 12.
142 ISOFORM 2 OF MUCIN AND CADHERIN-LIKE PROTEIN PRECURSOR.
143 ISOFORM 2 OF PROBABLE UBIQUITIN CARBOXYL-TERMINAL
HYDROLASE CYLD.
ISOFORM 2 OF TRANSMEMBRANE PROTEIN 34.
ISOFORM 2 OF TUMOR PROTEIN P73-LIKE.
ISOFORM 2 OF UPF0424 PROTEIN C1orf128 HOMOLOG.
ISOFORM 3 OF UBQUITIN CARBOXYL-TERMINAL HYDROLASE 2.
ISOFORM 5 OF PERIPHERAL PLASMA MEMBRANE PROTEIN CASK.
ISOFORM A OF ANION EXCHANGE PROTEIN 2.
ISOFORM ALPHA OF ATP-DEPENDENT DNA HELICASE Q1.
ISOFORM LONG OF MDM4 PROTEIN.
KIAA1409.
MEIOTIC RECOMBINATION PROTEIN REC8-LIKE 1.
MEMBRANE-ASSOCIATED TYROSINE- AND THREONINE-SPECIFIC CDC2-INHIBITORY KINASE.
MHC CLASS I H2 ANTIGEN GENE (FRAGMENT).
MITOCHONDRIAL CARNITINE/ACYLCARNITINE CARRIER PROTEIN.
MKIAA0315 PROTEIN (FRAGMENT).
NOD-DERIVED CD11C +VE DENDRITIC CELLS CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:F630116G04 PRODUCT:EPIDERMAL GROWTH FACTOR RECEPTOR PATHWAY SUBSTRATE 8, FULL INSERT SEQUENCE.
NOVEL PROTEIN SIMILAR TO OBSCURIN, CYTOSKELETAL CALMODULIN AND TITIN- INTERACTING RHOGEF.
NUCLEAR CAP-BINDING PROTEIN SUBUNIT 2.
OLFACORY RECEPTOR MOR108-4.
OLFACORY RECEPTOR OLFR224.
OLFACORY RECEPTOR OLFR624.
ORIGIN RECOGNITION COMPLEX SUBUNIT 3.
OSTEOCLAST-LIKE CELL CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:I420001D24 PRODUCT:SH3 DOMAIN AND TETRATRICOPEPTIDE REPEATS 1, FULL INSERT SEQUENCE.
OSTEOCLAST-LIKE CELL CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:I420025L17 PRODUCT:CYCLIN M2, FULL INSERT SEQUENCE.
POTASSIUM CHANNEL TETRAMERISATION DOMAIN CONTAINING 9.
PREDICTED.
PROBABLE HISTONE-LYSINE N-METHYLTRANSFERASE ASH1L.
PROLACTIN-LIKE PROTEIN B.
PROTEIN DISULFIDE-ISOMERASE A3 PRECURSOR.
PROTEIN DOM3Z.
PROTEIN TRANSPORT PROTEIN SEC61 SUBUNIT ALPHA ISOFORM 2.
PROTCADHERIN 19.
PUTATIVE ATP-DEPENDENT RNA HELICASE PL10.
RAN GTPASE-ACTIVATING PROTEIN 1.
REGULATOR OF G-PROTEIN SIGNALING 18.
REPLICATION FACTOR C SUBUNIT 2.
RETICULON-4 RECEPTOR PRECURSOR.
RHO GUANINE NUCLEOTIDE EXCHANGE FACTOR (GEF) 10-LIKE.
RHO-ASSOCIATED PROTEIN KINASE 2.
RHOMBOID 5 HOMOLOG 2.
RIKEN CDNA 4921506M07 GENE.
RIKEN CDNA E030041M21 GENE.
RPS16 PROTEIN.
SEC6-LIKE PROTEIN C14orf73 HOMOLOG.
SENTRIN 14.
SERINE/THREONINE-PROTEIN KINASE MARK1.
SERINE/THREONINE-PROTEIN KINASE TOUSLED-LIKE 1.
SERINE-PROTEIN KINASE ATM.
SERPIN H1 PRECURSOR.
SERUM ALBUMIN PRECURSOR.
SIMILAR TO 40S RIBOSOMAL PROTEIN S4, X ISOFORM.
SIMILAR TO ALPHA 3 TYPE VI COLLAGEN ISOFORM 3 PRECURSOR ISOFORM 2.
SIMILAR TO EC1-V2R PHEROMONE RECEPTOR.
SIMILAR TO MYOSIN-18B.
SIMILAR TO MYOTUBULARIN-RELATED PROTEIN 2.
SIMILAR TO STAM-BINDING PROTEIN.
SURVIVAL OF MOTOR NEURON.
TBC1 DOMAIN FAMILY MEMBER 7.
TESTIS EXPRESSED GENE 15.
THIOREDOXIN-LIKE PROTEIN 1.
TRANSCRIPTION FACTOR ETV6.
TRANSCRIPTION FACTOR MEL1.
TRANSFORMING, ACIDIC COILED-COIL CONTAINING PROTEIN 2 ISOFORM C.
TRANSMEMBRANE PROTEIN 24.
TRIPARTITE MOTIF FAMILY-LIKE PROTEIN 1.
UBIQUITIN CARBOXYL-TERMINAL HYDROLASE 26.
VASCULAR ENDOThELIAL GROWTH FACTOR RECEPTOR 2 PRECURSOR.
VGFR NERVE GROWTH FACTOR INDUCIBLE.
VOLTAGE-DEPENDENT ANION-SELECTIVE CHANNEL PROTEIN 2.
ZINC FINGER AND BTB DOMAIN CONTAINING 10 ISOFORM 1.
ZINC FINGER CCHC DOMAIN-CONTAINING PROTEIN 6.
Appendix A

227 13 DAYS EMBRYO LIVER CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:2510040B16 PRODUCT:HEMOGLOBIN, BETA ADULT MAJOR CHAIN, FULL INSERT SEQUENCE.

228 7 DAYS NEONATE CEREBELLUM CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:A730017H24 PRODUCT:HYPOTHETICAL SERINE-RICH REGION PROFILE CONTAINING PROTEIN, FULL INSERT SEQUENCE.

229 ADULT MALE CORPORA QUADRIGEMINA CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:B230341F08 PRODUCT:HYPOTHETICAL PROTEIN, FULL INSERT SEQUENCE.

230 CHLORIDE CHANNEL, NUCLEOTIDE-SENSITIVE, 1A.

231 CYTOSOLIC PHOSPHOLIPASE A2 BETA.

232 ELONGATION FACTOR 1-ALPHA 2.

233 EOSINOPHIL-ASSOCIATED, RIBONUCLEASE A FAMILY, MEMBER 11.

234 HEME-BINDING PROTEIN 2.

235 HEMOGLOBIN SUBUNIT BETA-1.

236 HISTONE H2B TYPE 1-F/J/L.

237 ISOFORM 3 OF YTH DOMAIN FAMILY PROTEIN 3.

238 MYOSIN-VIIA.

239 PCDHB18 PROTEIN.

240 PLEXIN-A1 PRECURSOR.

241 SIMILAR TO LYSOCARDIOLIPIN ACYLTRANSFERASE ISOFORM 1 ISOFORM 1.

242 SIMILAR TO TBP-ASSOCIATED FACTOR 1 ISOFORM 1 ISOFORM 13.

243 SIMILAR TO ZINC FINGER PROTEIN 217.

244 SOLUTE CARRIER FAMILY 4, SODIUM BICARBONATE TRANSPORTER-LIKE, MEMBER 11.

245 SURFEIT LOCUS PROTEIN 5.

246 T-CELL IMMUNOGLOBULIN AND MUCIN DOMAIN-CONTAINING PROTEIN 2 PRECURSOR.

247 TRANSCRIPTION FACTOR MEL1.

248 UBIQUITIN CARBOXYL-TERMINAL HYDROLASE 26.

Fraction 4

249 0 DAY NEONATE LUNG CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:E030013O11 PRODUCT:WEAKLY SIMILAR TO CDNA: FLJ21120 FIS, CLONE CAS05691.

250 0 DAY NEONATE LUNG CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:E030026H08 PRODUCT:HYPOTHETICAL PROTEIN, FULL INSERT SEQUENCE.

251 10 DAYS EMBRYO WHOLE BODY CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:2610027B07 PRODUCT:HISTONE 4 PROTEIN, FULL INSERT SEQUENCE.

252 12 DAYS EMBRYO EMBRYONIC BODY BETWEEN DIAPHRAGM REGION AND NECK CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:9430034P22 PRODUCT:GLUTATHIONE S-TRANSFERASE YB-3 (EC 2.5.1.18) (CHAIN 4) (GST YB3) (GST CLASS-MU 3) HOMOLOG.

253 13 DAYS EMBRYO LIVER CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:2510040B16 PRODUCT:HEMOGLOBIN, BETA ADULT MAJOR CHAIN, FULL INSERT SEQUENCE.
Appendix A

14.5 DAYS EMBRYO DF/DF RATHKE'S POCHES CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:K820003H17 PRODUCT:NUCLEAR FACTOR OF ACTIVATED T-CELLS, CYTOPLASMIC, CALCINEURIN-DEPENDENT 4, FULL INSERT SEQUENCE.

3 DAYS NEONATE THYMUS CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:A630059M15 PRODUCT:HYPOTHETICAL PROTEIN, FULL INSERT SEQUENCE (FRAGMENT).

353 KDA PROTEIN.

40 KDA PROTEIN.

40S RIBOSOMAL PROTEIN S19.

40S RIBOSOMAL PROTEIN S9.

47 KDA PROTEIN.

4930432K09RIK PROTEIN.

60S RIBOSOMAL PROTEIN L22.

7 DAYS NEONATE CEREBELLUM CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:A730099C22 PRODUCT:COATOMER ALPHA SUBUNIT.

78 KDA GLUCOSE-REGULATED PROTEIN PRECURSOR.

78 KDA PROTEIN.

ACETYL-COA CARBOXYLASE 1.

ACTIN, ALPHA SKELETAL MUSCLE.

ACTIN, CYTOPLASMIC 1.

ADAMTS-1 PRECURSOR.

ADULT MALE KIDNEY CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:F520013M09 PRODUCT:WEAKLY SIMILAR TO ORNITHINE DECARBOXYLASE 1.

ADULT MALE LIVER TUMOR CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:C730002J02 PRODUCT:FIBRINOGEN, GAMMA POLYPEPTIDE, FULL INSERT SEQUENCE.

ADULT MALE TESTIS CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:4932415D10 PRODUCT:HYPOTHETICAL PROTEIN, FULL INSERT SEQUENCE.

ADULT MALE THYMUS CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:5830445I05 PRODUCT:G1 TO PHASE TRANSITION 1, FULL INSERT SEQUENCE.

ANKYRIN REPEAT AND SOCS BOX PROTEIN 1.

ANNEXIN A5.

AP-3 COMPLEX SUBUNIT SIGMA-1.

ARYLACETAMIDE DEACETYLASE-LIKE 4.

ATP SYNTHASE SUBUNIT ALPHA, MITOCHONDRIAL PRECURSOR.

ATP SYNTHASE SUBUNIT BETA, MITOCHONDRIAL PRECURSOR.

AXONEMAL DYNEIN HEAVY CHAIN.

CELL DIVISION PROTEIN KINASE 10.

CELLULAR RETINOIC ACID-BINDING PROTEIN 2.

CENTROMERE/KINETOCHEORE PROTEIN ZW10 HOMOLOG.

CENTROSOMAL PROTEIN 78.

COLLAGEN ALPHA-1(III) CHAIN PRECURSOR.

COLLAGEN TRIPLE HELIX REPEAT-CONTAINING PROTEIN 1 PRECURSOR.

CPN10-LIKE PROTEIN.

CYCLIN-LIKE PROTEIN 1.

CYTOCHROME C OXIDASE POLYPEPTIDE VIC.

CYTOCHROME P450 11A1, MITOCHONDRIAL PRECURSOR.

DEAH.
Appendix A

292 ES CELLS CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:C330018J07 PRODUCT: HYPOTHETICAL LEUCINE-RICH REPEAT, OUTLIERS/LEUCINE-RICH REPEAT/LEUCINE-RICH REPEAT, TYPICAL SUBTYPE CONTAINING PROTEIN, FULL INSERT SEQUENCE.
293 GAG.
294 GLYCINE RECEPTOR SUBUNIT BETA PRECURSOR.
295 GUANYLATE CYCLASE SOLUBLE SUBUNIT ALPHA-3.
296 HELICASE-LIKE TRANSCRIPTION FACTOR.
297 HEMOGLOBIN SUBUNIT BETA-2.
298 HISTONE H1.2.
299 HISTONE H1.5.
300 HYDROXYMETHYLGLUTARYL-COA SYNTHASE, MITOCHONDRIAL PRECURSOR.
301 HYPOTHETICAL PROTEIN LOC320291.
302 HYPOTHETICAL PROTEIN LOC72371 ISOFORM 1.
303 HYPOTHETICAL PROTEIN.
304 ISOFORM 1 OF GC-RICH SEQUENCE DNA-BINDING FACTOR.
305 ISOFORM 1 OF INTERSECTIN-1.
306 ISOFORM 1 OF NEUROGENIC LOCUS NOTCH HOMOLOG PROTEIN 1 PRECURSOR.
307 ISOFORM 1 OF PERIAXIN.
308 ISOFORM 1 OF PROTEIN LMBR1L.
309 ISOFORM 1 OF RHO GTPASE-ACTIVATING PROTEIN 19.
310 ISOFORM 1 OF SUSHI-REPEAT-CONTAINING PROTEIN SRPX PRECURSOR.
311 ISOFORM 2 OF SERINE/THREONINE-PROTEIN PHOSPHATASE 4 REGULATORY SUBUNIT 1.
312 ISOFORM A OF TUBERIN.
313 ISOFORM CBF2 OF CCAAT/ENHANCER-BINDING PROTEIN ZETA.
314 LEUCINE-RICH REPEAT-CONTAINING PROTEIN 15 PRECURSOR.
315 LEUKOSIALIN PRECURSOR.
316 MULTIDRUG RESISTANCE PROTEIN 2.
317 MYOSIN LIGHT CHAIN, REGULATORY B-LIKE.
318 NUCLEOSIDE DIPHOSPHATE KINASE B.
319 PEROXISOMAL MULTIFUNCTIONAL ENZYME TYPE 2.
320 POGK PROTEIN.
321 POLYDUCTIN.
322 POTASSIUM/SODIUM HYPERPOLARIZATION-ACTIVATED CYCLIC NUCLEOTIDE-GATED CHANNEL 1.
323 PUTATIVE ATP-DEPENDENT RNA HELICASE PL10.
324 RIKEN CDNA 4930452B06 GENE.
325 SENTRIN 14.
326 SEPTIN 4.
327 SERUM ALBUMIN PRECURSOR.
328 SIMILAR TO 40S RIBOSOMAL PROTEIN S25.
329 SIMILAR TO 60S RIBOSOMAL PROTEIN L6.
330 SIMILAR TO 60S RIBOSOMAL PROTEIN L7A.
331 SIMILAR TO COFILIN-1 (COFILIN, NON-MUSCLE ISOFORM) ISOFORM 1.
332 SIMILAR TO DEVELOPMENT- AND DIFFERENTIATION-ENHANCING FACTOR 2.
333 SIMILAR TO DNA-DIRECTED RNA POLYMERASE III LARGEST SUBUNIT.
334 SIMILAR TO DUAL SPECIFICITY PHOSPHATASE AND PRO ISOMERASE DOMAIN CONTAINING 1.
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<td>SIMILAR TO GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE.</td>
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<td>SIMILAR TO LAMININ ALPHA-3 CHAIN PRECURSOR.</td>
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<td>SIMILAR TO MYOSIN HEAVY CHAIN, CARDIAC MUSCLE ALPHA ISOFORM.</td>
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<td>SIMILAR TO PROTEIN S-MYC.</td>
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<td>SIMILAR TO RHO GUANINE NUCLEOTIDE EXCHANGE FACTOR 5 ISOFORM 1.</td>
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<td>SMALL NUCLEAR RIBONUCLEOPROTEIN SM D3.</td>
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<td>SOLUTE CARRIER ORGANIC ANION TRANSPORTER FAMILY MEMBER 2B1.</td>
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<td>SPlicing FACTOR, ARGININE/SERINE-RICH 4.</td>
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<td>SYNAPTODBREVIN-LIKE PROTEIN.</td>
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<td>T-COMPLEX PROTEIN 1 SUBUNIT BETA.</td>
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<td>TOLL-LIKE RECEPTOR 12 PRECURSOR.</td>
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<td>TRANSCRIPTION ELONGATION FACTOR A PROTEIN 3.</td>
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<td>348</td>
<td>TRANSCRIPTION FACTOR MEL1.</td>
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<td>UNCHARACTERIZED PROTEIN C2ORF29 HOMOLOG.</td>
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<td>VOLTAGE-DEPENDENT P/Q-TYPE CALCIUM CHANNEL SUBUNIT ALPHA-1A.</td>
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<td>ZINC FINGER CCCH DOMAIN-CONTAINING PROTEIN 10.</td>
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<td>ZINC FINGER PROTEIN 40.</td>
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<td>0 DAY NEONATE HEAD CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:4833415F11 PRODUCT:HYPOTHETICAL BTB/POZ DOMAIN/METHYLATED-DNA-- PROTEIN-CYSTEINE METHYLMETRANSFERASE CONTAINING PROTEIN, FULL INSERT SEQUENCE.</td>
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<td>0 DAY NEONATE KIDNEY CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:D630003M21 PRODUCT:HYPOTHETICAL CELLULAR RETINALDEHYDE-BINDING PROTEIN (CRAL)/TRIPLE FUNCTION DOMAIN (TRIO) CONTAINING PROTEIN, FULL INSERT SEQUENCE.</td>
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<td>10 DAYS EMBRYO WHOLE BODY CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:2610027B07 PRODUCT:HISTONE 4 PROTEIN, FULL INSERT SEQUENCE.</td>
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<td>10 DAYS EMBRYO WHOLE BODY CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:2610524G09 PRODUCT:HYPOTHETICAL PROTEIN, FULL INSERT SEQUENCE.</td>
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<td>10, 11 DAYS EMBRYO WHOLE BODY CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:2810406K13 PRODUCT:HYPOTHETICAL BTB/POZ DOMAIN/KELCH REPEAT CONTAINING PROTEIN, FULL INSERT SEQUENCE.</td>
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<td>101 KDA PROTEIN.</td>
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<td>12 DAYS EMBRYO EMBRYONIC BODY BETWEEN DIAPHRAGM REGION AND NECK CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:9430034P22 PRODUCT:GLUTATHIONE S-TRANSFERASE YB-3 (EC 2.5.1.18) (CHAIN 4) (GST YB3) (GST CLASS-MU 3) HOMOLOG.</td>
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<td>12 DAYS EMBRYO SPINAL GANGLION CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:D130061015 PRODUCT:HYPOTHETICAL PROTEIN, FULL INSERT SEQUENCE.</td>
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<td>13 DAYS EMBRYO FORELIMB CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:5930413M14 PRODUCT:ANNEXIN A6, FULL INSERT SEQUENCE.</td>
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<td>13 KDA PROTEIN.</td>
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<td>15 DAYS EMBRYO MALE TESTIS CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:8030485A06 PRODUCT:SIMILAR TO CENTROSOMAL PROTEIN 2 HOMOLOG.</td>
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<td>16 DAYS EMBRYO HEAD CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:C130025D20 PRODUCT:SPlicing FACTOR ARGinine/seriNE-RiCH 11 (ARGinine-RiCH 54 KDA NUCLEAR PROTEIN) (P54) HOMOLOG.</td>
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<td>16 DAYS EMBRYO HEAD CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:C130060K24 PRODUCT:SPlicing Factor ARGinine/SeriNE-RiCH 11 (ARGinine-RiCH 54 KDA NUCLEAR PROTEIN) (P54) HOMOLOG.</td>
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<td>25 KDA PROTEIN.</td>
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<td>25 KDA PROTEIN.</td>
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<td>368</td>
<td>39S RIBOSOMAL PROTEIN L53, MITOCHONDRIAL PRECURSOR.</td>
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<td>40S RIBOSOMAL PROTEIN S3.</td>
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<td>40S RIBOSOMAL PROTEIN S9.</td>
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<td>43 KDA PROTEIN.</td>
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<td>5-AMINOLEVULINATE SYNTHASE, ERYTHROID-SPECIFIC, MITOCHONDRIAL PRECURSOR.</td>
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<td>6 DAYS NEONATE HEAD CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:5430411C19 PRODUCT:HYPOThETICAL PROTEIN, FULL INSERT SEQUENCE.</td>
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<td>60 KDA SS-A/RO RIBONUCLEOPROTEIN.</td>
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<td>60S RIBOSOMAL PROTEIN L26.</td>
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<td>88 KDA PROTEIN.</td>
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<td>ACSBG2.</td>
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<td>ACTIN, ALPHA SKELETAL MUSCLE.</td>
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<td>ADULT MALE CORPORA QUADRIGEMINA CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:B230341F08 PRODUCT:HYPOThETICAL PROTEIN, FULL INSERT SEQUENCE.</td>
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<td>ADULT MALE EYEBALL CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:7530414N08 PRODUCT:MICROSOMAL TRIGLYCERIDE TRANSFER PROTEIN, FULL INSERT SEQUENCE (FRAGMENT).</td>
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<td>ADULT MALE TESTIS CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:4932408F18 PRODUCT:HYPOThETICAL ARM REPEAT STRUCTURE CONTAINING PROTEIN, FULL INSERT SEQUENCE.</td>
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<td>ADULT MALE THYMUS CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:5830487H05 PRODUCT:HYPOThETICAL ARGinine-RiCH REGION PROFILE CONTAINING PROTEIN, FULL INSERT SEQUENCE.</td>
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<td>AID (FRAGMENT).</td>
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<td>AMINE OXIDASE [FLAVIN-CONTAINING] A.</td>
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<td>ANKYRIN REPEAT DOMAIN-CONTAINING PROTEIN 57.</td>
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<td>ASPARTYL-TRNA SYNTHETASE, MITOCHONDRIAL PRECURSOR.</td>
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389 ATP SYNTHASE SUBUNIT ALPHA, MITOCHONDRIAL PRECURSOR.
390 ATP-DEPENDENT DNA HELICASE 2 SUBUNIT 2.
391 ATP-DEPENDENT RNA HELICASE DDX3Y.
392 C1QTNF9.
393 CAMP-DEPENDENT PROTEIN KINASE REGULATORY SUBUNIT.
394 CENTROSOMAL PROTEIN 78.
395 COILED-COIL DOMAIN-CONTAINING PROTEIN 39.
396 COLLAGEN ALPHA-1(III) CHAIN PRECURSOR.
397 COLLAGEN TRIPLE HELIX REPEAT-CONTAINING PROTEIN 1 PRECURSOR.
398 COMPONENT OF OLIGOMERIC GOLGI COMPLEX 3.
399 CYTOCHROME P450 11A1, MITOCHONDRIAL PRECURSOR.
400 CYTOSKELETON ASSOCIATED PROTEIN 5.
401 DEDICATOR OF CYTOKINESIS 8.
402 DULLARD HOMOLOG.
403 DYNEIN, AXONEMAL, HEAVY CHAIN 10.
404 E3 UBQUITIN-PROTEIN LIGASE TRAF7.
405 ELAV (EMBRYONIC LETHAL, ABNORMAL VISION, DROSOPHILA)-LIKE 4.
406 ENDOPLASMIN PRECURSOR.
407 ERYTHROID DIFFERENTIATION-RELATED FACTOR 1.
408 ES CELLS CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:2410127E16 PRODUCT:LIPRIN-ALPHA3 HOMOLOG (FRAGMENT).
409 EXTRACELLULAR MATRIX PROTEIN 2 PRECURSOR.
410 FATTY ACID-BINDING PROTEIN, EPIDERMAL.
411 F-BOX ONLY PROTEIN 16.
412 FIBRILLIN-1 PRECURSOR.
413 FORKHEAD-RELATED TRANSCRIPTION FACTOR 10.
414 HEMOGLOBIN SUBUNIT BETA-2.
415 HISTONE H1.4.
416 HISTONE H2B TYPE 1-F/J/L.
417 HISTONE H3.2.
418 HISTONE H4.
419 HYDROXYACYL-COENZYME A DEHYDROGENASE/3-KETOACYL-COENZYME A THIOLASE/ENOYL-COENZYME A HYDRATASE (TRIFUNCTIONAL PROTEIN), ALPHA SUBUNIT.
420 HYPOTHETICAL PROTEIN LOC216393.
421 HYPOTHETICAL PROTEIN LOC228361 ISOFORM 1.
422 IN VITRO FERTILIZED EGGS CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:7420443F16 PRODUCT:SIMILAR TO ALPHA TUBULIN.
423 INTERFERON-RELATED DEVELOPMENTAL REGULATOR 1.
424 ISOFORM 1 OF 60 KDA HEAT SHOCK PROTEIN, MITOCHONDRIAL PRECURSOR.
425 ISOFORM 1 OF CAP-GLY DOMAIN-CONTAINING LINKER PROTEIN 1.
426 ISOFORM 1 OF CASKIN-1.
427 ISOFORM 1 OF CYCLIN FOLD PROTEIN 1.
428 ISOFORM 1 OF FOLATE TRANSPORTER 1.
429 ISOFORM 1 OF FORMIN-LIKE PROTEIN 1.
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430 ISOFORM 1 OF HETEROGENEOUS NUCLEAR RIBONUCLEOPROTEIN K.
431 ISOFORM 1 OF HIGH MOBILITY GROUP PROTEIN 20A.
432 ISOFORM 1 OF JOUBERIN.
433 ISOFORM 1 OF JUNCTOPHILIN-4.
434 ISOFORM 1 OF LAYILIN PRECURSOR.
435 ISOFORM 1 OF NESPRIN-3.
436 ISOFORM 1 OF PRE-MRNA-SPlicing FACTOR 18.
437 ISOFORM 1 OF PROTEIN FAM59A.
438 ISOFORM 1 OF SYNAPTOTAGMIN-LIKE PROTEIN 3.
439 ISOFORM 1 OF TESTIS-EXPRESSED PROTEIN 14.
440 ISOFORM 1 OF TRANSMEMBRANE PROTEIN 16E.
441 ISOFORM 1 OF TRNA MODIFICATION GTPASE GTPBP3, MITOCHONDRIAL PRECURSOR.
442 ISOFORM 2 OF PROTEIN BICAUDAL D HOMOLOG 2.
443 ISOFORM 3 OF ENGULFMENT AND CELL MOTILITY PROTEIN 2.
444 ISOFORM 7 OF INOSITOL 1,4,5-TRISPHOSPHATE RECEPTOR TYPE 1.
445 ISOFORM KAP3B OF KINESIN-ASSOCIATED PROTEIN 3.
446 ISOFORM RII-2 OF TGF-BETA RECEPTOR TYPE-2 PRECURSOR.
447 LAMININ SUBUNIT ALPHA-1 PRECURSOR.
448 LEUCINE-RICH REPEAT-CONTAINING PROTEIN 17 PRECURSOR.
449 MAMMARY GLAND RCB-0527 JYG-MC(B) CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:G930023K03 PRODUCT:X-LINKED MYOTUBULAR MYOPATHY GENE 1, FULL INSERT SEQUENCE.
450 MAX-INTERACTING PROTEIN.
451 MUSCULIN.
452 N-ACETYLLACTOSAMINIDE BETA-1,6-N-ACETYLGLUCOSAMINYL-TRANSFERASE.
453 NEBL PROTEIN.
454 NOVEL PROTEIN CONTAINING HEAT REPEATS.
455 NOVEL PROTEIN SIMILAR TO HUMAN PDZ DOMAIN CONTAINING GUANINE NUCLEOTIDE EXCHANGE FACTOR (GEF) 2 PDZGEF2.
456 NOVEL PROTEIN.
457 OLFACTORY RECEPTOR MOR187-1.
458 OLFACTORY RECEPTOR MOR264-6.
459 PLA2G4C PROTEIN.
460 PLASMA ALPHA-L-FUCOSIDASE PRECURSOR.
461 PLASMA KALLIKREIN-LIKE PROTEIN 4 PRECURSOR.
462 PREGNANCY SPECIFIC GLYCOPROTEIN 17.
463 PROBABLE HISTONE-LYSINE N-METHYLTRANSFERASE ASH1L.
464 PROTEIN BEX1.
465 PROTEIN FAM109B.
466 PROTOCADHERIN ALPHA 10.
467 PUTATIVE DEOXYRIBOSE-PHOSPHATE ALDOLASE.
468 RBM6 PROTEIN.
469 RECEPTOR-TYPE TYROSINE-PROTEIN PHOSPHATASE KAPPA PRECURSOR.
470 RELA-ASSOCIATED INHIBITOR.
471 RIBOSOME BIOGENESIS REGULATORY PROTEIN HOMOLOG.
472 RIKEN CDNA 1810013L24 GENE.
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473 RIKEN CDNA C230095G01 GENE.
474 RNA BINDING PROTEIN GENE WITH MULTIPLE SPLICING ISOFORM 2.
475 SAC DOMAIN-CONTAINING PROTEIN 3.
476 SEMAPHORIN-3A PRECURSOR.
477 SENTRIN-SPECIFIC PROTEASE 3.
478 SHORT TRANSIENT RECEPTOR POTENTIAL CHANNEL 6.
479 SIMILAR TO 60S RIBOSOMAL PROTEIN L29.
480 SIMILAR TO 60S RIBOSOMAL PROTEIN L35.
481 SIMILAR TO BUTYROPHILIN SUBFAMILY 1 MEMBER A1 PRECURSOR.
482 SIMILAR TO CENTAURIN-Delta 1.
483 SIMILAR TO CG31790-PA.
484 SIMILAR TO CG8866-PB, ISOFORM B ISOFORM 4.
485 SIMILAR TO CYCLIN-DEPENDENT KINASES REGULATORY SUBUNIT 1.
486 SIMILAR TO DYNEIN HEAVY CHAIN AT 89D CG1842-PA.
487 SIMILAR TO FERM DOMAIN-CONTAINING PROTEIN 6.
488 SIMILAR TO H3 HISTONE, FAMILY 3B.
489 SIMILAR TO LEIOMODIN 3.
490 SIMILAR TO MSX2-INTERACTING PROTEIN.
491 SIMILAR TO MYOSIN IXA ISOFORM 3.
492 SIMILAR TO PDZ DOMAIN-CONTAINING RING FINGER PROTEIN 4.
493 SIMILAR TO RAN, MEMBER RAS ONCOGENE FAMILY ISOFORM 1.
494 SIMILAR TO RIBOSOMAL PROTEIN L27A.
495 SIMILAR TO SPECTRIN BETA CHAIN, BRAIN 4.
496 SIMILAR TO SPERM ASSOCIATED ANTIGEN 7.
497 SIMILAR TO SPERM PROTEIN 3111.
498 SIMILAR TO UBIQUITIN A-52 RESIDUE RIBOSOMAL PROTEIN FUSION PRODUCT 1.
499 SIMILAR TO VITAMIN D-BINDING PROTEIN PRECURSOR.
500 SOLUTE CARRIER ORGANIC ANION TRANSPORTER FAMILY, MEMBER 5A1.
501 SPATA18 PROTEIN (FRAGMENT).
502 SPlicing FACTOR, PROLINE- AND GLUTAMINE-RICH.
503 STATHMIN 1 (STMN1) PSEUDOGENE.
504 TANKYRASE 1 BINDING PROTEIN 1.
505 TBC1 DOMAIN FAMILY MEMBER 5.
506 TCP-10.
507 TETRARICICOPEPTIDE REPEAT, ANKYRIN REPEAT AND COILED-COIL CONTAINING 2.
508 TFIIH BASAL TRANSCRIPTION FACTOR COMPLEX HELICASE SUBUNIT.
509 THYROID HORMONE RECEPTOR-ASSOCIATED PROTEIN 3.
510 TITIN ISOFORM N2-A.
511 TUBULIN ALPHA-1 CHAIN.
512 TUMOR NECROSIS FACTOR, ALPHA-INDUCED PROTEIN 8-LIKE PROTEIN 2.
513 TYROSYL-DNA PHOSPHODIESTERASE 1.
514 U4/U6 SMALL NUCLEAR RIBONUCLEOPROTEIN PRP3.
515 UNCHARACTERIZED PROTEIN KIAA0174.
UNCHARACTERIZED PROTEIN KIAA0427 HOMOLOG.

UTROPHIN.

VGFR NERVE GROWTH FACTOR INDUCIBLE.

VOLTAGE-DEPENDENT ANION CHANNEL 3.

ZFP503 PROTEIN.

ZINC FINGER PROTEIN 91 ISOFORM B.

ZINC FINGER PROTEIN ZFPM1.

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<td>10 DAYS EMBRYO WHOLE BODY CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:2610027B07 PRODUCT:HISTONE 4 PROTEIN, FULL INSERT SEQUENCE.</td>
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<td>2 DAYS PREGNANT ADULT FEMALE OVIDUCT CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:E230019M04 PRODUCT:HYPOTHETICAL PROTEIN, FULL INSERT SEQUENCE.</td>
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<td>9 DAYS EMBRYO WHOLE BODY CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:D030055P10 PRODUCT:WEAKLY SIMILAR TO DEPHOSPHO-COA KINASE.</td>
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<td>ACTIN, ALPHA SKELETAL MUSCLE.</td>
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<td>ADULT MALE COLON CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:9030417P20 PRODUCT:ECOTROPIC VIRAL INTEGRATION SITE 1, FULL INSERT SEQUENCE.</td>
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<td>ADULT MALE TESTIS CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:4933407K04 PRODUCT:HYPOTHETICAL PROTEIN, FULL INSERT SEQUENCE.</td>
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INORGANIC PYROPHOSPHATASE.
INTEGRIN ALPHA-5 PRECURSOR.
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ISOFORM 1 OF NEUROGENIC LOCUS NOTCH HOMOLOG PROTEIN 1 PRECURSOR.
ISOFORM 1 OF PEPTIDYL-PROLYL CIS-TRANS ISOMERASE-LIKE 3.
ISOFORM 1 OF SODIUM-DEPENDENT PHOSPHATE TRANSPORTER 1.
ISOFORM 1 OF ZINC FINGER RAN-BINDING DOMAIN-CONTAINING PROTEIN 2.
ISOFORM 12 OF POTASSIUM VOLTAGE-GATED CHANNEL SUBFAMILY KQT MEMBER 2.
ISOFORM 2 OF DYNAMIN-1-LIKE PROTEIN.
ISOFORM 3 OF BIFUNCTIONAL PROTEIN NCOAT.
ISOFORM 3 OF HETERogeneous NUCLEAR RIBONUCLEOPROTEINS A2/B1.
ISOFORM 3 OF U2-ASSOCIATED PROTEIN SR140.
ISOFORM C2 OF LAMIN-A/C.
ISOFORM PTX2B OF PITUITARY HOMEBOX 2.
LEM DOMAIN-CONTAINING PROTEIN 2.
LUPUS LA PROTEIN HOMOLOG.
MICROSOMAL GLUTATHIONE S-TRANSFERASE 1.
MICROSOMAL TRIGLYCERIDE TRANSFER PROTEIN LARGE SUBUNIT PRECURSOR.
MON1 HOMOLOG A.
MYOSIN, LIGHT POLYPEPTIDE KINASE 2, SKELETAL MUSCLE.
NADP-DEPENDENT MALIC ENZYME.
NOD-DERIVED CD11C +VE DENDRITIC CELLS CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:F630111F22 PRODUCT:ZINC FINGER PROTEIN 512, FULL INSERT SEQUENCE.
NOD-DERIVED CD11C +VE DENDRITIC CELLS CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:F630118K07 PRODUCT:TNF RECEPTOR-ASSOCIATED FACTOR 1, FULL INSERT SEQUENCE.
NOD-DERIVED CD11C +VE DENDRITIC CELLS CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:F630209K10 PRODUCT:HYPOTHETICAL PROLINE-RICH REGION PROFILE CONTAINING PROTEIN, FULL INSERT SEQUENCE.
PHD FINGER PROTEIN 3.
PHOSPHATIDYLETHANOLAMINE-BINDING PROTEIN 1.
PLECKSTRIN HOMOLOGY DOMAIN CONTAINING, FAMILY M (WITH RUN DOMAIN) MEMBER 2.
POLYPEPTIDE N-ACETYLGLALACTOSAMINYLTRANSFERASE 1.
PROTEIN DISULFIDE-ISOMERASE A3 PRECURSOR.
PYRIDOXAL PHOSPHATE PHOSPHATASE.
RAS-RELATED PROTEIN RAB-8A.
RHO GTPASE ACTIVATING PROTEIN 10.
RIBOSOMAL PROTEIN S6 KINASE ALPHA-3.
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SIMILAR TO 40S RIBOSOMAL PROTEIN S2.
SIMILAR TO 60S RIBOSOMAL PROTEIN L7A.
SIMILAR TO ALPHA 3 TYPE VI COLLAGEN ISOFORM 3 PRECURSOR ISOFORM 2.
SIMILAR TO GLUCOCORTICOID INDUCED TRANSCRIPT 1 ISOFORM 1.
SIMILAR TO GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE.
SIMILAR TO GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE.
SIMILAR TO HEMOGLOBIN, BETA ADULT MAJOR CHAIN.
SIMILAR TO NUCLEOLAR PRERIBOSOMAL-ASSOCIATED PROTEIN 1 ISOFORM 2.
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SPlicing FACTOR 3B SUBUNIT 1.
STEROL O-ACYLTRANSFERASE 1.
TCFL5 PROTEIN.
THYROID HORMONE RECEPTOR-ASSOCIATED PROTEIN 2.
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TRANSKETOLASE.
TRANSMEMBRANE PROTEIN 88.
TREACLE PROTEIN.
TUMOR SUSCEPTIBILITY GENE 101 PROTEIN.
URIDINE 5’-MONOPHOSPHATE SYNTHASE.
VGFG2573 ISOFORM 1.
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ZINC FINGER PROTEIN 22.
ZINC FINGER, FYVE DOMAIN CONTAINING 26.

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ISOFORM 1 OF EQUILIBRATIVE NUCLEOSIDE TRANSPORTER 1.
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0 DAY NEONATE HEAD CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:4833412E22 PRODUCT:HYPOTHETICAL AMINOACYL-TRANSFER RNA SYNTHETASES CLASS-II CONTAINING PROTEIN, FULL INSERT SEQUENCE.
0 DAY NEONATE LUNG CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:E030024J03 PRODUCT:ISOCITRATE DEHYDROGENASE 1 (NADP+), SOLUBLE, FULL INSERT SEQUENCE.
0 DAY NEONATE LUNG CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:E030024J03 PRODUCT:ISOCITRATE DEHYDROGENASE 1 (NADP+), SOLUBLE, FULL INSERT SEQUENCE.
10 DAY OLD MALE PANCREAS CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:1810009A15 PRODUCT:SIMILAR TO UNKNOWN.
10 DAY OLD MALE PANCREAS CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:1810009J06 PRODUCT:TRYPSIN IV (EC 3.4.21.4) (PRETRYPSINOGEN IV) HOMOLOG.

10 DAYS EMBRYO WHOLE BODY CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:3426406K10 PRODUCT:HYPOTHETICAL PROTEIN, FULL INSERT SEQUENCE.

10 DAYS NEONATE CORTEX CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:A830093E13 PRODUCT:HYPOTHETICAL PROTEIN, FULL INSERT SEQUENCE.

10 DAYS NEONATE SKIN CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:4732401E24 PRODUCT:HYPOTHETICAL PHOSPHOLIPASE D/NUCLEASE STRUCTURE CONTAINING PROTEIN, FULL INSERT SEQUENCE.

11 KDA PROTEIN.

12 DAYS EMBRYO EYEBALL CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:D230011M17 PRODUCT:HYPOTHETICAL PROTEIN, FULL INSERT SEQUENCE.

13 DAYS EMBRYO FORELIMB CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:5930413M14 PRODUCT:ANNEXIN A6, FULL INSERT SEQUENCE.

13 DAYS EMBRYO MALE TESTIS CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:6030457M03 PRODUCT:C321D2.2 (NOVEL PROTEIN SIMILAR TO REPLICATION FACTORS) HOMOLOG.

136 KDA PROTEIN.

14-3-3 PROTEIN ZETA/Delta.

15 DAYS EMBRYO MALE TESTIS CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:8030402P03 PRODUCT:SIMILAR TO HYDROXYMETHYLGLUTARYL-COA SYNTHASE.

15 DAYS PREGNANT ADULT FEMALE PLACENTA CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:I530025D16 PRODUCT:HYPOTHETICAL FIBRONECTIN, TYPE III SUBDOMAIN/FIBRONECTIN, TYPE III/FIBRONECTIN, TYPE III-LIKE FOLD CONTAINING PROTEIN, FULL INSERT SEQUENCE.

16 DAYS EMBRYO HEAD CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:C130093J22 PRODUCT:HYPOTHETICAL PROTEIN, FULL INSERT SEQUENCE.

18 KDA PROTEIN.

18-4-3 PROTEIN ZETA/Delta.

19 KDA PROTEIN.
Appendix A

2 DAYS NEONATE THYMUS THYMIC CELLS CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:C920007H02
PRODUCT: NEUGRIN, FULL INSERT SEQUENCE.

2 DAYS PREGNANT ADULT FEMALE OVARY CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:E330031I04
PRODUCT: HYPOTHETICAL CYSTEINYL-tRNA SYNTHETASE CONTAINING PROTEIN, FULL INSERT SEQUENCE.

2156 KDA PROTEIN.
25 KDA PROTEIN.
26 KDA PROTEIN.
26S PROTEASE REGULATORY SUBUNIT 4.
3 DAYS NEONATE THYMUS CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:A630052K13
PRODUCT: HYPOTHETICAL PROTEIN, FULL INSERT SEQUENCE.

34 KDA PROTEIN.
39 KDA PROTEIN.
3-KETOACYL-COA THIOLASE, MITOCHONDRIAL.
3-KETO-STEROID REDUCTASE.
40S RIBOSOMAL PROTEIN S14.
40S RIBOSOMAL PROTEIN S19.
40S RIBOSOMAL PROTEIN S3.
40S RIBOSOMAL PROTEIN S3A.
40S RIBOSOMAL PROTEIN S4, X ISOFORM.
40S RIBOSOMAL PROTEIN S6.
40S RIBOSOMAL PROTEIN S9.
4930579E17RIK PROTEIN.
564 KDA PROTEIN.
5-HYDROXYTRYPTAMINE 1A RECEPTOR.
5-OXOPRONASE.
6 KDA PROTEIN.
60S ACIDIC RIBOSOMAL PROTEIN P2.
60S ACIDIC RIBOSOMAL PROTEIN P2.
60S RIBOSOMAL PROTEIN L10A.
60S RIBOSOMAL PROTEIN L13A.
60S RIBOSOMAL PROTEIN L14.
60S RIBOSOMAL PROTEIN L17.
60S RIBOSOMAL PROTEIN L19.
60S RIBOSOMAL PROTEIN L3.
60S RIBOSOMAL PROTEIN L4.
60S RIBOSOMAL PROTEIN L5.
60S RIBOSOMAL PROTEIN L9.
7 DAYS NEONATE CEREBELLUM CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:A730099C22
PRODUCT: COATOMER ALPHA SUBUNIT.

78 KDA GLUCOSE-REGULATED PROTEIN PRECURSOR.
9.5 DAYS EMBRYO PARTHENOCENOTE CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:B130008D24
PRODUCT: HYPOTHETICAL ARGinine-RICH REGION/TyPe I ANTIFREEZE PROTEIN CONTAINING PROTEIN, FULL INSERT SEQUENCE (FRAGMENT).

A KINASE (PRKA) ANCHOR PROTEIN (YOTIAO) 9.
ABHYDROLASE DOMAIN CONTAINING 9.
ACONITATE HYDRATASE, MITOCHONDRIAL PRECURSOR.
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<td>677</td>
<td>ACTIN, ALPHA SKELETAL MUSCLE.</td>
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<td>ACTIVATED SPLNE CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:F830011G01 PRODUCT:SIMILAR TO PUTATIVE DEOXYRIBONUCLEASE KIAA0218.</td>
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<td>ACTIVATED SPLNE CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:F830201B12 PRODUCT:PYRUVATE CARBOXYLASE, FULL INSERT SEQUENCE.</td>
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<td>ADULT MALE KIDNEY CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:F520006M13 PRODUCT:HYPO&quot;ETICAL PROTEINASE INHIBITITOR 14, SERPIN CONTAINING PROTEIN, FULL INSERT SEQUENCE.</td>
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<td>ADULT MALE LUNG CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:1200003C15 PRODUCT:HYPO&quot;ETICAL PROTEIN, FULL INSERT SEQUENCE.</td>
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<tr>
<td>695</td>
<td>ADULT MALE LUNG CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:1200014P05 PRODUCT:HYPO&quot;ETICAL ACYL-COA DEHYDROGENASE CONTAINING PROTEIN, FULL INSERT SEQUENCE.</td>
</tr>
<tr>
<td>696</td>
<td>ADULT MALE MEDULLA OBLONGATA CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:6330408P06 PRODUCT:PROLINE SYNTHETASE CO-TRANSCRIBED, FULL INSERT SEQUENCE.</td>
</tr>
<tr>
<td>697</td>
<td>ADULT MALE MEDULLA OBLONGATA CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:6332414D09 PRODUCT:HYPO&quot;ETICAL NOL1/NOP2/SUN FAMILY CONTAINING PROTEIN, FULL INSERT SEQUENCE.</td>
</tr>
<tr>
<td>698</td>
<td>ADULT MALE TESTIS CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:4921517P09 PRODUCT:SOLUTE CARRIER ORGANIC ANION TRANSPORTER FAMILY, MEMBER 6C1, FULL INSERT SEQUENCE.</td>
</tr>
</tbody>
</table>
ADULT MALE TESTIS CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:4922501L14 PRODUCT:HYPOTHETICAL SERINE-RICH REGION CONTAINING PROTEIN, FULL INSERT SEQUENCE.

ADULT MALE TESTIS CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:4922505I17 PRODUCT:HYPOTHETICAL PROTEIN, FULL INSERT SEQUENCE.

ADULT MALE TESTIS CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:4932422I21 PRODUCT:WEAKLY SIMILAR TO VITAMIN A-DEFICIENT TESTICULAR PROTEIN 11-LIKE PROTEIN.

ADULT MALE TESTIS CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:4933412E24 PRODUCT:HYPOTHETICAL PROTEIN, FULL INSERT SEQUENCE.

ADULT MALE TESTIS CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:4933439B08 PRODUCT:HYPOTHETICAL AAA ATPASE SUPERFAMILY CONTAINING PROTEIN, FULL INSERT SEQUENCE.

ADULT MALE TESTIS CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:4933439G10 PRODUCT:TETRATRICOPEPTIDE REPEAT PROTEIN 9 (TPR REPEAT PROTEIN 9) HOMOLOG.

ADULT MALE THYMUS CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:5830428C01 PRODUCT:FERREDOXIN REDUCTASE, FULL INSERT SEQUENCE.

ADULT MALE THYMUS CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:5830428C01 PRODUCT:FERREDOXIN REDUCTASE, FULL INSERT SEQUENCE.

ADULT MALE TONGUE CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:2310034O17 PRODUCT:MITOCHONDRIAL CARRIER HOMOLOG 1, FULL INSERT SEQUENCE.

ADULT RETINA CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:A930013E17 PRODUCT:ZAP3 PROTEIN, FULL INSERT SEQUENCE.

AGRIN.

AHNAK NUCLEOPROTEIN ISOFORM 1.

ALANYL-TRNA SYNTHETASE, CYTOPLASMIC.

ALDOSE REDUCTASE-RELATED PROTEIN 2.

ALPHA-1-ANTITRYSIN 1-6 PRECURSOR.

ALPHA-1D ADRENERGIC RECEPTOR.

ALPHA-ACTININ-3.

AMPHOTERIN-INDUCED PROTEIN 2 PRECURSOR.

AN1-TYPE ZINC FINGER PROTEIN 1.

ANNEXIN A2.

ANTI-PORCINE VCAM MAB 2A2 HEAVY CHAIN VARIABLE REGION (FRAGMENT).

APOLIPOPROTEIN A-I PRECURSOR.

APOLIPOPROTEIN A-IV PRECURSOR.

ARACHIDONATE 15-LIPOOXYGENASE TYPE II.

ASPARTYL-TRNA SYNTHETASE, MITOCHONDRIAL PRECURSOR.

AT-HOOK TRANSCRIPTION FACTOR.

ATP SYNTHASE SUBUNIT ALPHA, MITOCHONDRIAL PRECURSOR.
ATP SYNTHASE SUBUNIT BETA, MITOCHONDRIAL PRECURSOR.

ATP-DEPENDENT CLP PROTEASE ATP-BINDING SUBUNIT CLPX-LIKE, MITOCHONDRIAL PRECURSOR.

B/K PROTEIN.

B6-DERIVED CD11 +VE DENDRITIC CELLS CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:F730221H19 PRODUCT:HYPOTHETICAL PROTEIN, FULL INSERT SEQUENCE (FRAGMENT).

BACULOVIRAL IAP REPEAT-CONTAINING 6.

BAP28 PROTEIN.

BARRIER-TO-AUTOINTEGRATION FACTOR.

BASEMENT MEMBRANE-SPECIFIC HEPARAN SULFATE PROTEOGLYCAN CORE PROTEIN PRECURSOR.

BAT2 DOMAIN CONTAINING 1 ISOFORM 4.

B-CELL LYMPHOMA 9 PROTEIN.

B-CELL RECEPTOR-ASSOCIATED PROTEIN 31.

BIFUNCTIONAL 3'-PHOSPHOADENOSINE 5'-PHOSPHOSULFATE SYNTHETASE 2.

BONE MARROW MACROPHAGE CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:G530019G22 PRODUCT:HYPOTHETICAL PROTEIN, FULL INSERT SEQUENCE.

BONE MARROW MACROPHAGE CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:I830045N07 PRODUCT:HETEROGENEOUS NUCLEAR RIBONUCLEOPROTEIN R, FULL INSERT SEQUENCE.

BONE MARROW MACROPHAGE CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:I830120C02 PRODUCT:HETEROGENEOUS NUCLEAR RIBONUCLEOPROTEIN C, FULL INSERT SEQUENCE.

BONE MARROW STROMA CELL CRL-2028 SR-4987 CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:G430067B09 PRODUCT:HISTONE H1.0, FULL INSERT SEQUENCE.

BONE MARROW STROMA CELL CRL-2028 SR-4987 CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:G430067B09 PRODUCT:HISTONE H1.0, FULL INSERT SEQUENCE.

BREAKPOINT CLUSTER REGION PROTEIN.

BROMODOMAIN ADJACENT TO ZINC FINGER DOMAIN, 2A.

CALREITININ.

CANCER SUSCEPTIBILITY CANDIDATE 3.

CARBAMOYL-PHOSPHATE SYNTHETASE 2, ASPARTATE TRANSCARBAMYLASE, AND DIHYDROOROTASE.

CARBOHYDRATE SULFOTRANSFERASE 11.

CARBOHYDRATE SULFOTRANSFERASE 2.

CARBONIC ANHYDRASE 6 PRECURSOR.

CARNITINE O-PALMITOYLTRANSFERASE 1, LIVER ISOFORM.

CASEIN KINASE I ISOFORM GAMMA-1.

CASPASE-4 PRECURSOR.

CATENIN ALPHA-3.

CDK5 REGULATORY SUBUNIT-ASSOCIATED PROTEIN 2.

CDNA SEQUENCE BC014805.

CDNA SEQUENCE BC021891.

CDNA SEQUENCE BC048502.

CDNA SEQUENCE BC049702.
| 760 | CENTROMERE ASSOCIATED PROTEIN-E. |
| 761 | CHARGED MULTIVESICULAR BODY PROTEIN 1B-1. |
| 762 | CHROMOBOX PROTEIN HOMOLOG 2. |
| 763 | CHROMOBOX PROTEIN HOMOLOG 3. |
| 764 | CLASP2 PROTEIN. |
| 765 | CLATHRIN, HEAVY POLYPEPTIDE. |
| 766 | COFILIN-2. |
| 767 | COILED-COIL DOMAIN CONTAINING 100. |
| 768 | COILED-COIL DOMAIN CONTAINING 125. |
| 769 | COLLAGEN ALPHA-1(V) CHAIN PRECURSOR. |
| 770 | COLLAGEN, TYPE XX, ALPHA 1. |
| 771 | CRL-1722 L5178Y-R CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:I730077L17 PRODUCT:THIOREDOXIN DOMAIN CONTAINING 7, FULL INSERT SEQUENCE. |
| 772 | CUB DOMAIN-CONTAINING PROTEIN 1 PRECURSOR. |
| 773 | CULLIN-5. |
| 774 | CYTOCHROME P450 11A1, MITOCHONDRIAL PRECURSOR. |
| 775 | CYTOCHROME P450, FAMILY 51. |
| 776 | CYTOPLASMIC FMR1-INTERACTING PROTEIN 2. |
| 777 | D11WSU47E PROTEIN. |
| 778 | DDHD DOMAIN CONTAINING 1 ISOFORM 3. |
| 779 | DEATH EFFECTOR DOMAIN-CONTAINING PROTEIN. |
| 780 | DESTRIN. |
| 781 | DIACYLGlycerol KINASE KAPPA. |
| 782 | DNA HELICASE-LIKE PROTEIN. |
| 783 | DUAL SPECIFICITY PROTEIN PHOSPHATASE 10. |
| 784 | E3 UBQUITIN-PROTEIN LIGASE BRE1B. |
| 785 | E3 UBQUITIN-PROTEIN LIGASE SIAH2. |
| 786 | EF-HAND DOMAIN-CONTAINING FAMILY MEMBER A1. |
| 787 | ELECTRON TRANSFER FLAVOPROTEIN SUBUNIT BETA. |
| 788 | ELECTRON TRANSFER FLAVOPROTEIN-UBIQUINONE OXIDOREDUCTASE, MITOCHONDRIAL PRECURSOR. |
| 789 | ELONGATION FACTOR 1-ALPHA 1. |
| 790 | ELONGATION FACTOR 1-BETA. |
| 791 | ELONGATION FACTOR 2. |
| 792 | ELONGATION FACTOR RNA POLYMERASE II 2. |
| 793 | ENVELOPE PROTEIN. |
| 794 | EPIDERMAL GROWTH FACTOR RECEPTOR SUBSTRATE 15. |
| 795 | ES CELLS CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:C330003B14 PRODUCT:HYPOTHETICAL HOMEobox DOMAIN CONTAINING PROTEIN, FULL INSERT SEQUENCE. |
| 796 | EXPORTIN-2. |
| 797 | EXPRESSED SEQUENCE AA409316. |
| 798 | EXTRACELLULAR MATRIX PROTEIN 2 PRECURSOR. |
| 799 | FASCIN HOMOLOG 2, ACTIN-BUNDLING PROTEIN, RETINAL. |
| 800 | FORKHEAD-ASSOCIATED (FHA) PHOSPHOPEPTIDE BINDING DOMAIN 1. |
| 801 | FRUCTOSE-BISPHOSPHATE ALDOLASE A. |
| 802 | GLUTAMINASE LIVER ISOFORM, MITOCHONDRIAL PRECURSOR. |
| 803 | GLUTATHIONE S-TRANSFERASE MU 1. |
| 804 | GRM2 PROTEIN. |
Appendix A

805  GTP-BINDING NUCLEAR PROTEIN RAN, TESTIS-SPECIFIC ISOFORM.

806  GUANINE NUCLEOTIDE EXCHANGE FACTOR DBS.
807  H-2 CLASS I HISTOCOMPATIBILITY ANTIGEN, D-D ALPHA CHAIN PRECURSOR.
808  HDGFRP2 PROTEIN.
809  HEAT SHOCK 70 KDA PROTEIN 4.
810  HEAT SHOCK COGNATE 71 KDA PROTEIN.
811  HEAT SHOCK PROTEIN 84B.
812  HECT DOMAIN CONTAINING 1.
813  HEDGEHOG-INTERACTING PROTEIN PRECURSOR.
814  HEMOGLOBIN SUBUNIT BETA-1.
815  HEMOGLOBIN SUBUNIT BETA-1.
816  HEMOGLOBIN SUBUNIT BETA-2.
817  HEMOGLOBIN SUBUNIT BETA-2.
818  HIGH MOBILITY GROUP PROTEIN B1.
819  HISTAMINE N-METHYLTRANSFERASE.
820  HISTONE H3.2.
821  HIV-1 INDUCED PROTEIN HIN-1.
822  HOMEBOX PROTEIN HOX-A6.
823  HOMEBOX PROTEIN NKX-2.2.
824  HYPOTHETICAL PROTEIN ISOFORM 1.
825  HYPOTHETICAL PROTEIN LOC320226.
826  HYPOTHETICAL PROTEIN LOC381522.
827  HYPOTHETICAL PROTEIN LOC75690.
828  HYPOTHETICAL PROTEIN LOC77011.
829  HYPOTHETICAL PROTEIN.
830  IMMUNOGLOBULIN SUPERFAMILY MEMBER 10 PRECURSOR.
831  IN VITRO FERTILIZED EGGS CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:7420422L18 PRODUCT:HYPOTHETICAL PROTEIN, FULL INSERT SEQUENCE.
832  INSULIN RECEPTOR SUBSTRATE-3 (FRAGMENT).
833  INTEGRAL MEMBRANE PROTEIN 2C.
834  INTEGRIN ALPHA-L PRECURSOR.
835  INTEGRIN ALPHA-X PRECURSOR.
836  INTEGRIN BETA-1 PRECURSOR.
837  INTRAFLAGELLAR TRANSPORT 80 HOMOLOG.
838  IQ MOTIF CONTAINING GTPASE ACTIVATING PROTEIN 3.
839  IRON-RESPONSIVE ELEMENT-BINDING PROTEIN 2.
840  ISLET CELL AUTOANTIGEN 1-LIKE PROTEIN.
841  ISOFORM 1 OF 60 KDA HEAT SHOCK PROTEIN, MITOCHONDRIAL PRECURSOR.
842  ISOFORM 1 OF 60 KDA HEAT SHOCK PROTEIN, MITOCHONDRIAL PRECURSOR.
843  ISOFORM 1 OF ALDO-KETO REDUCTASE FAMILY 1 MEMBER C18.
844  ISOFORM 1 OF ALDO-KETO REDUCTASE FAMILY 1 MEMBER C18.
845  ISOFORM 1 OF B-CELL CLL/LYMPHOMA 7 PROTEIN FAMILY MEMBER B.
846  ISOFORM 1 OF CALCIUM/CALMODULIN-DEPENDENT PROTEIN KINASE KINASE 2.
847  ISOFORM 1 OF CILIARY DYNEIN HEAVY CHAIN 8.
Appendix A

848 ISOFORM 1 OF CORE HISTONE MACRO-H2A.1.
849 ISOFORM 1 OF DESMOGLEIN-3 PRECURSOR.
850 ISOFORM 1 OF EPOXIDE HYDROLASE 2.
851 ISOFORM 1 OF EUKARYOTIC TRANSLATION INITIATION FACTOR 2-ALPHA KINASE 4.
852 ISOFORM 1 OF FATTY ACYL-COA REDUCTASE 1.
853 ISOFORM 1 OF FORKHEAD BOX PROTEIN J3.
854 ISOFORM 1 OF GPI MANNOSYLTRANSFERASE 2.
855 ISOFORM 1 OF HETEROGENEOUS NUCLEO Ribonucleoprotein Q.
856 ISOFORM 1 OF HISTONE-LYSINE N-METHYLTRANSFERASE NSD3.
857 ISOFORM 1 OF INVERSIN.
858 ISOFORM 1 OF KINETOCHORE PROTEIN NUF2.
859 ISOFORM 1 OF MACROPHAGE ERYTHROBLAST ATTACHER.
860 ISOFORM 1 OF MYOSIN-11.
861 ISOFORM 1 OF N-ACETYL-BETA-GLUCOSAMINYL-GLYCOPROTEIN 4-BETA-N-ACETYLGLACTOSAMINYLTRANSFERASE 1.
862 ISOFORM 1 OF NADPH OXIDASE ORGANIZER 1.
863 ISOFORM 1 OF PARKIN.
864 ISOFORM 1 OF PHD FINGER PROTEIN 10.
865 ISOFORM 1 OF PHOSPHORYLASE B KINASE REGULATORY SUBUNIT ALPHA, SKELETAL MUSCLE ISOFORM.
866 ISOFORM 1 OF POLY(ADP-RIBOSE) GLYCOHYDROLASE.
867 ISOFORM 1 OF POLY(RC)-BINDING PROTEIN 2.
868 ISOFORM 1 OF PROTEIN SET.
869 ISOFORM 1 OF PUTATIVE GTP-BINDING PROTEIN 9.
870 ISOFORM 1 OF RAB GDP DISSOCIATION INHIBITOR BETA.
871 ISOFORM 1 OF RHO GTPASE-ACTIVATING PROTEIN RICH2.
872 ISOFORM 1 OF RHO/RAC GUANINE NUCLEOTIDE EXCHANGE FACTOR 2.
873 ISOFORM 1 OF RNA-BINDING PROTEIN MUSASHI HOMOLOG 1.
874 ISOFORM 1 OF ROOTLETIN.
875 ISOFORM 1 OF SERINE/THREONINE-PROTEIN KINASE MRCK ALPHA.
876 ISOFORM 1 OF SEROLOGICALLY DEFINED COLON CANCER ANTIGEN 1.
877 ISOFORM 1 OF SH2 DOMAIN-CONTAINING PROTEIN 3C.
878 ISOFORM 1 OF SH3 AND MULTIPLE ANKYRIN REPEAT DOMAINS PROTEIN 2.
879 ISOFORM 1 OF SH3 AND PX DOMAIN-CONTAINING PROTEIN 2A.
880 ISOFORM 1 OF SOLUTE CARRIER FAMILY 12 MEMBER 6.
881 ISOFORM 1 OF SYNAPTOTAGMIN-8.
882 ISOFORM 1 OF TYROSINE-PROTEIN KINASE TEC.
883 ISOFORM 1 OF UNCHARACTERIZED PROTEIN C6orf190 Homolog.
884 ISOFORM 1 OF UNCHARACTERIZED PROTEIN KIAA0776.
885 ISOFORM 1 OF UNCHARACTERIZED PROTEIN KIAA1267.
886 ISOFORM 1 OF ZINC FINGER PROTEIN 62.
887 ISOFORM 1 OF ZINC FINGER UBR1-TYPE PROTEIN 1.
ISOFORM 2 OF 6-PHOSPHOFRUCTO-2-KINASE/FRUCTOSE-2,6-BIPHOSPHATASE 1.

ISOFORM 2 OF ALPHA-TAXILIN.

ISOFORM 2 OF BCL-2-ASSOCIATED TRANSCRIPTION FACTOR 1.

ISOFORM 2 OF CLEAVAGE STIMULATION FACTOR 64 KDA SUBUNIT.

ISOFORM 2 OF COLLAGEN ALPHA-1(II) CHAIN PRECURSOR.

ISOFORM 2 OF DUAL SPECIFICITY MITOGEN-ACTIVATED PROTEIN KINASE KINASE 3.

ISOFORM 2 OF GLUCOCORTICOID RECEPTOR.

ISOFORM 2 OF JMJC DOMAIN-CONTAINING HISTONE DEMETHYLATION PROTEIN 3A.

ISOFORM 2 OF LATENT-TRANSFORMING GROWTH FACTOR BETA-BINDING PROTEIN 4 PRECURSOR.

ISOFORM 2 OF LEUCINE-RICH REPEAT-CONTAINING PROTEIN 49.

ISOFORM 2 OF LIN-9 HOMOLOG.

ISOFORM 2 OF MONOGLYCERIDE LIPASE.

ISOFORM 2 OF MYELIN TRANSCRIPTION FACTOR 1-LIKE PROTEIN.


ISOFORM 2 OF PROBABLE E3 UBQUITIN-PROTEIN LIGASE HERC2.

ISOFORM 2 OF PROBABLE E3 UBQUITIN-PROTEIN LIGASE MGRN1.

ISOFORM 2 OF PROTEIN SMG5.

ISOFORM 2 OF PROTEIN YIPF4.

ISOFORM 2 OF RECEPTOR-TYPE TYROSINE-PROTEIN PHOSPHATASE T PRECURSOR.

ISOFORM 2 OF SERINE/THREONINE-PROTEIN PHOSPHATASE 2B CATALYTIC SUBUNIT BETA ISOFORM.

ISOFORM 2 OF SWI/SNF-RELATED MATRIX-ASSOCIATED ACTIN-DEPENDENT REGULATOR OF CHROMATIN SUBFAMILY A CONTAINING DEAD/H BOX 1.

ISOFORM 2 OF TRANSCRIPTION FACTOR 4.

ISOFORM 2 OF TRIPARTITE MOTIF-CONTAINING PROTEIN 9.

ISOFORM 2 OF TUMOR SUPPRESSOR P53-BINDING PROTEIN 1.

ISOFORM 2 OF UBQUITIN CARBOXYL-TERMINAL HYDROLASE 34.

ISOFORM 3 OF HISTONE-LYSINE N-METHYLTRANSFERASE SUV420H1.

ISOFORM 3 OF SCAVENGER RECEPTOR CLASS A MEMBER 5.

ISOFORM 3 OF SPICING FACTOR, ARGinine/SERine-RICH 16.

ISOFORM 3 OF UNCHARACTERIZED PROTEIN KIAA0350.

ISOFORM 4 OF 2-OXOGLUTARATE DEHYDROGENASE E1 COMPONENT, MITOCHONDRIAL PRECURSOR.

ISOFORM 5 OF SORBIN AND SH3 DOMAIN-CONTAINING PROTEIN 1.

ISOFORM 5G OF PERIPHERIN.

ISOFORM A OF TUBERIN.

ISOFORM CBF2 OF CCAAT/ENHANCER-BINDING PROTEIN ZETA.

ISOFORM GLT-1A OF EXCITATORY AMINO ACID TRANSPORTER 2.

ISOFORM KV3.3B OF POTASSIUM VOLTAGE-GATED CHANNEL SUBFAMILY C MEMBER 3.

ISOFORM LONG OF COMPLEMENT C3 PRECURSOR.

ISOFORM LONG OF EXTRACELLULAR MATRIX PROTEIN 1 PRECURSOR.

ISOFORM LONG OF SERINE/THREONINE-PROTEIN KINASE PCTAIRE-1.

ISOFORM LONG OF TRANSCRIPTION FACTOR COE3.

ISOFORM PLEC-1I OF PLECTIN-1.

ISOFORM PL-VDAC1 OF VOLTAGE-DEPENDENT ANION-SELECTIVE CHANNEL PROTEIN 1.

ISOFORM SERCA2B OF SARCOPLASMIC/ENDOPLASMIC RETICULUM CALCIUM ATPASE 2.

ISOFORM SHORT OF G2/MITOTIC-SPECIFIC CYCLIN-F.

ISOFORM SHORT OF TRANSCRIPTIONAL ENHANCER FACTOR TEF-3.

ISOFORM SERCA2B OF SARCOPLASMIC/ENDOPLASMIC RETICULUM CALCIUM ATPASE 2.

ISOFORM SHORT OF G2/MITOTIC-SPECIFIC CYCLIN-F.

ISOFORM SHORT OF TRANSCRIPTIONAL ENHANCER FACTOR TEF-3.

ISOFORM SERCA2B OF SARCOPLASMIC/ENDOPLASMIC RETICULUM CALCIUM ATPASE 2.
MITOCHONDRIAL 2-OXOGLUTARATE/MALATE CARRIER PROTEIN.

MITOGEN-ACTIVATED PROTEIN KINASE 3.
MITOGEN-ACTIVATED PROTEIN KINASE 6.
MITOGEN-ACTIVATED PROTEIN KINASE KINASE 2.
MKIAA0988 PROTEIN (FRAGMENT).
MKIAA1030 PROTEIN.
MKIAA1040 PROTEIN (FRAGMENT).
MKL/MYOCARDIN-LIKE PROTEIN 2.
MOESIN.
MYB-BINDING PROTEIN 1A.
MYELOID BACTENECIN.
MYELOID/LYMPHOID OR MIXED-LINEAGE LEUKEMIA 3 ISOFORM 4.
MYOSIN-9.
MYOSIN-XV.
NACHT-, LRR-, AND PYD-CONTAINING PROTEIN 1 PARALOG B.
NAD-DEPENDENT MALIC ENZYME, MITOCHONDRIAL PRECURSOR.
NADP-DEPENDENT MALIC ENZYME.
NADPH OXIDASE 1 ALPHA.
NIT PROTEIN 2.
NOD-DERIVED CD11C +VE DENDRITIC CELLS CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:F630026O15 PRODUCT:RING FINGER PROTEIN 150, FULL INSERT SEQUENCE (FRAGMENT).
NOD-DERIVED CD11C +VE DENDRITIC CELLS CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:F630109N12 PRODUCT:PEROXISOMAL CA-DEPENDENT SOLUTE CARRIER HOMOLOG, FULL INSERT SEQUENCE.
NOD-DERIVED CD11C +VE DENDRITIC CELLS CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:F630226G03 PRODUCT:HYPOTHETICAL PROTEIN, FULL INSERT SEQUENCE.
NOTCH GENE HOMOLOG 4.
NOVEL PROTEIN CONTAINING HEAT REPEATS.
NOVEL PROTEIN SIMILAR TO CHROMODOMAIN HELICASE DNA BINDING PROTEIN 7.
NOVEL PROTEIN SIMILAR TO PRAME PROTEINS.
NOVEL PROTEIN.
NOVEL PROTEIN.
NOVEL PROTEIN.
NOVEL ZINC FINGER PROTEIN.
NUCLEAR FACTOR OF KAPPA LIGHT POLYPEPTIDE GENE ENHANCER IN B-CELLS INHIBITOR-LIKE 2.
NUCLEAR RECEPTOR SUBFAMILY 4, GROUP A, MEMBER 2.
NUCLEOLAR PROTEIN 10.
NUCLEOPHOSMIN.
NUCLEOPORIN 188.
NUCLEOPORIN NUP53.
NUCLEOSIDE DIPHOSPHATE KINASE B.
OLFACTORY RECEPTOR MOR162-7.
Appendix A

997  Olfactory receptor MOR223-2.
998  Olfactory receptor OLFR169.
999  Olfactory receptor OLFR209.
1000 Olfactory receptor OLFR814.
1001 OL-protecadherin isoform.
1002 Osteoclast-like cell cDNA, Riken full-length enriched library, clone:i420031B14 product: weakly similar to UDP-N-acetylglucosamine pyrophosphorylase 1, full insert sequence.
1003 Osteoclast-like cell cDNA, Riken full-length enriched library, clone:i420039N16 product: heterogeneous nuclear ribonucleoprotein U, full insert sequence.
1004 PCNA-associated factor.
1005 PDZ domain containing 3 isoform 2.
1006 Pejvakin.
1007 Pendrin.
1008 Peptidylprolyl isomerase B.
1009 Peroxiredoxin-1.
1010 Peroxiredoxin-4.
1011 Phosphatidylethanolamine-binding protein 1.
1012 Phosphatidylinositol 3-kinase gamma isoform.
1013 Phosphoglycerate mutase 1.
1014 Phospholipase C delta-1.
1015 PIH1D1 protein.
1016 Piwi-like protein 2.
1017 Pleckstrin homology domain containing, family G (with rhoGEF domain) member 6.
1018 Predicted.
1019 Prefoldin subunit 2.
1020 Pre-mRNA cleavage complex II protein PCF11.
1021 Pre-mRNA-processing-splicing factor 8.
1022 Probable phospholipid-transporting ATPase IH.
1023 Progesterone-induced blocking factor 1 isoform B.
1024 Proteasome activator complex subunit 1.
1025 Proteasome subunit beta type 8 precursor.
1026 Protein disulfide-isomerase A3 precursor.
1027 Protein disulfide-isomerase A3 precursor.
1028 Protein kinase C binding protein 1.
1029 Protein phosphatase 1 regulatory subunit 3A.
1030 Protein S100-A10.
1031 Protein S100-A4.
1032 Protein.
1033 Protecadherin gamma A1.
1034 Proton myo-inositol cotransporter.
1035 Putative homeodomain transcription factor 2.
1036 Putative polycomb group protein ASXL1.
1037 RALGPS2 protein.
1038 Ras P21 protein activator 4 isoform 2.
1039 RB-associated Krab repressor.
1040 Retinal dehydrogenase 1.
1041 RFC1 protein.
1042 Ribosomal protein S27a.
1043 Riken cDNA 3110023B02 gene.
Appendix A

1044 RIKEN CDNA A630038E17 GENE.
1045 RING FINGER PROTEIN 151.
1046 RING FINGER PROTEIN 168.
1047 RNA-BINDING MOTIF PROTEIN, X-LINKED 2.
1048 RPS16 PROTEIN.
1049 RST.
1050 SECRETOGRAIN-3 PRECURSOR.
1051 SEMAPHORIN-4G PRECURSOR.
1052 SERINE/THREONINE-PROTEIN KINASE LATS1.
1053 SERINE/THREONINE-PROTEIN KINASE PAK 2.
1054 SERINE/THREONINE-PROTEIN KINASE TAO1.
1055 SERINE-PROTEIN KINASE ATM.
1056 SEROTRANSFERRIN PRECURSOR.
1057 SERUM ALBUMIN PRECURSOR.
1058 SIMILAR TO 40S RIBOSOMAL PROTEIN S6.
1059 SIMILAR TO 60S RIBOSOMAL PROTEIN L17.
1060 SIMILAR TO 60S RIBOSOMAL PROTEIN L18.
1061 SIMILAR TO 60S RIBOSOMAL PROTEIN L23A.
1062 SIMILAR TO ABSENT IN MELANOMA 1 PROTEIN.
1063 SIMILAR TO ACETYL-COENZYME A CARBOXYLASE BETA.
1064 SIMILAR TO ADAMTS-LIKE 3.
1065 SIMILAR TO ALPHA-1-ANTIPROTEINASE PRECURSOR.
1066 SIMILAR TO ATP SYNTHASE, H+ TRANSPORTING, MITOCHONDRIAL F0 COMPLEX, SUBUNIT D ISOFORM 1.
1067 SIMILAR TO BREFELDIN A-INHIBITED GUANINE NUCLEOTIDE-EXCHANGE PROTEIN 1 ISOFORM 5.
1068 SIMILAR TO CADHERIN-18 PRECURSOR.
1069 SIMILAR TO CG10889-PA.
1070 SIMILAR TO CG31790-PA.
1071 SIMILAR TO DYNEIN, AXONEMAL, HEAVY POLYPEPTIDE 9 ISOFORM 2 ISOFORM 5.
1072 SIMILAR TO FAS APOPTOTIC INHIBITORY MOLECULE 1.
1073 SIMILAR TO FIBROUS SHEATH INTERACTING PROTEIN 2.
1074 SIMILAR TO FIDGETIN-LIKE 1.
1075 SIMILAR TO FLJ44048 PROTEIN.
1076 SIMILAR TO GLE1 RNA EXPORT MEDIATOR-LIKE (YEAST.
1077 SIMILAR TO H3 HISTONE, FAMILY 3B.
1079 SIMILAR TO IG HEAVY CHAIN V REGION PJ14 PRECURSOR.
1080 SIMILAR TO KELCH-LIKE 9.
1081 SIMILAR TO LAMIN-B2.
1082 SIMILAR TO LOW DENSITY LIPOPROTEIN RECEPTOR-RELATED PROTEIN 1.
1083 SIMILAR TO MELANOMA ANTIGEN, FAMILY B, 1.
1084 SIMILAR TO MLCK PROTEIN.
1085 SIMILAR TO NANCE-HORAN SYNDROME PROTEIN.
1086 SIMILAR TO NEBULIN.
1087 SIMILAR TO NEUREXIN 2.
1088 SIMILAR TO ODD OZ/TEN-M HOMOLOG 1.
1089 SIMILAR TO P2Y PURINOCEPTOR 2.
SIMILAR TO PHOSPHODIESTERASE 3B, CGMP-INHIBITED.
SIMILAR TO PR DOMAIN CONTAINING 14.
SIMILAR TO PROBABLE ATP-DEPENDENT RNA HELICASE DDX10.
SIMILAR TO PROTEIN C6ORF136.
SIMILAR TO PROTEIN KIAA0934.
SIMILAR TO RAS AND EF HAND DOMAIN CONTAINING.
SIMILAR TO RHO GTPASE ACTIVATING PROTEIN 21 ISOFORM 2.
SIMILAR TO RIBOSOMAL PROTEIN L9.
SIMILAR TO RIBOSOMAL PROTEIN S12.
SIMILAR TO SEC14-LIKE 1 ISOFORM 2.
SIMILAR TO SET BINDING FACTOR 1 ISOFORM A ISOFORM 16.
SIMILAR TO SET BINDING FACTOR 1 ISOFORM A ISOFORM 16.
SIMILAR TO SPECTRIN BETA CHAIN, BRAIN 4.
SIMILAR TO STRETCHIN-MLCK CG18255-PA, ISOFORM A.
SIMILAR TO TBP-ASSOCIATED FACTOR 4.
SIMILAR TO THIOREDOXIN DOMAIN-CONTAINING PROTEIN 6.
SIMILAR TO U1 SMALL NUCLEAR RIBONUCLEOPROTEIN C.
SIMILAR TO UBIQUITIN A-52 RESIDUE RIBOSOMAL PROTEIN FUSION PRODUCT 1.
SIMILAR TO UBIQUITIN SPECIFIC PROTEASE 31 ISOFORM 2.
SIMILAR TO VACUOLAR PROTEIN SORTING 13D ISOFORM 2 ISOFORM 5.
SIMILAR TO ZINC FINGER PROTEIN 217.
SIMILAR TO ZINC FINGER PROTEIN 267.
SIMILAR TO ZINC FINGER PROTEIN 292 ISOFORM 4.
SIMILAR TO ZINC FINGER PROTEIN 709.
SIMILAR TO ZINC FINGER PROTEIN RLF.
SIMILAR TO ZINC FINGER PROTEIN ZFP ISOFORM 1.
SLIT HOMOLOG 1 PROTEIN PRECURSOR.
SPECTRIN BETA 4.
SPERM FLAGELLAR PROTEIN 1.
SPHINGOSINE KINASE 1.
SPlicing FACTOR U2AF 35 KDA SUBUNIT.
STERILE ALPHA MOTIF DOMAIN-CONTAINING PROTEIN 4A.
SUPPRESSOR OF CYTOKINE SIGNALING 3.
SYNAPTIC NUCLEAR ENVELOPE 1 ISOFORM 3.
SYNAPTOBREVIN HOMOLOG YKT6.
SYNAPTOTAGMIN-5.
SYNTENIN-1.
TC10/CDC42 GTPASE-ACTIVATING PROTEIN.
T-COMPLEX PROTEIN 1 SUBUNIT GAMMA.
TELOMERASE-BINDING PROTEIN EST1A.
TENSIN 1.
THIOREDOXIN.
THIOREDOXIN-DEPENDENT PEROXIDE REDUCTASE, MITOCHONDRIAL PRECURSOR.
TIB-55 BB88 CDNA, RIKEN FULL-LENGTH ENRICHED LIBRARY, CLONE:I730061N04 PRODUCT:NEUROLYSIN (METALLOPEPTIDASE M3 FAMILY), FULL INSERT SEQUENCE.
TRANSCOBALAMIN-2 PRECURSOR.
TRANSCRIPTION ELONGATION FACTOR B POLYPEPTIDE 1.
TRANSGLSELIN.
TRANSIENT RECEPTOR POTENTIAL CATION CHANNEL SUBFAMILY C MEMBER 2 LONG CHANNEL SPLICE FORM.
TRANSITIONAL ENDOPLASMIC RETICULUM ATPASE.
TRANSMEMBRANE PROTEIN 132D PRECURSOR.
TRANSSTHYRETIN PRECURSOR.
TRIPARTITE MOTIF-CONTAINING PROTEIN 32.
TRIPARTITE MOTIF-CONTAINING PROTEIN 69.
TROPOMYSIN ALPHA-4 CHAIN.
TRYSIN 4.
TRYSINOGEN 16.
TRYSINOGEN 16.
TUBULIN ALPHA-1 CHAIN.
TUBULIN FOLDING COFACTOR B.
TUBULIN, GAMMA COMPLEX ASSOCIATED PROTEIN 5.
TYROSINE-PROTEIN KINASE RECEPTOR TIE-1 PRECURSOR.
U6 SNRNA-ASSOCIATED SM-LIKE PROTEIN LSM7.
UBIQUITIN LIGASE FWD1.
UBIQUITOUSLY TRANSCRIBED Y CHROMOSOME TETRATRICOPEPTIDE REPEAT PROTEIN.
UDP-GLCNAC:BETAGAL BETA-1,3-N-ACETYLGLUCOSAMINYLT XFERASE 4.
UNCHARACTERIZED PROTEIN C1ORF51 HOMOLOG.
VIMENTIN.
VOMERONASAL RECEPTOR V1RI9.
WD REPEAT PROTEIN 24.
ZFP-57.
ZINC FINGER CCCH DOMAIN-CONTAINING PROTEIN 10.
ZINC FINGER PROTEIN 407.
ZINC FINGER RNA BINDING PROTEIN.
### Appendix B: Isolated Oocyte Proteomic Identifications

#### B.1 2D LC-MS/MS analysis of Isolated Whole Oocytes

**Table B1. Mascot Protein identities obtained for 2D LC-MS/MS analysis of whole oocyte peptides**

<table>
<thead>
<tr>
<th>No.</th>
<th>GENE</th>
<th>DESCRIPTION</th>
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<tbody>
<tr>
<td>1</td>
<td>1433E</td>
<td>14-3-3 protein epsilon (14-3-3E)</td>
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<td>2</td>
<td>1433G</td>
<td>14-3-3 protein gamma</td>
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<td>3</td>
<td>1433T</td>
<td>14-3-3 protein theta (14-3-3 protein tau)</td>
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<td>4</td>
<td>1433Z</td>
<td>14-3-3 protein zeta/delta (Protein kinase C inhibitor protein 1) (KCIP-1) (SEZ-2)</td>
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<td>5</td>
<td>4ET</td>
<td>Eukaryotic translation initiation factor 4E transporter (eIF4E transporter) (4E-T) (Eukaryotic translation initiation factor 4E nuclear import factor 1)</td>
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<td>6</td>
<td>6PGL</td>
<td>6-phosphogluconolactonase (EC 3.1.1.31) (6PGL)</td>
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<tr>
<td>7</td>
<td>AASS</td>
<td>Alpha-aminoadipic semialdehyde synthase, mitochondrial precursor (LKR/SDH) [Includes: Lysine ketoglutarate reductase (EC 1.5.1.8) (LOR) (LKR); Saccharopine dehydrogenase (EC 1.5.1.9) (SDHI)]</td>
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<tr>
<td>8</td>
<td>ACOT8</td>
<td>Acyl-coenzyme A thiosterase 8 (EC 3.1.2.27) (Choloyl-coenzyme A thiosterase) (Acyl-CoA thiosterase 8) (Peroxisomal acyl-coenzyme A thioster hydrolase 1) (PTE-1) (Peroxisomal long-chain acyl-coa thiosterase 1) (Peroxisomal acyl-coa thiesterase)</td>
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<td>9</td>
<td>ACOX3</td>
<td>Acyl-coenzyme A oxidase 3, peroxisomal (EC 1.3.3.6) (Pristanoyl-CoA oxidase) (Branched-chain acyl-CoA oxidase) (BRCACox)</td>
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<td>10</td>
<td>ACTB</td>
<td>Actin, cytoplasmic 1 (Beta-actin)</td>
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<td>11</td>
<td>ACTN4</td>
<td>Alpha-actinin-4 (Non-muscle alpha-actinin 4) (F-actin cross-linking protein)</td>
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<td>12</td>
<td>ADT2</td>
<td>ADP/ATP translocase 2 (Adenine nucleotide translocator 2) (ANT 2) (ADP,ATP carrier protein 2) (Solute carrier family 25 member 5)</td>
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<td>13</td>
<td>AFAD</td>
<td>Afdalin (Protein Af-6) (Fragment)</td>
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<td>AFG32</td>
<td>AFG3-like protein 2 (EC 3.4.24.-)</td>
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<td>AIFM1</td>
<td>Apoptosis-inducing factor 1, mitochondrial precursor (EC 1.-.-.-) (Programmed cell death protein 8)</td>
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<td>16</td>
<td>AIP</td>
<td>AH receptor-interacting protein (AIP) (Aryl-hydrocarbon receptor-interacting protein)</td>
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<td>17</td>
<td>AK1A1</td>
<td>Alcohol dehydrogenase [NADP+] (EC 1.1.1.2) (Aldehyde reductase) (Aldo-keto reductase family 1 member A1)</td>
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<td>18</td>
<td>AL7A1</td>
<td>Alpha-aminoadipic semialdehyde dehydrogenase (EC 1.2.1.31) (Alpha-AASA dehydrogenase) (Delta1-piperideine-6-carboxylate dehydrogenase) (P6c dehydrogenase) (Aldehyde dehydrogenase family 7 member A1) (Antiquitin-1)</td>
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<td>19</td>
<td>AL9A1</td>
<td>4-trimethylaminobutyraldehyde dehydrogenase (EC 1.2.1.47) (TMABADH) (Aldehyde dehydrogenase 9A1) (EC 1.2.1.3)</td>
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<td>ALBU</td>
<td>Serum albumin precursor</td>
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<td>ALDH2</td>
<td>Aldehyde dehydrogenase, mitochondrial precursor (EC 1.2.1.3) (ALDH class 2) (AHD-M1) (ALDH1) (ALDH-E2)</td>
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<td>22</td>
<td>ALDOA</td>
<td>Fructose-bisphosphate aldolase A (EC 4.1.2.13) (Muscle-type aldolase) (Aldolase 1)</td>
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<td>23</td>
<td>ALDR</td>
<td>Aldose reductase (EC 1.1.1.21) (AR) (Aldehyde reductase)</td>
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<td>24</td>
<td>ANXA2</td>
<td>Annexin A2 (Annexin II) (Lipocortin II) (Calpain I heavy chain) (Chromobindin-8) (p36) (Protein I) (Placental anticoagulant protein IV) (PAP-IV)</td>
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<td>ANXA7</td>
<td>Annexin A7 (Annexin VII) (Synexin)</td>
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<td>26</td>
<td>AP1B1</td>
<td>AP-1 complex subunit beta-1 (Adapter-related protein complex 1 beta-1 subunit) (Beta-adapatin 1) (Adaptor protein complex AP-1 beta-1 subunit) (GolgI adaptor HA1/AP1 adapatin beta subunit) (Clathrin assembly protein complex 1 beta large chain) - Mus m</td>
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<td>27</td>
<td>APOO</td>
<td>Apolipoprotein O (Protein FAM121B)</td>
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<td>28</td>
<td>ARF1</td>
<td>ADP-ribosylation factor 1</td>
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Appendix B

<table>
<thead>
<tr>
<th>Number</th>
<th>Gene Name</th>
<th>Description</th>
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<tr>
<td>29</td>
<td>ARGI2</td>
<td>Arginase-2, mitochondrial precursor (EC 3.5.3.1) (Arginase II) (Non-hepatic arginase) (Kidney-type arginase)</td>
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<td>ARL3</td>
<td>ADP-ribosylation factor-like protein 3</td>
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<td>31</td>
<td>ASPC1</td>
<td>Tether containing UBX domain for GLUT4 (Alveolar soft part sarcoma chromosome region candidate 1 homolog)</td>
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<td>ASTL</td>
<td>Astacin-like metalloendopeptidase precursor (EC 3.4.-.-) (Oocyte astacin) (Ovastacin)</td>
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<td>33</td>
<td>AT1A1</td>
<td>Sodium/potassium-transporting ATPase alpha-1 chain precursor (EC 3.6.3.9) (Sodium pump 1) (Na(+)/K(+) ATPase 1)</td>
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<td>34</td>
<td>AT2A2</td>
<td>Sarcoplasmic/endoplasmic reticulum calcium ATPase 2 (EC 3.6.3.8) (Calcium pump 2) (SERCA2) (SR Ca(2+)-ATPase 2) (Calcium-transporting ATPase sarcoplasmic reticulum type, slow twitch skeletal muscle isoform) (Endoplasmic reticulum class 1/2 Ca(2+) ATPase)</td>
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<td>35</td>
<td>ATPA</td>
<td>ATP synthase subunit alpha, mitochondrial precursor (EC 3.6.3.14)</td>
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<td>36</td>
<td>ATPB</td>
<td>ATP synthase subunit beta, mitochondrial precursor (EC 3.6.3.14)</td>
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<td>37</td>
<td>B2L10</td>
<td>Apoptosis regulator Bcl-2 (Bcl-2-like 10 protein) (Bcl2-L10) (Anti-apoptotic protein Boo) (Bcl-2 homolog Diva)</td>
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<td>38</td>
<td>BLK</td>
<td>Tyrosine-protein kinase BLK (EC 2.7.10.2) (B lymphocyte kinase) (p55-BLK)</td>
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<td>39</td>
<td>BUB1B</td>
<td>Mitotic checkpoint serine/threonine-protein kinase BUB1 beta (EC 2.7.11.1) (MAD3/BUB1-related protein kinase) (Mitotic checkpoint kinase MAD3L)</td>
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<td>Putative C10 protein (B-cell receptor-associated protein 37)</td>
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<td>Calreticulin precursor (CRP55) (Calregulin) (HACBP) (ERp60)</td>
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<td>Cullin-associated NEDD8-dissociated protein 1 (Cullin-associated and neddylation-dissociated protein 1) (p120 CAND1)</td>
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<td>CATD</td>
<td>Cathepsin D precursor (EC 3.4.23.5)</td>
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<td>F-actin capping protein subunit alpha-1 (CapZ alpha-1)</td>
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<td>Uncharacterized protein C3orf29 homolog precursor</td>
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<td>CDC37</td>
<td>Hsp90 co-chaperone Cdc37 (Hsp90 chaperone protein kinase-targeting subunit) (p50Cdc37)</td>
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<td>CG024</td>
<td>Uncharacterized protein C7orf24 homolog</td>
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<td>CH10</td>
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<td>Charged multivesicular body protein 4b (Chromatin-modifying protein 4b) (CHMP4b)</td>
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<td>Citrate synthase, mitochondrial precursor (EC 2.3.3.1)</td>
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<td>CLCF1</td>
<td>Cardiotrophin-like cytokine factor 1 precursor (B cell-stimulating factor 3) (BSF-3) (Novel neurotrophin-1) (NTI-1)</td>
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<td>CLPB</td>
<td>Caseinolytic peptidase B protein homolog (Suppressor of potassium transport defect 3)</td>
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<td>CNN3</td>
<td>Calponin-3 (Calponin, acidic isoform)</td>
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<td>COF2</td>
<td>Coflin-2 (Cofilin, muscle isoform)</td>
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<td>Coatomer subunit beta-2 (Beta-coat protein) (Beta-coat protein) (p102)</td>
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<td>Coatomer subunit epsilon (Epsilon-coat protein) (Epsilon-COP)</td>
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<td>66</td>
<td>CSN3</td>
<td>COP9 signalosome complex subunit 3 (Signalosome subunit 3) (SGN3) (JAB1-containing signalosome subunit 3)</td>
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<td>Cullin-1 (CUL-1)</td>
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<td>Probable ATP-dependent RNA helicase DDX6 (EC 3.6.1.-) (DEAD box protein 6) (ATP-dependent RNA helicase p54) (Oncogene RCK homolog)</td>
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<td>Estradiol 17-beta-dehydrogenase 8 (EC 1.1.1.62) (17-beta-HSD 8) (17-beta-hydroxysteroid dehydrogenase 8) (Protein Ke6) (Ke-6)</td>
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<td>Dihydropteridine reductase (EC 1.5.1.34) (DHPR) (Quinoid dihydropteridine reductase)</td>
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<td>Destrin (Actin-depolymerizing factor) (ADF) (Sid 23)</td>
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<td>DDLB</td>
<td>Dynemin light chain roadblock-type 1 (Dynemin light chain 2A, cytoplasmic)</td>
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<td>DNNH2</td>
<td>DnaJ homolog subfamily A member 2 (mDj3)</td>
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<td>DnaJ homolog subfamily A member 3, mitochondrial precursor (Tumorous imaginal discs protein Tid56 homolog) (DnaJ protein Tid-1) (mTid-1)</td>
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<td>DnaJ homolog subfamily C member 9</td>
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<td>DNA (cytosine-5)-methyltransferase 1 (EC 2.1.1.37) (Dnm1) (DNA methyltransferase MmuI) (DNA MTase MmuI) (MCMT) (M.MmuI) (Met-1)</td>
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<td>DPP3</td>
<td>Dipeptidyl-peptidase 3 (EC 3.4.14.4) (Dipeptidyl-peptidase III) (DPP III) (Dipeptidyldipeptidase III) (Dipeptidyl aminopeptidase III)</td>
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<td>82</td>
<td>DTX2</td>
<td>Protein deltex-2 (Deltex-2) (Deltex2) (mDTX2)</td>
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<td>DHC1</td>
<td>Dynein heavy chain, cytosolic (DYHC) (Cytoplasmic dynein heavy chain 1) (DHC1) (Dynein heavy chain 1, cytoplasmic 1)</td>
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<td>84</td>
<td>DYL2</td>
<td>Dynein light chain 2, cytoplasmic (Dynein light chain LC8-type 2) (8 kDa dynein light chain) (DLC8) (DLC8b)</td>
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<td>Dihydrofolate reductase (EC 1.5.1.3)</td>
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<td>ECM29</td>
<td>Proteasome-associated protein ECM29 homolog (Ecm29)</td>
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<td>EDC4</td>
<td>Enhancer of mRNA-decapping protein 4</td>
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<td>Early endosome antigen 1</td>
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<td>EF1A1</td>
<td>Elongation factor 1-alpha 1 (EF-1-alpha-1) (Elongation factor 1 A-1) (eEF1A-1) (Elongation factor Tu) (EF-Tu)</td>
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<td>Elongation factor 1-delta (EF-1-delta)</td>
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<td>Elongation factor 2 (EF-2)</td>
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<td>EF-hand domain-containing family member A1</td>
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<td>EFTU</td>
<td>Elongation factor Tu, mitochondrial precursor</td>
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<td>ELL2</td>
<td>ELAV-like protein 2 (Hu-antigen B) (HuB) (ELAV-like neuronal protein 1) (Nervous system-specific RNA-binding protein Mel-N1)</td>
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<td>ELOC</td>
<td>Transcription elongation factor B polypeptide 1 (RNA polymerase II transcription factor SII subunit C) (SII p15) (Elongin-C) (EloC) (Elongin 15 kDa subunit) (Stromal membrane-associated protein SMAP1B homolog)</td>
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<td>Alpha-enolase (EC 4.2.1.11) (2-phospho-D-glycerate hydro-lyase) (Non-neural enolase) (NNE) (Enolase 1)</td>
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<td>98</td>
<td>ENPL</td>
<td>Endoplasmin precursor (Heat shock protein 90 kDa beta member 1) (94 kDa glucose-regulated protein) (GRP94) (ERP99) (Polymorphic tumor rejection antigen 1) (Tumor rejection antigen gp96)</td>
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<td>99</td>
<td>ERP29</td>
<td>Endoplasmic reticulum protein ERP29 precursor</td>
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<tr>
<td>100</td>
<td>ETFA</td>
<td>Electron transfer flavoprotein subunit alpha, mitochondrial precursor (Alpha-ETF)</td>
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<tr>
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<tr>
<td>101</td>
<td>ETFB</td>
<td>Electron transfer flavoprotein subunit beta (Beta-ETF)</td>
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<tr>
<td>102</td>
<td>F10A1</td>
<td>Hsc70-interacting protein (Hip) (Protein ST13 homolog) (Protein FAM10A1)</td>
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<td>103</td>
<td>FAM3C</td>
<td>Protein FAM3C precursor</td>
</tr>
<tr>
<td>104</td>
<td>FIP1</td>
<td>Pre-mRNA 3''-end-processing factor FIP1 (FIP1-like 1)</td>
</tr>
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<td>105</td>
<td>FKBP2</td>
<td>FK506-binding protein 2 precursor (EC 5.2.1.8) (Peptidyl-prolyl cis-trans isomerase) (PPlase) (Rotamase) (13 kDa FKBP) (FKBP-13)</td>
</tr>
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<td>106</td>
<td>FLNB</td>
<td>Filamin-B (FLN-B) (Beta-filamin) (Actin-binding-like protein) (ABP-280-like protein)</td>
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<td>107</td>
<td>G3P</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12) (GAPDH)</td>
</tr>
<tr>
<td>108</td>
<td>G6PD1</td>
<td>Glucose-6-phosphate 1-dehydrogenase X (EC 1.1.1.49) (G6PD)</td>
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<td>109</td>
<td>GALM</td>
<td>Aldose 1-epimerase (EC 5.1.3.3) (Galactose mutarotase)</td>
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<td>110</td>
<td>GDIB</td>
<td>Rab GDP dissociation inhibitor beta (Rab GDI beta) (Guanosine diphosphate dissociation inhibitor 2) (GDI-2) (GDI-3)</td>
</tr>
<tr>
<td>111</td>
<td>GDIR</td>
<td>Rho GDP-dissociation inhibitor 1 (Rho GDI 1) (Rho-GDI alpha) (GDI-1)</td>
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<td>112</td>
<td>GDPD1</td>
<td>Glycerophosphodiester phosphodiesterase domain-containing protein 1 (EC 3.1.-) (Glycerophosphodiester phosphodiesterase 4)</td>
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<td>113</td>
<td>GFPT1</td>
<td>Glucosamine--fructose-6-phosphate aminotransferase [isomerizing] 1 (EC 2.6.1.16) (Hexosephosphate aminotransferase 1) (D-fructose-6-phosphate amidotransferase 1) (GFAT 1) (GFAT1)</td>
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<td>114</td>
<td>GLGB</td>
<td>1,4-alpha-glucan branching enzyme (EC 2.4.1.18) (Glycogen branching enzyme) (Brancher enzyme)</td>
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<td>115</td>
<td>GLRX1</td>
<td>Glutaredoxin-1 (Thioltransferase-1) (TTase-1)</td>
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<td>116</td>
<td>GLRX5</td>
<td>Glutaredoxin-related protein 5</td>
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<td>117</td>
<td>GLU2B</td>
<td>Glucosidase 2 subunit beta precursor (Glucosidase II subunit beta) (Protein kinase C substrate, 60.1 kDa protein, heavy chain) (PKCSH) (80K-H protein)</td>
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<td>118</td>
<td>GLYG</td>
<td>Glycogenin-1 (EC 2.4.1.186)</td>
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<td>119</td>
<td>GMDS</td>
<td>GDP-mannose 4.6 dehydratase (EC 4.2.1.47) (GDP-D-mannose dehydratase) (GMD)</td>
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<td>120</td>
<td>GRP75</td>
<td>Stress-70 protein, mitochondrial precursor (75 kDa glucose-regulated protein) (GRP 75) (Peptide-binding protein 74) (PBP74) (P66 MOT) (Mortalin)</td>
</tr>
<tr>
<td>121</td>
<td>GRP78</td>
<td>78 kDa glucose-regulated protein precursor (GRP 78) (Heat shock 70 kDa protein 5) (Immunoglobulin heavy chain-binding protein) (BiP)</td>
</tr>
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<td>122</td>
<td>GUAA</td>
<td>GMP synthase [glutamine-hydrolyzing] (EC 6.3.5.2) (Glutamine amidotransferase) (GMP synthetase)</td>
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<tr>
<td>123</td>
<td>GUAD</td>
<td>Guanine deaminase (EC 3.5.4.3) (Guanase) (Guanine aminase) (Guanine aminohydrolase) (GAH)</td>
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<td>124</td>
<td>GYS1</td>
<td>Glycogen [starch] synthase, muscle (EC 2.4.1.11)</td>
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<td>125</td>
<td>H2A1F</td>
<td>Histone H2A type 1-F</td>
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<td>126</td>
<td>H2B1A</td>
<td>Histone H2B type 1-A (Histone H2B, testis) (Testis-specific histone H2B)</td>
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<td>127</td>
<td>H31</td>
<td>Histone H3.1</td>
</tr>
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<td>128</td>
<td>HINT1</td>
<td>Histidine triad nucleotide-binding protein 1 (Adenosine 5'--monophosphoramidase) (Protein kinase C inhibitor 1) (Protein kinase C-interacting protein 1) (PKCI-1)</td>
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<td>129</td>
<td>HNRL2</td>
<td>Heterogeneous nuclear ribonucleoprotein U-like protein 2 (MLF1-associated nuclear protein)</td>
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<td>130</td>
<td>HNRPK</td>
<td>Heterogeneous nuclear ribonucleoprotein K</td>
</tr>
<tr>
<td>131</td>
<td>HNRPQ</td>
<td>Heterogeneous nuclear ribonucleoprotein Q (hnRNP Q) (hnRNP-Q) (Synaptotagmin-binding, cytoplasmic RNA-interacting protein) (Glycine-and tyrosine-rich RNA-binding protein) (GRY-RBP) (NS1-associated protein 1) (pp68)</td>
</tr>
<tr>
<td>132</td>
<td>HS105</td>
<td>Heat-shock protein 105 kDa (Heat shock-related 100 kDa protein E7I) (HSP-E7I) (Heat shock 110 kDa protein) (42 degrees C-HSP)</td>
</tr>
<tr>
<td>No.</td>
<td>Gene Symbol</td>
<td>Description</td>
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<td>167</td>
<td>MAP4</td>
<td>Microtubule-associated protein 4 (MAP 4)</td>
</tr>
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<td>168</td>
<td>MARH5</td>
<td>E3 ubiquitin-protein ligase MARCH5 (EC 6.3.2.-) (Membrane-associated RING finger protein 5) (Membrane-associated RING-CH protein V) (MARCH-V)</td>
</tr>
<tr>
<td>169</td>
<td>MDHC</td>
<td>Malate dehydrogenase, cytoplasmic (EC 1.1.1.37) (Cytosolic malate dehydrogenase)</td>
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<tr>
<td>170</td>
<td>MDHM</td>
<td>Malate dehydrogenase, mitochondrial precursor (EC 1.1.1.37)</td>
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<td>171</td>
<td>MECR</td>
<td>Trans-2-enoyl-CoA reductase, mitochondrial precursor (EC 1.3.1.38)</td>
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<td>172</td>
<td>MTPN</td>
<td>Myotrophin (Protein V-1) (Granule cell differentiation protein)</td>
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<td>173</td>
<td>MVP</td>
<td>Major vault protein (MVP)</td>
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<tr>
<td>174</td>
<td>MYH11</td>
<td>Myosin-11 (Myosin heavy chain 11) (Myosin heavy chain, smooth muscle isoform) (SMMHC)</td>
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<tr>
<td>175</td>
<td>MYH9</td>
<td>Myosin-9 (Myosin heavy chain 9) (Nonmuscle myosin heavy chain Ila) (NMMHC IIa) (Cellular myosin heavy chain A) (NMMHC-A)</td>
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<td>176</td>
<td>MYL6B</td>
<td>Myosin light polypeptide 6B (Smooth muscle and nonmuscle myosin light chain alkali 6B)</td>
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<tr>
<td>177</td>
<td>NACA</td>
<td>Nascent polypeptide-associated complex subunit alpha (Alpha-NAC) (Alpha-NAC/1.9.2)</td>
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<td>178</td>
<td>NALP5</td>
<td>NACHT, LRR and PYD-containing protein 5 (Maternal antigen that embryos require) (Ooplasm-specific protein 1) (OP1)</td>
</tr>
<tr>
<td>179</td>
<td>NDU2A</td>
<td>NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 2 (EC 1.6.5.3) (EC 1.6.99.3) (NADH-ubiquinone oxidoreductase B8 subunit) (Complex I-B8) (CI-B8)</td>
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<tr>
<td>180</td>
<td>NDUS3</td>
<td>NADH dehydrogenase [ubiquinone] iron-sulfur protein 3, mitochondrial precursor (EC 1.6.5.3) (EC 1.6.99.3) (NADH-ubiquinone oxidoreductase 30 kDa subunit) (Complex I-30kD) (CI-30kD)</td>
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<td>181</td>
<td>NKX32</td>
<td>Homeobox protein Nkx-3.2 (Bagpipe homeobox protein homolog 1)</td>
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<td>182</td>
<td>NP1L4</td>
<td>Nucleosome assembly protein 1-like 4</td>
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<td>183</td>
<td>NPM2</td>
<td>Nucleoplasm-2</td>
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<tr>
<td>184</td>
<td>NSF</td>
<td>Vesicle-fusing ATPase (EC 3.6.4.6) (Vesicular-fusion protein NSF) (N-ethylmaleimide sensitive fusion protein) (NEM-sensitive fusion protein) (Suppressor of K(+) transport growth defect 2) (Protein SKD2)</td>
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<td>185</td>
<td>NSF1C</td>
<td>NSF1 acfator p47 (p97 cofactor p47)</td>
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<tr>
<td>186</td>
<td>NUDC</td>
<td>Nuclear migration protein nudC (Nuclear distribution protein C homolog) (Silica-induced gene 92 protein) (SIG-92)</td>
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<td>187</td>
<td>NUDT5</td>
<td>ADP-sugar pyrophosphatase (EC 3.6.1.13) (EC 3.6.1.13) (Nucleoside diphosphate-linked moiety X motif 5) (Nudix motif 5)</td>
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<tr>
<td>188</td>
<td>ODO1</td>
<td>2-oxoglutarate dehydrogenase E1 component, mitochondrial precursor (EC 1.2.4.2) (Alpha-ketoglutarate dehydrogenase)</td>
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<tr>
<td>189</td>
<td>ODO2</td>
<td>Dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex, mitochondrial precursor (EC 2.3.1.61) (Dihydrolipoamide succinyltransferase component of 2-oxoglutarate dehydrogenase complex) (E2) (E2K) - Mus muscul</td>
</tr>
<tr>
<td>190</td>
<td>ODPA</td>
<td>Pyruvate dehydrogenase E1 component alpha subunit, somatic form, mitochondrial precursor (EC 1.2.4.1) (PDHE1-A type I)</td>
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<td>191</td>
<td>OM34</td>
<td>Mitochondrial import receptor subunit TOM34 (Translocase of outer membrane 34 kDa subunit)</td>
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<tr>
<td>192</td>
<td>OST48</td>
<td>Dolichyl-diphospho-hexose:protein glycosyltransferase 48 kDa subunit precursor (EC 2.4.1.119) (Oligosaccharyl transferase 48 kDa subunit) (DDOST 48 kDa subunit)</td>
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<td>193</td>
<td>OTUB1</td>
<td>Ubiquitin thioesterase OTUB1 (EC 3.1.2.-) (Otubain-1) (OTU domain-containing ubiquitin aldehyde-binding protein 1) (Ubiquitin-specific-processing protease OTUB1) (Deubiquitinating enzyme OTUB1)</td>
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<td>194</td>
<td>PA1B3</td>
<td>Platelet-activating factor acetylhydrolase IB subunit gamma (EC 3.1.1.47) (PAF acetylhydrolase 29 kDa subunit) (PAF-AH 29 kDa subunit) (PAFAH subunit gamma) (PAFAH subunit gamma)</td>
</tr>
<tr>
<td>Protein ID</td>
<td>Description</td>
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<tr>
<td>PADI6</td>
<td>Protein-arginine deiminase type-6 (EC 3.5.3.15) (Protein-arginine deiminase type VI) (Peptidylarginine deiminase VI) (Arginine deiminase-like protein) (Egg and embryo abundant PAD) (ePAD)</td>
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<tr>
<td>PAOX</td>
<td>Peroxisomal N1-acetyl-spermine/spermidine oxidase (EC 1.5.3.11) (Polyamine oxidase)</td>
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<td>PARK7</td>
<td>Protein DJ-1 (Parkinson disease protein 7 homolog)</td>
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<td>PCBP1</td>
<td>Poly(C)-binding protein 1 (Alpha-CP1) (hnRNP-E1)</td>
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<td>PCBP2</td>
<td>Poly(C)-binding protein 2 (Alpha-CP2) (Putative heterogeneous nuclear ribonucleoprotein X) (hnRNP X) (CTBP) (CBP)</td>
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<td>PCCB</td>
<td>Propionyl-CoA carboxylase beta chain, mitochondrial precursor (EC 6.4.1.3) (PCCase subunit beta) (Propionyl-CoA:carbon dioxide ligase subunit beta)</td>
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<td>PCNA</td>
<td>Proliferating cell nuclear antigen (PCNA) (Cyclin)</td>
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<td>PDC6I</td>
<td>Programmed cell death 6-interacting protein (ALG-2-interacting protein X) (ALG-2-interacting protein 1) (E2F1-inducible protein) (Eig2)</td>
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<td>PDCD5</td>
<td>Programmed cell death protein 5 (Protein TFAR19) (TF-1 cell apoptosis-related gene 19 protein)</td>
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<td>PDIA1</td>
<td>Protein disulfide-isomerase precursor (EC 5.3.4.1) (PDI) (Prolyl 4-hydroxylase subunit beta) (Cellular thyroid hormone-binding protein) (p55) (Erp59)</td>
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<td>PDIA3</td>
<td>Protein disulfide-isomerase A3 precursor (EC 5.3.4.1) (Disulfide isomerase ER-60) (ERp60) (58 kDa microsomal protein) (p58) (ERp57)</td>
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<td>PDIA4</td>
<td>Protein disulfide-isomerase A4 precursor (EC 5.3.4.1) (Protein ERp-72) (ERp72)</td>
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<td>PDIA5</td>
<td>Protein disulfide-isomerase A5 precursor (EC 5.3.4.1) (Protein disulfide isomerase-related protein)</td>
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<td>PDIA6</td>
<td>Protein disulfide-isomerase A6 precursor (EC 5.3.4.1) (Thioredoxin domain-containing protein 7)</td>
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<td>PEBP1</td>
<td>Phosphatidylethanolamine-binding protein 1 (PEBP-1) (HCNPpp) [Contains: Hippocampal cholinergic neurostimulating peptide (HCNP)]</td>
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<td>PEPR</td>
<td>Peroxisomal trans-2-enoyl-CoA reductase (EC 1.3.1.38)</td>
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<td>PFD1</td>
<td>Prefoldin subunit 1</td>
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<tr>
<td>PFD2</td>
<td>Prefoldin subunit 2</td>
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<td>PHB</td>
<td>Prohibitin (B-cell receptor-associated protein 32) (BAP 32)</td>
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<td>PHB2</td>
<td>Prohibitin-2 (B-cell receptor-associated protein BAP37) (Repressor of estrogen receptor activity)</td>
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<td>PICA</td>
<td>Phosphatidylinositol-binding clathrin assembly protein (Clathrin assembly lymphoid myeloid leukemia) (CALM)</td>
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<td>PLAP</td>
<td>Phospholipase A-2-activating protein (PLAP)</td>
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<td>PLK1</td>
<td>Serine/threonine-protein kinase PLK1 (EC 2.7.11.21) (Polo-like kinase 1) (PLK-1) (Serine/threonine-protein kinase 13) (STPK13)</td>
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<td>PLSL</td>
<td>Plastin-2 (L-plastin) (Lymphocyte cytosolic protein 1) (LCP-1) (65 kDa macrophage protein) (pp65)</td>
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<td>PMG</td>
<td>Phosphoglycerate mutase (EC 5.4.2.4) (2,3-bisphosphoglycerate mutase, erythrocyte) (2,3-bisphosphoglycerate synthase) (BPGM) (EC 5.4.2.1) (EC 3.1.3.13) (BPG-dependent PGAM)</td>
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<td>PPIA</td>
<td>Peptidyl-prolyl cis-trans isomerase A (EC 5.2.1.8) (PPlase A) (Rotamase A) (Cyclophilin A) (Cyclophilin A-binding protein) (SP18)</td>
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<td>PRDX1</td>
<td>Peroxiredoxin-1 (EC 1.11.1.15) (Thioredoxin peroxidase 2) (Thioredoxin-dependent peroxide reductase 2) (Osteoblast-specific factor 3) (OSF-3) (Macrophage 23 kDa stress protein)</td>
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<tr>
<td>PRDX2</td>
<td>Peroxiredoxin-2 (EC 1.11.1.15) (Thioredoxin peroxidase 1) (Thioredoxin-dependent peroxide reductase 1) (Thiol-specific antioxidant protein) (TSA)</td>
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<tr>
<td>PRDX5</td>
<td>Peroxiredoxin-5, mitochondrial precursor (EC 1.11.1.15) (Prx-V) (Peroxisomal antioxidant enzyme) (PLP) (Thioredoxin reductase) (Thioredoxin peroxidase PMP20) (Antioxidant enzyme B166) (AOE166) (Liver tissue 2D-page spot 2D-0014IV) - Mus musculus (M)</td>
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<tr>
<td>Number</td>
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<td>Description</td>
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<tr>
<td>224</td>
<td>PRDX6</td>
<td>Peroxiredoxin-6 (EC 1.11.1.15) (Antioxidant protein 2) (1-Cys peroxiredoxin) (1-Cys PRX) (Acidic calcium-independent phospholipase A2) (EC 3.1.1.-) (aPLA2) (Non-selenium glutathione peroxidase) (EC 1.11.1.7) (NSGPx)</td>
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<td>225</td>
<td>PREP</td>
<td>Presequence protease, mitochondrial precursor (EC 3.4.24.-) (Pitrilysin metalloproteinase 1)</td>
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<td>226</td>
<td>PROF1</td>
<td>Proliin-1 (Proliin)</td>
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<td>227</td>
<td>PRS6A</td>
<td>26S protease regulatory subunit 6A (Proteasome 26S subunit ATPase 3) (TAT-binding protein 1) (TBP-1)</td>
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<td>228</td>
<td>PSA</td>
<td>Puromycin-sensitive aminopeptidase (EC 3.4.11.-) (PSA)</td>
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<td>229</td>
<td>PSMD5</td>
<td>26S proteasome non-ATPase regulatory subunit 5 (26S proteasome subunit S5B) (26S protease subunit S5 basic)</td>
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<td>230</td>
<td>PSMD9</td>
<td>26S proteasome non-ATPase regulatory subunit 9 (26S proteasome regulatory subunit p27)</td>
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<td>231</td>
<td>PUR8</td>
<td>Adenylosuccinate lyase (EC 4.3.2.2) (Adenylosuccinase) (ASL) (ASASE)</td>
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<td>232</td>
<td>PUR9</td>
<td>Bifunctional purine biosynthesis protein PURH [Includes: Phosphoribosylaminomimidazolecarboxamide formyltransferase (EC 2.1.2.3) (5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase) (AICAR transformylase); IMP cyclohydrolase (EC 3.5.4.10) ]</td>
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<td>233</td>
<td>PURA2</td>
<td>Adenylosuccinate synthetase isozyme 2 (EC 6.3.4.4) (Adenylosuccinate synthetase, non-muscle isozyme) (Adenylosuccinate synthetase, acidic isozyme) (IMP--aspartate ligase 2) (AdSS 2) (AMPSase 2)</td>
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<tr>
<td>234</td>
<td>RAB14</td>
<td>Ras-related protein Rab-14</td>
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<tr>
<td>235</td>
<td>RAB1A</td>
<td>Ras-related protein Rab-1A (YPT1-related protein)</td>
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<td>236</td>
<td>RAB2A</td>
<td>Ras-related protein Rab-2A</td>
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<tr>
<td>237</td>
<td>RAB3A</td>
<td>Ras-related protein Rab-3A</td>
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<td>238</td>
<td>RAB5A</td>
<td>Ras-related protein Rab-5A</td>
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<tr>
<td>239</td>
<td>RAB7A</td>
<td>Ras-related protein Rab-7a</td>
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<td>RAD1</td>
<td>Radixin (ESP10)</td>
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<td>241</td>
<td>RAN</td>
<td>GTP-binding nuclear protein Ran (GTPase Ran) (Ras-like protein TC4)</td>
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<td>242</td>
<td>RANG</td>
<td>Ran-specific GTPase-activating protein (Ran-binding protein 1) (RANBP1) (HpaII tiny fragments locus 9a protein)</td>
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<td>243</td>
<td>RB11A</td>
<td>Ras-related protein Rab-11A (Rab-11)</td>
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<td>244</td>
<td>RFA1</td>
<td>Replication protein A 70 kDa DNA-binding subunit (RP-A) (RF-A) (Replication factor-A protein 1) (p70)</td>
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<td>245</td>
<td>RIB1</td>
<td>Dolichyl-diphosphooligosaccharide--protein glycosyltransferase 67 kDa subunit precursor (EC 2.4.1.119) (Ribophorin I) (RPN-I)</td>
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<td>246</td>
<td>RL11</td>
<td>60S ribosomal protein L11</td>
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<td>247</td>
<td>RL12</td>
<td>60S ribosomal protein L12</td>
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<tr>
<td>248</td>
<td>RL13</td>
<td>60S ribosomal protein L13 (A52)</td>
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<td>60S ribosomal protein L22 (Heparin-binding protein HBp15)</td>
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<td>60S ribosomal protein L6 (TAX-responsive enhancer element-binding protein 107) (TAXREB107)</td>
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<td>RL7</td>
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<td>RL10</td>
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<td>RPB4</td>
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<td>RPE</td>
<td>Ribulose-phosphate 3-epimerase (EC 5.1.3.1) (Ribulose-5-phosphate-epimerase)</td>
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<td>262 RPN2</td>
<td>Dolichyl-diphosphooligosaccharide–protein glycosyltransferase 63 kDa subunit precursor (EC 2.4.1.119) (Ribophorin II) (RPN-II)</td>
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<td>40S ribosomal protein S9</td>
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<td>40S ribosomal protein SA (p40) (34/67 kDa laminin receptor)</td>
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<td>270 RUFY1</td>
<td>RUN and FYVE domain-containing protein 1 (Rab4-interacting protein)</td>
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<td>272 Sep-15</td>
<td>15 kDa selenoprotein precursor</td>
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<td>273 SKP1</td>
<td>S-phase kinase-associated protein 1A (Cyclin A/CDK2-associated protein p19) (p19A) (p19skp1)</td>
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<td>Histone RNA hairpin-binding protein (Histone stem-loop-binding protein)</td>
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<td>Sorting nexin-12 (SDP8 protein)</td>
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<td>276 SODC</td>
<td>Superoxide dismutase [Cu-Zn] (EC 1.15.1.1)</td>
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<td>277 SODM</td>
<td>Superoxide dismutase [Mn], mitochondrial precursor (EC 1.15.1.1)</td>
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<td>278 SPD2A</td>
<td>SH3 and PX domain-containing protein 2A (SH3 multiple domains protein 1) (Five SH3 domain-containing protein)</td>
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<td>279 SPIN1</td>
<td>Spindlin-1 (30000 Mr metaphase complex) (SSEC P)</td>
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<td>280 SPR6</td>
<td>Sepiapterin reductase (EC 1.1.1.153) (SPR)</td>
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<td>281 STIP1</td>
<td>Stress-induced-phosphoprotein 1 (STI1) (Hsc70/Hsp90-organizing protein) (Hop) (mSTI1)</td>
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<td>Stathmin (Phosphoprotein p19) (Oncoprotein 18) (Op18) (Leukemia-associated phosphoprotein p18) (pp17) (Prosolin) (Metablastin) (Pr22 protein) (Leukemia-associated gene protein)</td>
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<td>Arginyl-tRNA synthetase, cytoplasmic (EC 6.1.1.19)</td>
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<td>285 T126A</td>
<td>Transmembrane protein 126A</td>
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<td>Transfoming acidic coiled-coil-containing protein 3 (ARNT-interacting protein)</td>
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<td>Tubulin alpha-1 chain (Alpha-tubulin 1) (Alpha-tubulin isotype M-alpha-1)</td>
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<td>Tubulin alpha-3/alpha-7 chain (Alpha-tubulin isotype M-alpha-3/7)</td>
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<td>Tubulin beta-2C chain</td>
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<td>294 TBC15</td>
<td>TBC1 domain family member 15</td>
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<td>295 TBCD</td>
<td>Tubulin-specific chaperone D (Tubulin-folding cofactor D) (Beta-tubulin cofactor D)</td>
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<td>299 TCPF</td>
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<td>300 TCPQ</td>
<td>T-complex protein 1 subunit theta (TCP-1-theta) (CCT-theta)</td>
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<td>301 TCPZ</td>
<td>T-complex protein 1 subunit zeta (TCP-1-zeta) (CCT-zeta) (CCT-zeta-1)</td>
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<td>Acetyl-CoA acetyltransferase, mitochondrial precursor (EC 2.3.1.9) (Acetoacetyl-CoA thiolase)</td>
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<tr>
<td>Gene</td>
<td>Description</td>
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<td>Transketolase (EC 2.2.1.1) (TK) (P68)</td>
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<td>TMED4</td>
<td>Transmembrane emp24 domain-containing protein 4 precursor (p26)</td>
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<td>TNIK</td>
<td>Traf2 and NCK-interacting protein kinase (EC 2.7.11.1) (Fragments)</td>
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<td>TPA</td>
<td>Tissue-type plasminogen activator precursor (EC 3.4.21.68) (tPA) (t-PA) (t-plasminogen activator) [Contains: Tissue-type plasminogen activator chain A; Tissue-type plasminogen activator chain B]</td>
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<td>TRA2A</td>
<td>Transformer-2 protein homolog (TRA-2 alpha)</td>
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<td>TRXR1</td>
<td>Thioredoxin reductase 1, cytoplasmic (EC 1.8.1.9) (TR) (TR1)</td>
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<td>TTCX1</td>
<td>Tetranucleotide repeat protein 5 (TPR repeat protein 5)</td>
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<td>TXND5</td>
<td>Thioredoxin domain-containing protein 5 precursor (Thioredoxin-like protein p46) (Endoplasmic reticulum protein ERP46) (Plasma cell-specific thioredoxin-related protein) (PC-TRP)</td>
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<td>U5S1</td>
<td>116 kDa U5 small nuclear ribonucleoprotein component (U5 snRNP-specific protein, 116 kDa) (U5-116 kDa) (Elongation factor Tu GTP-binding domain protein 2)</td>
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<td>UB1L2</td>
<td>Ubiquitin-activating enzyme E1-like protein 2</td>
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<td>UB2D3</td>
<td>Ubiquitin-conjugating enzyme E2 D3 (EC 6.3.2.19) (Ubiquitin-protein ligase D3) (Ubiquitin carrier protein D3) (Ubiquitin-conjugating enzyme E2-17 kDa 3) (E2(17)K8 3)</td>
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<td>UBC12</td>
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<td>UBE1X</td>
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<td>UBE2N</td>
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<td>UBFD1</td>
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<td>UBIQ</td>
<td>Ubiquitin</td>
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<td>Ubiquitin carboxyl-terminal hydrolase isozyme L1 (EC 3.4.19.12) (EC 6.-.-.-.) (UCH-L1) (Ubiquitin thioesterase L1) (Neuron cytoplasmic protein 9.5) (PGP 9.5) (PGP9.5)</td>
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<td>UHRF1</td>
<td>E3 ubiquitin-protein ligase UHRF1 (EC 6.3.2.-) (Ubiquitin-like PHD and RING finger domain-containing protein 1) (Ubiquitin-like-containing PHD and RING finger domains protein 1) (Nuclear zinc finger protein Np95)</td>
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<td>VAMP3</td>
<td>Vesicle-associated membrane protein 3 (VAMP-3) (Synaptofuscin-3) (Cellubrevin) (CEB)</td>
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<td>VAT1</td>
<td>Synaptic vesicle membrane protein VAT-1 homolog (EC 1.-.-.-.)</td>
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<td>VATB2</td>
<td>Vacular ATP synthase subunit B, brain isofom (EC 3.6.3.14) (V-ATPase B2 subunit) (Vacular proton pump B isofom 2) (Endomembrane proton pump 58 kDa subunit)</td>
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<td>VDAC2</td>
<td>Voltage-dependent anion-selective channel protein 2 (VDAC-2) (mVDAC2) (mVDAC6) (Outer mitochondrial membrane protein porin 2)</td>
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<td>WDR1</td>
<td>WD repeat protein 1 (Actin-interacting protein 1) (AIP1)</td>
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<td>WD repeat protein 82</td>
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<td>Exportin-1 (Exp1) (Chromosome region maintenance 1 protein homolog)</td>
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<td>Exportin-7 (Exp7) (Ran-binding protein 16)</td>
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<td>YBOX2</td>
<td>Y-box-binding protein 2 (Germ cell-specific Y-box-binding protein) (FRGY2 homolog)</td>
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<tr>
<td>ZP2</td>
<td>Zona pellucida sperm-binding protein 2 precursor (Zona pellucida glycoprotein ZP2) (Zona pellucida protein A)</td>
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<td>ZP3</td>
<td>Zona pellucida sperm-binding protein 3 precursor (Zona pellucida glycoprotein ZP3) (Sperm receptor) (Zona pellucida protein C)</td>
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</table>
Appendix C: Buffers, Solutions and Media

**4X Resolving Buffer (for resolving gels)**

182 g Tris-base
4 g SDS
-Made up to 1 L with MilliQ-H₂O, pH 8.8

**4X Stacking Buffer (for stacking gel)**

60.5 g Tris-base
4 g SDS
-Made up to 1 L with MilliQ-H₂O, pH 6.8

**BWW Stock Diluent**

5.54 mg/mL sodium chloride
0.35 mg/mL potassium chloride
0.25 mg/mL calcium chloride dihydrate
0.162 mg/mL potassium dihydrogen orthophosphate
0.294 mg/mL magnesium sulphate heptahydrate
-Made up in MilliQ-H₂O

**BWW/PVA or BWW/BSA**

2.1 mg/mL sodium hydrogen carbonate
1.0 mg/mL D-glucose
0.03 mg/mL sodium pyruvate
3.7 µL/mL sodium lactate
1.0 % penicillin/streptomycin
2.0 % HEPES buffer
1.0 mg/mL polyvinyl alcohol, or
3 mg/mL BSA
-Made up with BWW Stock Diluent

**α-MEM**

-Purchased from Gibco (Invitrogen, Australia: Cat No. 32561)

**MOWIOL**

13% Mowiol4-88
33% glycerol
66mM Tris (pH 8.5)
2.5% 1,4diazobicyclo-[2.2.2]octane (DABCO)

**Oocyte Lysate Buffer**

1% Nonidet P-40
50 mM Tris-HCL

**PBS**

-PBS tablets dissolved in MilliQ-H₂O according to manufacturers instructions (Sigma-Aldrich)

**SDS-PAGE Loading buffer (reducing)**

0.2% SDS
0.375 M Tris
10% Sucrose
4% 2β-mercaptoethanol
-Made up in MilliQ-H₂O, pH 6.8.
-Add 3 – 5 crystals of bromophenol blue

**SDS-PAGE Running buffer (5X)**

1 mM Tris-base
1 M glycine
0.5% SDS
-Made up to volume in MilliQ-H₂O

**Sperm dilution fluid (for cell count)**

0.6 M sodium hydrogen carbonate
10 mL 37% formaldehyde
-Made up to 1 L with MilliQ-H₂O

**TBS**

0.1 M Tris
0.15 M sodium chloride
-Made up in MilliQ-H₂O

**TBS-T**

TBS supplemented with 0.1% Tween-20

**Western Blot Transfer buffer**

0.15 M Tris-base
0.9 M glycine
15% methanol
### Table C.1 Composition of 7.5% SDS-PAGE gels

<table>
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<th>Stock Solution</th>
<th>4% Stacking gel (mL)</th>
<th>7.5% Resolving gel (mL)</th>
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<tr>
<td>40% Polyacrylamide solution</td>
<td>0.49</td>
<td>2.8</td>
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<tr>
<td>4x Stacking buffer</td>
<td>1.25</td>
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<tr>
<td>4x Separating buffer</td>
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<tr>
<td>H₂O</td>
<td>3.20</td>
<td>8.4</td>
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<tr>
<td>10% APS</td>
<td>0.05</td>
<td>0.1</td>
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<tr>
<td>TEMED</td>
<td>0.01</td>
<td>0.02</td>
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<tr>
<td><strong>Total</strong></td>
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<td><strong>15</strong></td>
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### Table C.2 Composition of 10% SDS-PAGE gels

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<td>40% Polyacrylamide solution</td>
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<tr>
<td>H₂O</td>
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<td>7.5</td>
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<tr>
<td>10% APS</td>
<td>0.05</td>
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<td>0.01</td>
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<tr>
<td><strong>Total</strong></td>
<td><strong>5</strong></td>
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Appendix D: Antibodies, Enzymes and Cellular Probes

Table D1. Antibodies used during experimentation

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<th>Conjugate</th>
<th>Supplier</th>
<th>Catalogue #</th>
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<tr>
<td>Mouse anti-recomb CD160 (CL1-R2')</td>
<td>none</td>
<td>MBL</td>
<td>K0122-1</td>
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<td>Mouse anti-human LPL</td>
<td>none</td>
<td>Abcam</td>
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<td>Mouse anti-recomb Hyal2</td>
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<td>Abnova</td>
<td>H00008692-A01</td>
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<td>Produced and donated by Dr. Kunio Tsujimura</td>
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<tr>
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<td><strong>Tetraspanins:</strong></td>
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<td><strong>Secondary Antibodies</strong></td>
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<td>HRP</td>
<td>Chemicon</td>
<td>SA202</td>
</tr>
</tbody>
</table>

*HRP = horseradish peroxidase; Alk-Phos = alkaline phosphatase; FITC = fluorescein isothiocyanate*
### Table D2. Enzymes and cellular probes used during experimentation

<table>
<thead>
<tr>
<th>Name of Enzyme or Probe</th>
<th>Abbreviation</th>
<th>Supplier</th>
<th>Catalogue #</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphatidylinositol-specific phospholipase C</td>
<td>PI-PLC</td>
<td>Molecular Probes (Invitrogen)</td>
<td>P6466</td>
</tr>
<tr>
<td>-isolated from Bacillus cereus</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glycophosphatidylinositol-specific phospholipase D</td>
<td>GPI-PLD</td>
<td>Produced and donated by Prof. Peter Buetikofer</td>
<td></td>
</tr>
<tr>
<td>-isolated from bovine serum</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Angiotensin-converting enzyme</td>
<td>ACE</td>
<td>Sigma-Aldrich</td>
<td>A6778</td>
</tr>
<tr>
<td>-isolated from rabbit lung</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hyaluronidase (mouse embryo tested)</td>
<td>N/A</td>
<td>Sigma-Aldrich</td>
<td>H4272</td>
</tr>
<tr>
<td>-isolated from bovine testes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AlexaFluor 488 C₆-maleimide</td>
<td>N/A</td>
<td>Molecular Probes (Invitrogen)</td>
<td>A10254</td>
</tr>
</tbody>
</table>
Appendix E: Abstracts, Presentations and Publications

PUBLICATIONS


ABSTRACTS & PRESENTATIONS


* Indicates presenting author.