

The role of the oocyte activation factor PLC ζ in human male factor infertility

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Table of contents

List of abbreviations.....	vii
List of figures	xi
List of tables	xix

Abstract	1

Chapter 1: General introduction

1.1 Oocyte activation and calcium oscillations	3
1.2 PLCs at fertilisation and oocyte activation	12
1.3 PLCζ: the mammalian oocyte activation factor	16
1.4 PLCζ structure and function	19
1.5 Localisation of PLCζ in mammalian sperm.....	24
1.6 Links between PLCζ and human male factor infertility	29
1.7 Male factor infertility and assisted oocyte activation (AOA).....	35
1.8 Therapeutic and Diagnostic applications of PLCζ.....	42
1.9 The effects of clinician-induced (iatrogenic) damage on sperm proteins.....	43
1.10 Aims of this thesis.....	45

Chapter 2: General materials and methods

2.1 Ethics and patient selection.....	48
2.2 Genomic DNA extraction from buccal cells.....	49
2.3 Polymerase chain reaction (PCR) and direct DNA sequencing	50
2.4 Determination of DNA and RNA concentration by spectrophotometry	51
2.5 Agarose gel electrophoresis	52
2.6 Luria Bertani (LB)-Agar plates, LB-broth, and phosphate buffered solution (PBS)	52
2.7 Cloning of molecular constructs	53
2.8 Site directed mutagenesis.....	56
2.9 Plasmid DNA isolation for mammalian cell transfection	58
2.10 Mammalian cell culture and transfection using the jetPEI kit.....	59

2.11	Preparation of HEK293T cell lysates	60
2.12	cRNA synthesis.....	61
2.13	RNA agarose denaturing-gel electrophoresis	62
2.14	Density gradient washing (DGW) of human sperm samples	63
2.15	Determination of PLC ζ immunofluorescence in human sperm.....	64
2.16	Polyacrylamide gel electrophoresis and immunoblotting for protein analysis.....	65
2.17	Mouse oocyte collection, culture, and micro-injection.....	66
2.18	Ca ²⁺ imaging in mouse oocytes	67
2.19	Microscopy and digital imaging of transfected HEK293T cells	68
2.20	Statistical analyses	69

Chapter 3: Screening of the PLC ζ gene from genomic DNA, and immunofluorescent analysis of PLC ζ in sperm, from fertile controls and infertile patients

3.1	Introduction.....	70
3.2	Materials and Methods.....	74
3.2.1	Patient selection and genomic analysis	74
3.2.2	PCR and direct sequencing of PLC ζ active site coding exons.....	74
3.2.3	Sub-cloning of PLC ζ amplicons into the pCRII-TOPO vector	77
3.2.4	Sperm density gradient washing (DGW)	77
3.2.5	Sperm PLC ζ immunofluorescence	78
3.2.6	Statistical analysis	78
3.3	Results	80
3.3.1	PCR screening of PLC ζ exons from fertile and infertile human males	80
3.3.2	Quantitative total PLC ζ immunofluorescence in fertile control and infertile patient sperm.....	85
3.3.3	Proportions of sperm exhibiting PLC ζ immunofluorescence	87
3.3.4	Proportional analysis of PLC ζ localisation patterns in control sperm	89
3.3.5	Proportional analysis of PLC ζ localisation patterns in sperm from infertile males	95
3.3.6	Comparative proportional analysis of PLC ζ localisation pattern populations within sperm from fertile and infertile males	97
3.4	Discussion	99

3.4.1 Loss-of-activity mutations within the PLC ζ active site may be rare occurrences	99
3.4.2 Screening of exons coding for other functional domains of PLC ζ	102
3.4.3 Quantitative immunofluorescence analysis indicates a large degree of variability in the levels of total PLC ζ fluorescence in control fertile sperm	104
3.4.4 The predominant localisation pattern of PLC ζ varies in control sperm, with no predominant pattern present in sperm from infertile males	108
3.5 Conclusions and future work	110

Chapter 4: Functional characterisation and modes of inheritance of a novel PLC ζ mutation (H233L) identified in an infertile male

4.1 Introduction	112
4.2 Materials and Methods	115
4.2.1 Sample acquisition and processing	115
4.2.2 Molecular analysis of the PLC ζ gene.....	115
4.2.3 cRNA synthesis.....	116
4.2.4 Mouse oocyte collection, culture, micro-injection, and Ca ²⁺ imaging	117
4.2.5 Direct mutation detection using individual sperm cells.....	117
4.2.6 TaqMan SNP genotyping assay	119
4.3 Results	121
4.3.1 Identification of a novel second mutation in PLC ζ from patient ^{H398P}	121
4.3.2 Genetic modes of inheritance of the H233L and H398P PLC ζ mutations.....	122
4.3.3 Single sperm screening for H233L and H398P in patient ^{H398P}	129
4.3.4 Genetic screening of the prevalence of H233L and H398P in 100 fertile individuals and 8 patients diagnosed with oocyte activation deficiency	131
4.4 Discussion	134
4.4.1 A second novel loss-of-activity mutation in PLC ζ	134
4.4.2 A maternally inherited autosomal mutation contributes to male infertility	136
4.4.3 Mutations in PLC ζ may function in a recessive manner.....	139
4.5 Conclusions and future work	145

Chapter 5: Investigation of the potential clinician-induced (iatrogenic) damage upon levels of PLC ζ in human sperm by clinical ART protocols

5.1 Introduction.....	146
5.2 Materials and Methods.....	149
5.2.1 Fertile control semen samples.....	149
5.2.2 Sperm density gradient washing	149
5.2.3 Cryopreservation.....	149
5.2.4 Sperm PLC ζ immunofluorescence, microscopy, and fluorescence quantification	150
5.2.5 Statistical analysis.....	150
5.3 Results.....	151
5.3.1 Proportional analysis of PLC ζ immunofluorescence in fertile sperm following DGW	151
5.3.2 Total PLC ζ immunofluorescence following cryopreservation	151
5.4 Discussion	156
5.4.1 DGW increases the proportion of sperm exhibiting PLC ζ immunofluorescence	156
5.4.2 Cryopreservation reduces levels of total PLC ζ immunofluorescence in human sperm.....	157
5.4.3 Iatrogenic effects of novel ART techniques on gametes and markers of gamete function.....	159
5.5 Conclusions and future work.....	164

Chapter 6: Generation of active recombinant human PLC ζ protein using bacterial and mammalian expression systems

6.1 Introduction.....	166
6.2 Materials and Methods.....	171
6.2.1 DNA templates, vectors and constructs	171
6.2.2 Bacterial transformation and protein purification	173
6.2.3 Protein identification via Liquid Chromatography/Mass Spectrometry (LC/MS).....	174
6.2.4 Sea urchin egg homogenate (SUEH)	174
6.2.5 Mammalian cell culture	175
6.2.6 Calcium phosphate precipitation transfection.....	175
6.2.7 Fluorescence microscopy	175

6.2.8	Protein expression and purification using a mammalian system	176
6.2.9	Mouse oocyte injection of recombinant cRNA and human protein.....	177
6.2.10	Immunoblotting.....	177
6.2.11	Reverse transcription PCR (RT-PCR)	178
6.3	Results	180
6.3.1	Bacterial expression system	180
6.3.2	Mammalian expression system	183
6.3.3	Reverse transcription (RT)-PCR.....	185
6.3.4	Mouse oocyte microinjection.....	187
6.4	Discussion	191
6.4.1	Recombinant human PLC ζ protein is successfully expressed by a prokaryotic system in an inactive state, and expressed in an active form by a eukaryotic system.....	192
6.4.2	Human PLC ζ protein generated in a human cell line induces Ca ²⁺ oscillations in mouse oocytes, and may require post-translational modifications to obtain an active state.....	194
6.5	Conclusions and future work.....	198

Chapter 7: Functional characterisation of wild type and mutant isoforms of PLC ζ in HEK293T cells

7.1	Introduction.....	200
7.2	Materials and Methods.....	204
7.2.1	Molecular cloning of fluorescently tagged constructs	204
7.2.2	Mammalian cell culture	204
7.2.3	Imaging Ca ²⁺ release in live HEK293T cell preparations.....	206
7.2.4	Immunoblotting.....	208
7.2.5	Analysis of transfected HEK293T cells by fluorescent and confocal microscopy.....	208
7.3	Results	210
7.3.1	Live cell Ca ²⁺ imaging of transfected HEK293T cells	210
7.3.2	IP ₃ R-1 immunoblotting	215
7.3.3	Fluorescence quantification and intracellular localisation of mCherry-PLC ζ isoforms in HEK293T cells	216
7.4	Discussion	223

7.4.1	HEK293T cells transfected with PLC ζ^{WT} exhibit reduced responses to ATP	224
7.4.2	Loss-of-activity mutations in highly conserved regions of human PLC ζ may lead to overall protein instability	226
7.4.3	Human PLC ζ localises to the endoplasmic reticulum and not the plasma membrane in HEK293T cells.....	229
7.4.4	The regulatory EF hands may play a novel role in intracellular and potential nuclear localisation of human PLC ζ	230
7.5	Conclusions and future work	233
 Chapter 8: General discussion		
8.1	The role of PLC ζ in human male factor infertility	237
8.2	PLC ζ as a prognostic marker of male fertility	242
8.3	The clinical use of PLC ζ as a therapeutic agent within ART	247
8.4	PLC ζ : the chosen one, or one of many?	250
8.5	General conclusions	254
References		256
Supplementary Appendices		294
List of publications		304

List of abbreviations

The following is an explanation of commonly used abbreviations used throughout the text.

Other, less commonly used abbreviations are explained within the text where appropriate.

ΔYC2	Frameshift PLCζ mutation (deletion of partial Y and complete C2 domains)
ΔEF	PLCζ mutation (deletion of EF hands domain)
ANOVA	Analysis of variance
AOA	Artificial oocyte activators
ART	Assisted reproductive technologies
AUC	Area under the curve
BSA	Bovine serum albumin
Ca²⁺	Calcium ions
CaMKII (γ)	Calmodulin-dependent protein kinase II (gamma isoform)
CHO	Chinese hamster ovary
CHX	Cyclohexamide
CICR	Ca ²⁺ -induced Ca ²⁺ release
cRNA	Complementary RNA
DAG	Diacylglycerol
DAPI	4'-6-Diamidino-2-phenylindole
DGW	Density gradient washing
DMEM	Dulbecco's modified Eagle's medium
EDTA	Ethylenediaminetetraacetic acid
EF	EF hands domain of PLCζ
ER	Endoplasmic reticulum
FBS	Fetal bovine serum

FITC	Fluorescein isothiocyanate
GFP	Green fluorescent protein
GST	Glutathione sepharose tag
HBSS	Hank's buffered saline solution
H233L	Histidine to Leucine point mutant at position 233 of PLC ζ amino acid sequence
H398P	Histidine to Proline point mutant at position 398 of PLC ζ amino acid sequence
HEK293T	Human embryonic kidney cell line 293 (transformed)
HRP	Horseradish peroxidase
ICSI	Intracytoplasmic sperm injection
IVF	<i>In vitro</i> fertilisation
IP₃	Inositol 1,4,5-trisphosphate
IP₃R(-1)	Inositol 1,4,5-trisphosphate receptor (Type 1)
KO	Knock-out
LB	Luria Bertani
LC/MS	Liquid chromatography/ mass spectrometry
MII oocytes	Oocytes arrested at metaphase of the second meiotic division
MOPS	10x 3-N(morpholino) propane sulfonic acid solution
NLS	Nuclear localisation signal
OFU	Oxford Fertility Unit
ORF	Open reading frame
OAD	Oocyte activation deficient
MOAT%	Mouse oocyte activation test success (in %)
mCherry	Red fluorescent protein (excitation: 560nm)
Patient^{H398P}	Patient from whom the H398P PLC ζ mutation was first identified

PAWP	Post-acrosomal sheathWW domain-binding protein
PB	Polar body
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PH domain	Pleckstrin homology domain
PIP₂	Phosphatidylinositol 4,5-bisphosphate
PKC	Protein kinase C
PLC	Phospholipase C
PLCζ	Phospholipase C zeta (ζ)
PT-DGW	Post-thaw density gradient wash
PTM	Post-translational modification
RNAi	RNA interference
ROI	Region of interest
ROS	Reactive oxygen species
RT	Room temperature (°C)
RT-PCR	Reverse transcription-PCR
SEM	Standard error of the mean
SFK	Src family kinase
SH	Src Homology domain
SNP	Single nucleotide polymorphism
SUEH	Sea urchin egg homogenate
TAE	Tris-Acetate-EDTA
TG	Thapsigargin
TGCE	Thermal gradient capillary electrophoresis
TRITC	Tetraethyl rhodamine isothiocyanate

WGA	Wheat germ agglutinin
WHO	World Health Organisation
WT	Wild type
ZP	Zona Pellucida

List of figures

Chapter 1: General introduction

Figure 1.1:	Mouse oocyte arrested at the MII stage, and zygotes at the one-cell and two-cell stages.....	3
Figure 1.2:	Calcium wave in a <i>Xenopus</i> egg, and calcium transients in various species.....	4
Figure 1.3:	Schematic representation of the mechanism of the signalling cascade involved in mammalian oocyte activation	6
Figure 1.4:	Schematic representations of the Ca^{2+} conduit, membrane receptor, and sperm factor models of oocyte activation	11
Figure 1.5:	Schematic representation of the current understanding of mammalian PLC ζ mechanism of action	20
Figure 1.6:	Schematic representations of PLC ζ domain structure and quaternary folding.....	21
Figure 1.7:	Immunofluorescent localisation of PLC ζ in hamster and human sperm ..	29
Figure 1.8:	Immunofluorescent localisation of PLC ζ in oocyte activation deficient sperm.....	32
Figure 1.9:	Representative immunoblotting profile of PLC ζ in oocyte activation deficient and fertile human sperm, and microinjection of oocyte activation deficient and fertile human sperm in mouse oocytes	33
Figure 1.10:	Representative three-dimensional modelling of wild type and H398P PLC ζ and their corresponding Ca^{2+} oscillation profiles within mouse oocytes	34
Figure 1.11:	Primary causes of general and male factor infertility	38

Figure 1.12:	Representative Ca^{2+} oscillation profiles elicited by various agents of assisted oocyte activation (AOA)	40

Chapter 3: Screening of the PLC ζ gene from genomic DNA, and immunofluorescent analysis of PLC ζ in sperm, from fertile controls and infertile patients

Figure 3.1:	Representative 2% agarose gel of PCR amplicons for PLC ζ active site exons from patient 2	81
Figure 3.2:	Representative sequencing peaks showing successful and unsuccessful sequencing of PLC ζ active site exons.....	82
Figure 3.3:	BLAST alignment result of exon 6 using forward primer from patient ^{H398P} identifying the novel H233L mutation	83
Figure 3.4:	Representative 2% agarose gel showing restriction enzyme digested colonies following sub-cloning of PCR amplicons in the pCRII-TOPO vector	83
Figure 3.5:	Representative BLAST alignment of two different colonies for the same PCR generated amplicon sub-cloned into the pCRII-TOPO vector	84
Figure 3.6:	Mean relative total PLC ζ fluorescence levels of PLC ζ in human fertile control and infertile patient sperm	86
Figure 3.7:	Mean relative total PLC ζ fluorescence levels of PLC ζ exhibited by individual human fertile control and infertile patient sperm	86
Figure 3.8:	Representative confocal images of fertile control human sperm exhibiting various localisation patterns of PLC ζ immunofluorescence.....	87
Figure 3.9:	Mean proportions of sperm exhibiting PLC ζ immunofluorescence in human fertile control and infertile patient sperm.....	88
Figure 3.10:	Mean proportions of sperm exhibiting PLC ζ immunofluorescence from individual human fertile controls and infertile patients	88

Figure 3.11:	Mean proportions of sperm exhibiting PLC ζ immunofluorescence patterns from controls 1 and 2	90
Figure 3.12:	Mean proportions of sperm exhibiting PLC ζ immunofluorescence patterns from controls 3, 4, and 5	92
Figure 3.13:	Mean proportions of sperm exhibiting PLC ζ immunofluorescence patterns from controls 6 and 7	94
Figure 3.14:	Mean proportions of sperm exhibiting PLC ζ immunofluorescence patterns from patients 1 and 2.....	96
Figure 3.15:	Mean proportions of sperm exhibiting PLC ζ immunofluorescence patterns from human fertile control and infertile patients	97
 Chapter 4: Functional characterisation and modes of inheritance of a novel PLCζ mutation (H233L) identified in an infertile male		
Figure 4.1:	Schematic representation of the locations of H233L and H398P within the PLC ζ open reading frame, and three-dimensional modelling of the effects of H233L on local protein structure, along with the conservation of H233 among various taxa.....	123
Figure 4.2:	Representative Ca ²⁺ traces elicited following microinjection of cRNA corresponding to PLC ζ ^{WT} and PLC ζ ^{H233L} into mouse oocytes at concentrations of 344ng/ μ l and 352ng/ μ l respectively.....	124
Figure 4.3:	Representative Ca ²⁺ traces elicited following microinjection of cRNA corresponding to PLC ζ ^{WT} and PLC ζ ^{H233L} into mouse oocytes at concentrations of 172ng/ μ l and 176ng/ μ l respectively.....	125
Figure 4.4:	Analysis of Ca ²⁺ peaks following microinjection of cRNA corresponding to PLC ζ ^{WT} and PLC ζ ^{H233L} into mouse oocytes at high and low concentrations	126
Figure 4.5:	Representative mini-sequencing peaks of genomic DNA from patient ^{H398P} indicating presence of the H233L and H398P mutations	127

Figure 4.6:	Representative direct sequencing peaks of genomic DNA from patient ^{H398P} indicating presence of the H233L and H398P mutations in a heterozygous manner	128
Figure 4.7:	Pedigree illustrating the inheritance patterns of the H233L and H398P mutations in patient ^{H398P} and his direct family	129
Figure 4.8:	Representative mini-sequencing peaks of single sperm from patient ^{H398P} for PLC ζ ^{H233L}	130
Figure 4.9:	Representative mini-sequencing peaks of single sperm from patient ^{H398P} for PLC ζ ^{H398P}	130
Figure 4.10:	Representative scatter plot showing screening results for 100 fertile individuals for PLC ζ ^{H233L} and PLC ζ ^{H398P}	132
Figure 4.11:	Schematic illustrating a possible model of spermatogenesis in the father of patient ^{H398P} , indicating how both PLC ζ ^{H233L} and PLC ζ ^{H398P} DNA and protein may be divided amongst the sperm population assuming that human PLC ζ is first transcribed at the spermatid stage	141
Figure 4.12:	Schematic illustrating a possible model of spermatogenesis in patient ^{H398P} , indicating how both PLC ζ ^{H233L} and PLC ζ ^{H398P} DNA and protein may be divided amongst the sperm population assuming that human PLC ζ is first transcribed at the spermatid stage	142
Figure 4.13:	Schematic showing a possible model of spermatogenesis in the father of patient ^{H398P} , indicating how both PLC ζ ^{H233L} and PLC ζ ^{H398P} DNA and protein may be divided amongst the sperm population assuming that human PLC ζ is transcribed throughout the meiotic phases of spermatogenesis	143
Figure 4.14:	Schematics showing a possible model of spermatogenesis in patient ^{H398P} , indicating how both PLC ζ ^{H233L} and PLC ζ ^{H398P} DNA and protein may be divided amongst the sperm population assuming that human PLC ζ is transcribed throughout the meiotic phases of spermatogenesis	144

Chapter 5: Investigation of the potential clinician-induced (iatrogenic) damage occurred upon levels of PLC ζ in human sperm by clinical ART protocols

- Figure 5.1:** Representative bright-field and corresponding PLC ζ immunofluorescence of human sperm showing characteristic localisation patterns previously identified by Grasa *et al.* (2008)..... **152**
- Figure 5.2:** Relative proportions of sperm exhibiting PLC ζ fluorescence in raw and DGW fractions for donors 2 - 6. **153**
- Figure 5.3:** Representative bright-field and corresponding PLC ζ immunofluorescence of human sperm for all three sperm fractions, showing PLC ζ levels and localisation patterns..... **154**
- Figure 5.4:** Relative PLC ζ fluorescence in sperm from donors 1 - 7 before and after cryopreservation..... **155**

Chapter 6: Generation of active recombinant human PLC ζ protein using bacterial and mammalian expression systems

- Figure 6.1:** Schematic representation of the PLC ζ ORF illustrating the primer sets utilised for RT-PCR and their respective binding sites on the PLC ζ domain ORF..... **179**
- Figure 6.2:** Polyacrylamide gel electrophoresis and coomassie blue staining of PLC ζ^{WT} cultures before and after purification..... **180**
- Figure 6.3:** Polyacrylamide gel electrophoresis and coomassie blue staining of PLC ζ^{WT} cultures before and after cleavage of the GST tag following purification..... **181**
- Figure 6.4:** Representative Ca²⁺ peaks from the SUEH activity bioassay, and oocyte microinjection, with recombinant human PLC ζ proteins obtained from the bacterial expression system..... **182**
- Figure 6.5:** Representative fluorescence microscopy image taken across 8, 24, 36, 48, and 56 hour time points for HEK293T cells transfected with pTracer-PLC ζ constructs **184**

Figure 6.6:	Representative immunoblotting using an anti-His monoclonal antibody with purification samples from pcDNA and pTracer constructs, and using an anti-PLC ζ polyclonal antibody on lysates obtained from HEK293T cells transfected with pTracer-PLC ζ^{WT} , and -PLC ζ^{H398P}	184
Figure 6.7:	Relative densitometry values illustrating the intensity of PLC ζ bands obtained for HEK293T cells transfected with pTracer-PLC ζ^{WT} , and -PLC ζ^{H398P}	185
Figure 6.8:	Schematic illustrating the primer sets utilised for RT-PCR and their respective binding sites on the PLC ζ domain ORF, and representative 1% agarose gel pictures of RT-PCR using primer sets P1-P6	186
Figure 6.9:	Representative Ca $^{2+}$ oscillation profiles of mouse oocytes injected with lysates at low and high concentrations, prepared from HEK293T cells transfected with pTracer empty vector, pTracer-PLC ζ^{H398P} , pTracer-PLC ζ^{WT}	188
Figure 6.10:	Representative comparison of first Ca $^{2+}$ peak between mouse oocytes injected with the high concentration of lysate from HEK293T cells transfected with pTracer-PLC ζ^{WT} , and oocytes injected with human PLC ζ^{WT} cRNA	189
 Chapter 7: Functional characterisation of wild type and mutant isoforms of PLCζ in HEK293T cells		
Figure 7.1:	Representative Ca $^{2+}$ responses of individual HEK293T cells transfected with mCherry -empty vector, -PLC ζ^{WT} , -PLC ζ^{H398P} -PLC $\zeta^{\Delta\text{YC2}}$ constructs stimulated with 1 μM , 5 μM , and 100 μM of ATP	211
Figure 7.2:	Mean data illustrating the proportion of cells responding to different ATP concentrations, the amplitude of Ca $^{2+}$ responses, and the amount of Ca $^{2+}$ release for cells transfected with mCherry-PLC ζ constructs	212
Figure 7.3:	Representative Ca $^{2+}$ responses of individual HEK293T cells transfected with mCherry-empty vector, -PLC ζ^{WT} , -PLC ζ^{H398P} , and -PLC $\zeta^{\Delta\text{YC2}}$ constructs stimulated with 5 μM Thapsigargin (TG).....	213

Figure 7.4:	Mean data illustrating the proportion of cells responding to TG stimulation, and the amount of Ca^{2+} release in response to the different ATP concentrations, by HEK293T cells transfected with the various mCherry-PLC ζ constructs.....	214
Figure 7.5:	Representative immunoblotting using an IP ₃ R-1 antibody on lysates obtained from HEK293T cells transfected with pTracer-empty vector, -PLC ζ^{WT} , and -PLC ζ^{H398P}	215
Figure 7.6:	Representative fluorescence microscopy image taken across 8, 24, 36, 48, and 56 hour time points for HEK293T cells transfected with mCherry-PLC ζ constructs	216
Figure 7.7:	Mean mCherry fluorescence intensity per cell for HEK293T cells transfected with mCherry-PLC ζ^{WT} , -PLC ζ^{H398P} , -PLC ζ^{H233L} , -PLC $\zeta^{\text{H233L+H398P}}$, and -PLC $\zeta^{\Delta\text{YC2}}$ at 48 and 56 hour time points	217
Figure 7.8:	Representative single plane confocal microscopy images of 48 and 56 hour time points for HEK293T cells transfected with mCherry-PLC ζ constructs using a plasma membrane stain	218
Figure 7.9:	Representative single plane confocal microscopy images of cells transfected with mCherry-empty vector and negative transfected cells using a plasma membrane stain	219
Figure 7.10:	Representative 3D projections, obtained by reconstructing Z-stack images obtained by confocal microscopy, of HEK293T cells transfected with mCherry-PLC ζ^{WT} using a plasma membrane stain	219
Figure 7.11:	Representative single plane confocal microscopy images of 48 and 56 hour time points for HEK293T cells transfected with mCherry-PLC ζ^{WT} , -PLC ζ^{EF} , and -PLC $\zeta^{\Delta\text{EF}}$ constructs using a ER stain	220
Figure 7.12:	Representative single plane confocal microscopy images of cells transfected with mCherry-empty vector and negative transfected cells using a ER stain	221

Figure 7.13: Representative 3D projections, obtained by reconstructing Z-stack images obtained by confocal microscopy, of HEK293T cells transfected with mCherry-PLC ζ^{WT} and mCherry-PLC $\zeta^{\Delta\text{EF}}$ using a ER stain 222

List of tables

Chapter 2: General materials and methods

Table 2.1:	Summary of the World Health Organisation (WHO) 2010 parameters for normal sperm	49
Table 2.2:	PCR cycles utilised to amplify exons coding for the PLC ζ active site (5-12), as well as to amplify appropriate PLC ζ isoform sequences during cloning of molecular constructs	51
Table 2.3:	PCR cycles utilised for site directed mutagenesis	58

Chapter 3: Screening of the PLC ζ gene from genomic DNA, and immunofluorescent analysis of PLC ζ in sperm, from fertile controls and infertile patients

Table 3.1:	Criteria used for patient selection for the patient population from Belgium, Iran, and the UK	75
Table 3.2:	Criteria used for patient selection, and clinical outcomes, for the patient population recruited from the Oxford Fertility Unit.....	76
Table 3.3:	Sequences of PLC ζ amplicons amplified (exons 5-12), and the forward and reverse primers used to amplify them	76
Table 3.4:	Sequencing results for exons 5-12 of all patients and controls studied during genomic screening in this project	81

Chapter 4: Functional characterisation and modes of inheritance of a novel PLC ζ mutation (H233L) identified in an infertile male

Table 4.1:	Primer sequences used to perform direct mutation detection on individually-isolated sperm	118
Table 4.2:	Thermal cycling conditions for direct mutation detection in single sperm ..	119

Table 4.3:	Thermal cycling conditions for the TaqMan SNP genotyping assay	120
Table 4.4:	Direct mutation detection in twelve individually-isolated sperm from patient ^{H398P} of the H233L and H398P PLC ζ mutations.....	131
Table 4.5:	OAD patients screened for PLC ζ ^{H233L} and PLC ζ ^{H398P}	133

Chapter 6: Generation of active recombinant human PLC ζ protein using bacterial and mammalian expression systems

Table 6.1:	The various constructs created for the purposes of this Chapter, showing PLC ζ isoform, vector used, and system targeted.....	172
Table 6.2:	Summary of primer sequences and restriction enzymes used during molecular cloning	172
Table 6.3:	Primer names and sequences used for RT-PCR	179
Table 6.4:	Fate of all 26 injected oocytes with purified protein obtained from the prokaryotic system.....	183
Table 6.5:	Individual and total mouse oocytes injected with lysates prepared from HEK293T cells transfected with pTracer-empty vector, -PLC ζ ^{WT} , and -PLC ζ ^{H398P} exhibiting Ca ²⁺ oscillations and pronuclei formation as indicated on three separate occasions.....	190

Chapter 7: Functional characterisation of wild type and mutant isoforms of PLC ζ in HEK293T cells

Table 7.1:	The various constructs created for the purposes of this Chapter, showing PLC ζ isoform, vector used, and system targeted.....	205
Table 7.2:	Summary of constructs created via site-directed mutagenesis, and the templates and primers used to create them.....	205
Table 7.3:	Summary of primer sequences and restriction enzymes used during the molecular cloning of mCherry- PLC ζ ^{ΔEF} and mCherry-PLC ζ ^{EF} isoforms ...	206

Abstract

Title: The role of the oocyte activation factor PLC ζ in human male factor infertility

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General consensus agrees that the oscillations in intracellular calcium (Ca^{2+}) leading to oocyte activation are induced by a testis-specific phospholipase C, PLCzeta (PLC ζ). Strong evidence now indicates that certain types of human infertility are caused by failure of the sperm to activate the oocyte in an appropriate manner. Such sperm exhibit absent/reduced levels of PLC ζ , while molecular analysis of the PLC ζ gene in an infertile patient identified a substitution of a conserved histidine residue at position 398 of the PLC ζ protein sequence for a proline residue (H398P), leading to severely reduced PLC ζ function and oocyte activation efficiency. Collectively, these studies indicate the therapeutic and diagnostic potential use of PLC ζ for assisted reproductive technology (ART). In the present study, a novel mutation was identified in the patient exhibiting the H398P mutation, which caused histidine to be substituted for leucine at position 233 (H233L) of the PLC ζ protein sequence. The H233L mutation severely reduced PLC ζ functional ability, and investigation of the inheritance patterns of H233L and H398P revealed that the patient inherited H233L from his mother and H398P from his father. Additionally, data suggested that quantitative immunofluorescence of PLC ζ in human sperm may not be an informative indicator of oocyte activation capability, while proportional analysis of sperm exhibiting PLC ζ immunoreactivity may reflect fertility. Density gradient washing (DGW) increased the proportion of human sperm exhibiting PLC ζ immunofluorescence, while cryopreservation reduced total PLC ζ immunofluorescence within fertile human sperm. While recombinant human PLC ζ protein was successfully purified from a prokaryotic system, this proved to be inactive following mouse oocyte microinjection. Most importantly, the current study describes for the first time, the successful production of active recombinant human PLC ζ protein within lysates from transfected transformed human embryonic kidney (HEK293T) cells, which induced characteristic patterns of Ca^{2+} release when injected into mouse oocytes. The present data indicate that wild type PLC ζ was transcribed in an active state within a eukaryotic system, while loss-of-activity PLC ζ isoforms may contribute to overall protein instability, possibly resulting in increased rates of protein degradation. It is demonstrated that PLC ζ isoforms were localised to the endoplasmic reticulum (ER), with the EF-hand domain possibly determining the localisation of the enzyme. Collectively, these results not only assist with the therapeutic application of PLC ζ , but also illustrate the complex nature of the mechanisms underlying its functional activity.



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CHAPTER 1

General introduction

Sexual reproduction is a proliferative strategy employed by a multitude of species, and is characterised by the transfer of genetic material from each parent to produce a unique individual. In mammals, males produce vast numbers of sperm (~53-55µm in length) in the testes, which are deposited in the female reproductive tract in a liquid medium (semen) via the process of ejaculation. The female oocyte is much larger in comparison (~100µm in diameter), surrounded by a glycoprotein layer (the zona pellucida; ZP), and generated in follicles within the ovaries. Following maturation, oocytes are released and transported through the fallopian tube to the uterus. Sexual reproduction involves the concerted release of male and female gametes, which subsequently fuse to result in a new, genetically unique individual; a process termed '*fertilisation*'.

Mammalian oocytes remain arrested for most of their existence at the dictyate stage of prophase I (Jones, 2007). These then undergo their first meiotic division within the ovary, immediately after which, and just before ovulation, oocytes become arrested at metaphase of the second meiotic division (MII), following the exclusion of the first polar body (Jones, 2005; Jones, 2007) (Figure 1.1A). This arrest is maintained by stabilisation of M-Phase promoting factor (MPF), the universal driver for G2/M cell cycle transition. MPF is a heterodimer consisting of a regulatory cyclin subunit, cyclin B, and a catalytic subunit, Cdc2 kinase. Active Cdc2 drives entry into M-phase by phosphorylating substrates leading to nuclear envelope breakdown and spindle formation (Horner & Wolfner, 2008). Alleviation of MII arrest must be eliminated for cell division and subsequent embryogenesis (Figure 1.1B, C) to proceed, and this occurs following fertilisation through a series of concurrent events, collectively termed '*oocyte activation*'.

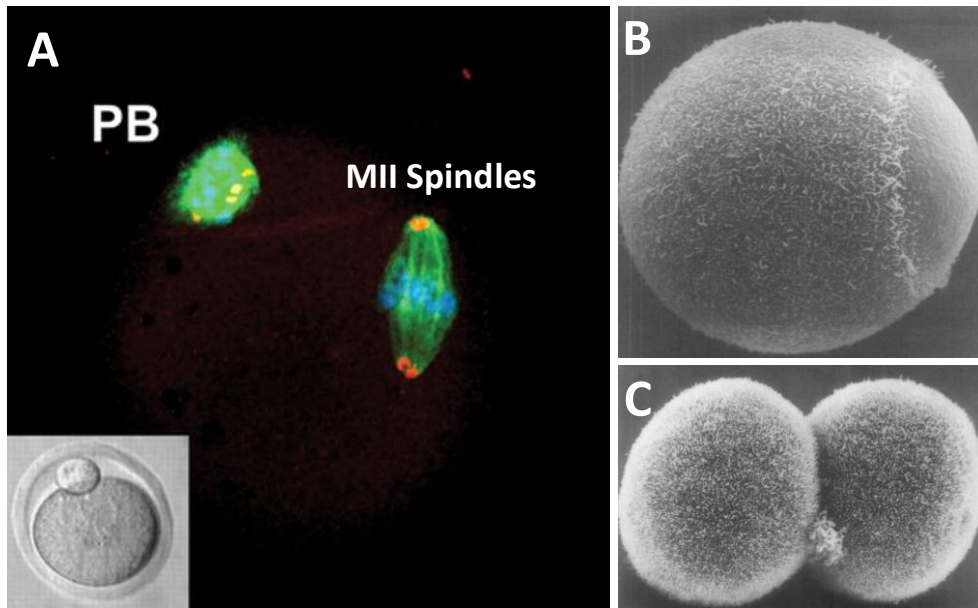


Figure 1.1: Fluorescence micrograph of an MII arrested mouse oocyte (A). Inset shows brightfield image of same oocyte. Green: polymerised microtubules; red/orange: microtubule organising centers; blue: chromatin. PB: Polar body. Scanning electron micrographs of (B) an unfertilised mouse oocyte, and (C) the early mouse embryo at the 2 cell stage following removal of the zona pellucida. Reproduced from Phillips & Shalgi (1980) and Swain *et al.* (2008) with permission.

1.1 Oocyte activation and calcium oscillations

Characterised by the formation of the second polar body (2PB) and the male and female pronuclei (Miyazaki & Ito, 2006), oocyte activation also involves cortical granule exocytosis, progression of the cell cycle, and maternal mRNA recruitment and embryonic gene expression, resulting in the initiation of embryogenesis (Kline & Kline, 1992; Swann & Ozil, 1994; Miyazaki & Ito, 2006; Publicover *et al.*, 2007; Swann & Yu, 2008). The sperm nuclear envelope is removed, and sperm-specific protamines or histones are replaced by maternal histone variants (Horner & Wolfner, 2008). Maternal mRNAs and proteins then undergo several dynamic changes, including regulated degradation, translation, or post-translational modification.

The exact mechanisms by which the sperm causes these changes in the mammalian oocyte were the subject of intense debate, until the utilisation of calcium (Ca^{2+}) sensitive dyes led to the discovery that in non-mammalian species such as sea urchins and frogs, activation was triggered by a single cytosolic Ca^{2+} transient which initiates from the sperm point of entry and traverses across the oocyte to the opposite pole (Fontanilla & Nuccitelli, 1998) (Figure 1.2A). In contrast, mammalian oocytes undergo a series of Ca^{2+} ‘pulses’, termed Ca^{2+} oscillations (Publicover *et al.*, 2007; Swann & Yu, 2008) (Figure 1.2B).

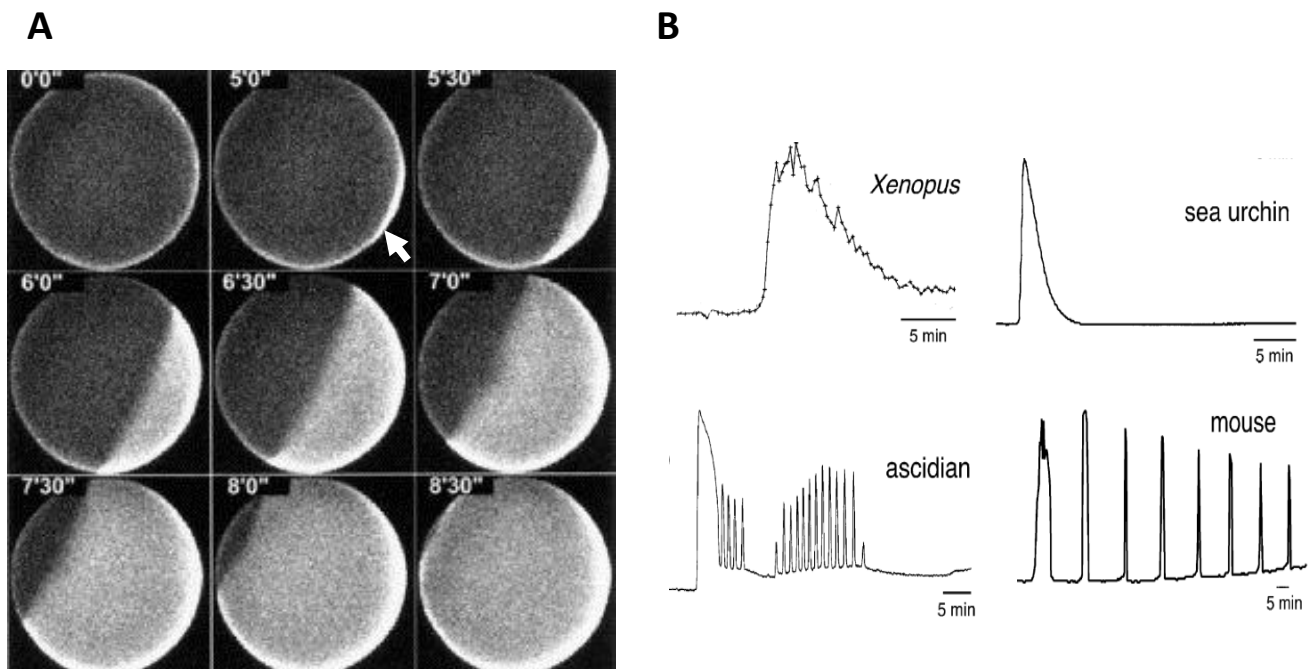


Figure 1.2: **A)** Calcium wave as observed using calcium-green-1-dextran in a *Xenopus* egg. Time 0 shows the egg's resting levels of calcium. The sperm-induced calcium wave was initiated by sperm entry (indicated by white arrow), and traversed the entire egg. **B)** Ca^{2+} responses at fertilisation in eggs and oocytes of several species. Reproduced from Fontanilla & Nuccitelli (1998), and Miyazaki (2006) with permission.

Microinjection of Ca^{2+} ions alone was sufficient to trigger embryo development to the blastocyst stage in mice (Fulton & Whittingham, 1978; Swann & Yu, 2008). Moreover the temporal pattern of Ca^{2+} oscillations was determined to be largely species-specific, with specific patterns of amplitude, duration, and frequency over time (Miyazaki *et al.*, 1993; Jones, 1998; Ducibella *et al.*, 2002; 2006). Ca^{2+} oscillations in mammalian oocytes are now generally acknowledged to be the direct result of inositol trisphosphate (IP_3)-mediated Ca^{2+} release (Parrington, 2001; Swann *et al.*, 2006; Whitaker, 2006; Parrington *et al.*, 2007; Saunders *et al.*, 2007; Swann & Yu, 2008). Blocking, down-regulating, or reducing the expression levels of IP_3 receptors (IP_3Rs) in mouse and hamster oocytes, all led to the inhibition of Ca^{2+} oscillations and oocyte activation (Miyazaki *et al.*, 1993; Brind *et al.*, 2000; Jellerette *et al.*, 2000; Xu *et al.*, 2003). Furthermore, cytosolic increases in IP_3 concentration during fertilisation in mammalian oocytes (Swann & Yu, 2008) provided further support for the importance of IP_3 levels and IP_3 -mediated Ca^{2+} release.

The alleviation of MII arrest in mammalian oocytes is thought to occur through the degradation of cyclin B1 proteolysis, mediated by ubiquitin/proteasome activation via Ca^{2+} oscillations (Miyazaki & Ito, 2006). Binding of Ca^{2+} to calmodulin activates calmodulin-dependent kinase II (CaMKII) (Lorca *et al.*, 1993; Miyazaki & Ito, 2006), a repetitive process which re-occurs coincident with each Ca^{2+} peak in fertilised mouse oocytes (Markoulaki *et al.*, 2004). Cyclin B1 is then poly-ubiquitinated by the anaphase promoting complex or cyclosome (APC/C), an E3 ubiquitin ligase, resulting in the degradation of cyclin B1. This process is prevented in unfertilised oocytes by cytosolic factor (CSF), which assists MPF in maintaining MII arrest (Hyslop *et al.*, 2004; Jones, 2004; Miyazaki & Ito, 2006). Persistent Ca^{2+} oscillations are thought to be responsible for pronucleus

formation via the reduction of mitogen-associated protein kinase (MAPK) activity (Ducibella *et al.*, 2002; Miyazaki & Ito, 2006) (Figure 1.3).

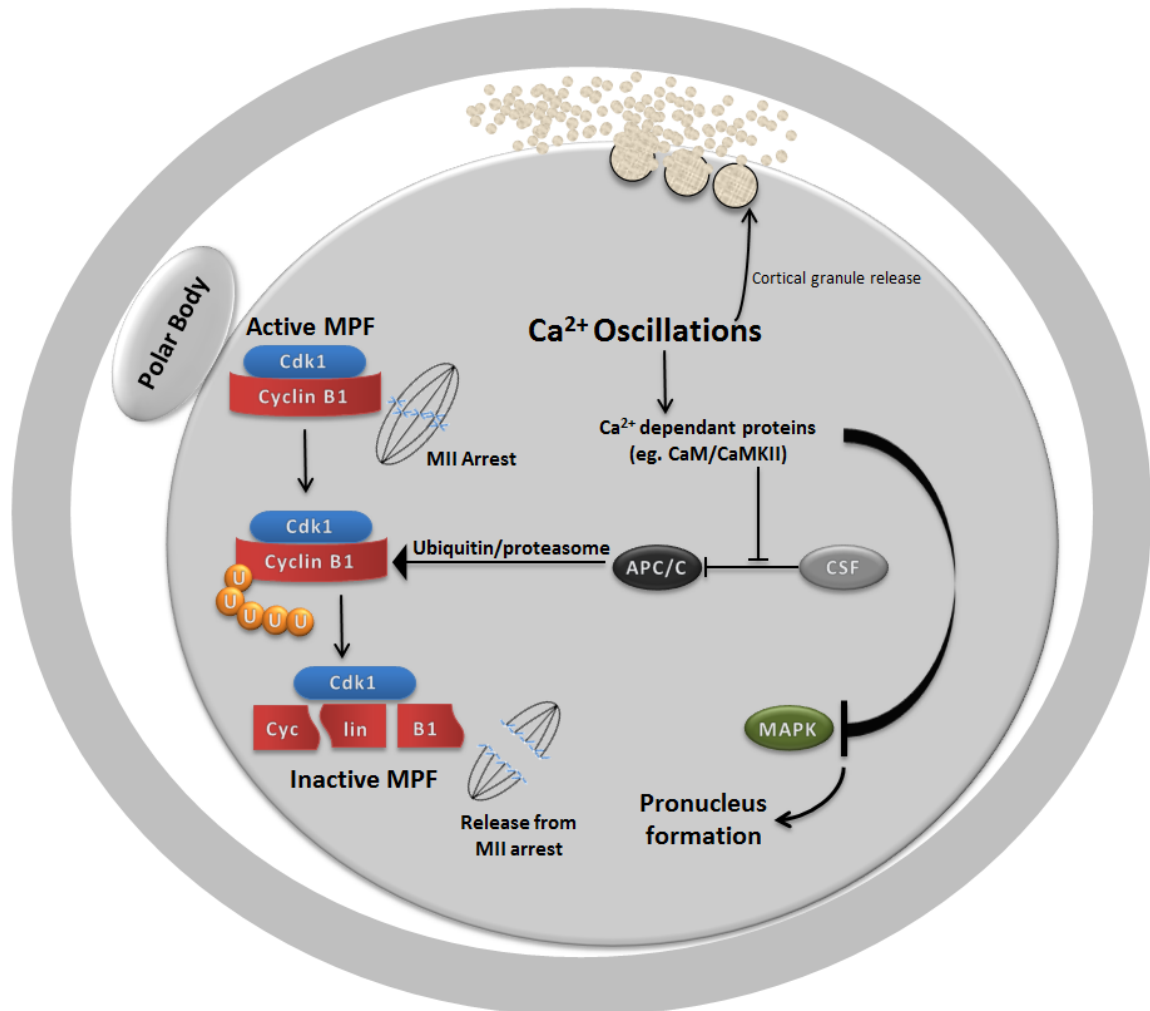


Figure 1.3: Schematic representation of the signalling mechanism downstream of Ca²⁺ release at fertilisation, thought to alleviate MII arrest in mammalian oocytes and leading to subsequent embryogenesis. MPF: M-Phase promoting factor; U: Ubiquitination; CaM: Calmodulin; CaMKII: calmodulin-dependent kinase II; APC/C: Anaphase promoting complex/cyclosome; CSF: Cytostatic factor; MAPK: mitogen-associated protein kinase. Figure created by JK.

A key role of the fertilization Ca^{2+} signal is thus to restart the cell cycle, via pathways that interact with the cell-cycle-control machinery. In mammals and ascidians, full activation at fertilization requires repetitive Ca^{2+} spikes (Whitaker, 2006). Most Ca^{2+} signals that rely on Ca^{2+} release from intracellular stores, however generated, have an oscillatory component (Berridge & Galione, 1988; Whitaker, 2006), involving Ca^{2+} -induced Ca^{2+} -release (CICR). Indeed, increased levels of cytoplasmic Ca^{2+} enhance the probability of Ca^{2+} channels within intracellular stores opening, while very high concentrations of cytoplasmic Ca^{2+} may have inhibitory effects on such channels (Ehrlich, 1995; Whitaker, 2006). In addition, the depletion of ER Ca^{2+} may exert a further negative-feedback element, while additional positive feedback can come from Ca^{2+} -stimulated hydrolysis of PIP_2 . Furthermore, data suggest that the oscillatory nature of Ca^{2+} release at mammalian oocyte activation may be governed by the basic feedback properties of IP_3Rs (Berridge & Galione, 1988; Adkins & Taylor, 1999; Williams, 2002; Malcuit *et al.*, 2006; Whitaker, 2006). These Ca^{2+} oscillations are then terminated following exit from cell cycle arrest, indicating that the oscillatory nature of Ca^{2+} release is due to the absence of Cdk activity (Whitaker, 2006).

Oocytes appear to be remarkably sensitive to each wave of oscillations, with early events such as cortical granule exocytosis requiring fewer oscillations than subsequent events such as the alleviation of MII arrest (Malcuit *et al.*, 2006; Stitzel & Seydoux, 2007). The frequency and amplitude of Ca^{2+} oscillations have been reported to affect protein expression profiles in early mouse embryos (Ducibella *et al.*, 2002), and embryonic development in rabbits, playing a role in compaction, blastocyst formation, and the rate of successful transplantation of 4-cell embryos to host mothers (Swann & Ozil, 1994;

Miyazaki & Ito, 2006). Frequencies and amplitudes of Ca^{2+} oscillations are also directly responsible for cell cycle progression, with varying Ca^{2+} transients resulting in different rates of cell cycle progression (Ducibella *et al.*, 2002). Considering that the rate of progression to the 2- and 4-cell stages of human oocytes following fertilisation has been proposed as an indicator of normal embryogenesis (Wong *et al.*, 2010), the profile of Ca^{2+} oscillations at fertilisation may not only be necessary for oocyte activation, but equally important for subsequent embryogenesis. Indeed, alterations in the Ca^{2+} oscillatory activity of vitrified mouse oocytes were found to affect oocyte quality and subsequent embryonic development directly (Kim *et al.*, 2011).

Spatial and temporal Ca^{2+} and IP_3 gradients assist in the establishment of the dorsal–ventral axis of various developing embryos (Ozil *et al.*, 2006; Web & Miller, 2006), with the velocity and duration of Ca^{2+} waves playing similar roles (Jaffe, 2008; Whitaker, 2008). The induction of a variety of Ca^{2+} release profiles did not universally result in good quality embryos (Fulton & Whittingham, 1978; Kubiak, 1989; Rogers *et al.*, 2006; Tóth *et al.*, 2006). Ca^{2+} transients at fertilisation are also responsible for the activation of Ca^{2+} -sensitive genes or proteins important in subsequent embryo development, such as CAMKII (De Koninck & Schulman, 1998; Hudmon & Schulman, 2002). Indeed, CaMKII γ (the predominant CaMKII isoform in mouse oocytes) is thought to control mouse oocyte activation specifically via cell cycle resumption (Backs *et al.*, 2010). Although it is well-established that Ca^{2+} oscillations are of the utmost importance for oocyte activation, the precise mechanism responsible, particularly in relation to the relative roles played by the respective gametes during fusion, has been subject to intense scrutiny. Three predominant models have been hypothesised: 1) the Ca^{2+} conduit model, 2)

the membrane receptor model, and 3) the soluble sperm factor model (Swann *et al.*, 2006; Saunders *et al.*, 2007; Parrington *et al.*, 2007).

The Ca^{2+} conduit model

This model hypothesised the introduction of a bolus of Ca^{2+} directly into the oocyte following gamete fusion, leading to Ca^{2+} -induced Ca^{2+} release (CICR; Jaffe, 1983; 1991), and was subsequently modified to suggest that sperm membrane channels acted as conduits following gamete fusion thereby facilitating Ca^{2+} influx into the oocyte (Figure 1.4A) (Créton & Jaffe, 1995; 2001). While initially an attractive model, particularly for sea urchin egg activation, experimental evidence strongly indicated that IP_3 -mediated Ca^{2+} release was an integral aspect of oocyte activation. Furthermore, studies failed to detect localised elevations in cytoplasmic Ca^{2+} during mouse gamete fusion (Jones *et al.*, 1998). Furthermore, sea urchin eggs can be activated by sperm in seawater lacking Ca^{2+} (Epel *et al.*, 1982; Whitaker, 2006), rendering it difficult to envisage how Ca^{2+} flux through the sperm could be sustained where Ca^{2+} concentrations are lower or similar to resting concentrations within the egg (Jones *et al.*, 1998; Parrington *et al.*, 2007).

The membrane receptor model

This model proposed that an interaction between a ligand on the sperm surface and a corresponding receptor on the oocyte triggered activation (Figure 1.4B), and was the dominant hypothesis for many years (Jaffe, 1990; Schultz & Kopf, 1995; Evans & Kopf, 1998; Parrington *et al.*, 2007). Adopting one of the most fundamental features of cell signalling in somatic cells, this model proposed that a signalling cascade triggered by

sperm/oocyte interaction, would lead to the activation of a phospholipase C (PLC) inside the oocyte (Jaffe, 1990; Schultz & Kopf 1995; Evans & Kopf, 1998). However, despite many targeted studies, such a sperm ligand/oocyte receptor remains to be characterised (Parrington *et al.*, 2007). Critically, the successful nature of microinjection techniques such as intracytoplasmic sperm injection (whereby sperm is directly microinjected into the oocyte cytosol; ICSI) casts significant doubt over such a mechanism as this method bypasses sperm/oocyte membrane interaction completely.

The soluble sperm factor model

This model proposed that oocyte activation was triggered by a soluble factor released from the sperm into the oocyte during, or immediately following, gamete fusion (Figure 1.4C). A series of studies, involving the injection of sperm extracts into eggs and oocytes of a variety of species, including marine worms, and ascidians, resulted in successful Ca^{2+} release and oocyte activation (Swann, 1990; Kyojuka *et al.*, 1998; Stricker, 1999). Sperm extracts from frogs, chickens, and tilapia fish, have also been shown to trigger Ca^{2+} oscillations in mouse oocytes (Dong *et al.*, 2000; Coward *et al.*, 2003; 2005), suggesting the existence of a similar sperm-based mechanism throughout a wide spectra of species. However, the precise identity of this ‘sperm factor’ remained a mystery for some time, although a considerable body of research suggested that the IP_3 -dependant nature of oocyte activation depended upon a PLC-mediated mechanism (Parrington *et al.*, 2007).

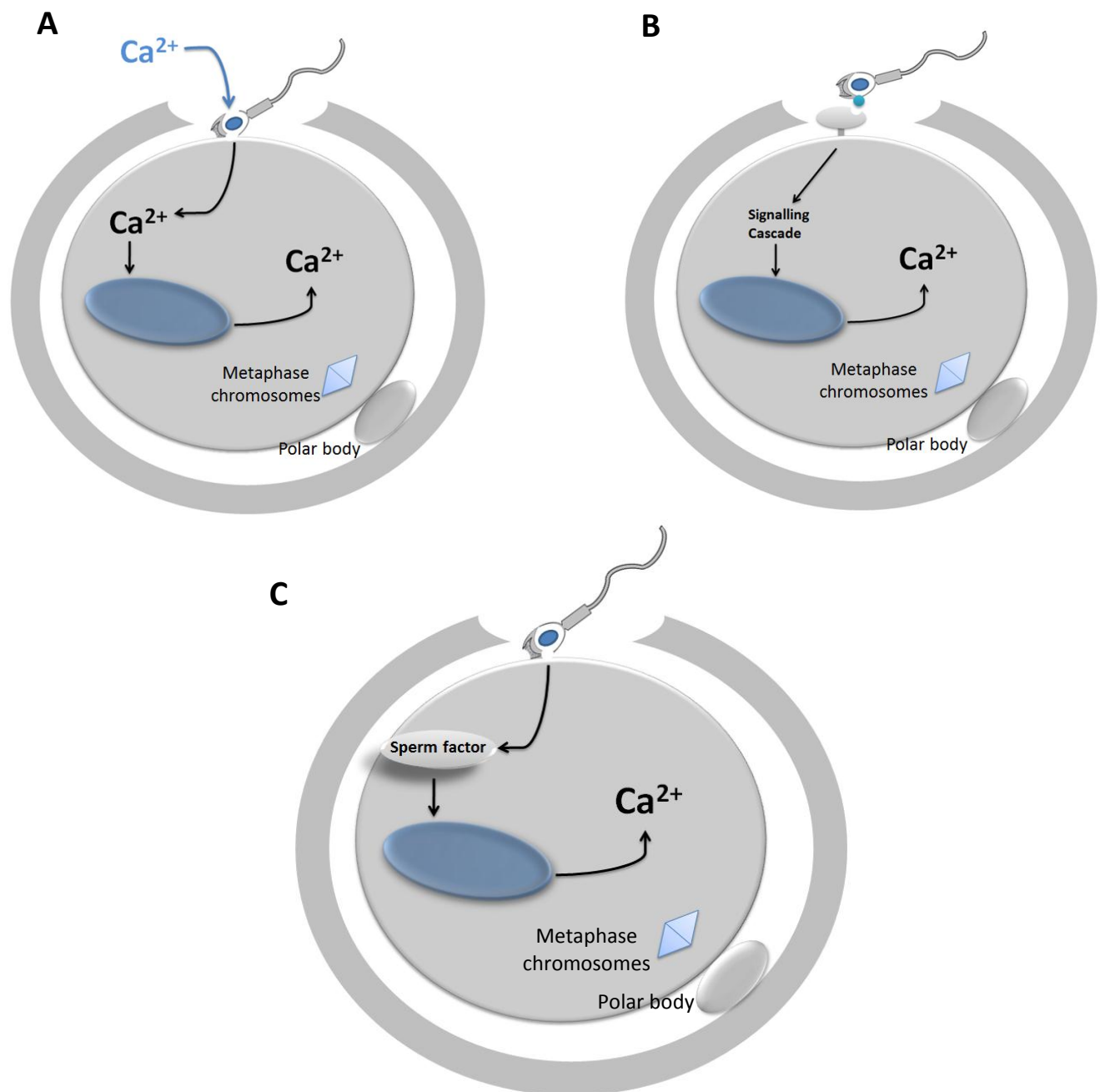


Figure 1.4: Schematic representations of **A)** Ca^{2+} conduit, **B)** membrane receptor, and **C)** sperm factor models of oocyte activation. Figure adapted from Parrington *et al.* (2007).

Data indicated that the mammalian sperm factor was a sperm-specific PLC possessing distinct enzymatic properties compared to other known PLCs (Jones *et al.*, 1998; 2000). Indeed, this correlated with the hypothesis that oocyte activation involved Ca^{2+} oscillations generated in an IP_3 -mediated manner, supporting the notion that the soluble sperm factor was a PLC which mediated the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) to IP_3 and diacylglycerol (DAG) which activates protein kinases, such as protein kinase C (PKC)-mediated signalling pathways (Miyazaki *et al.*, 1993; Saunders *et al.*, 2002; Swann & Yu, 2008). General consensus agreed that the soluble factor responsible for Ca^{2+} release within oocytes was sperm-specific, as extracts from other tissues did not result in Ca^{2+} induction upon oocyte injection (Swann, 1990; Wu *et al.*, 1997). However, the specific identity of such a PLC isozyme remained a mystery for some time.

1.2 PLCs at fertilisation and oocyte activation

Phosphoinositide metabolism, a vital intra-cellular signalling system, is involved in multiple cellular functions such as hormone secretion, neurotransmitter transduction, growth factor signalling, membrane trafficking, cytoskeletal regulation, and has also been linked to fertilisation and embryogenesis (Singal *et al.*, 2004; Janetopoulos & Devreotes, 2006; Cockcroft & Carvou, 2007; Nakamura & Fukami, 2009; Fukami *et al.*, 2010). PLC and IP_3R signalling mechanisms are also involved in sperm chemotaxis, where elevated levels of Ca^{2+} may modify flagellar bending and sperm motion paths (Hofmann & Majerus, 1984; Bahat & Eisenbach, 2006; Eisenbach & Giojalas, 2006; Bahat & Eisenbach, 2010).

Currently, there are 13 known mammalian PLC isozymes, categorised on the basis of structure and regulatory activation mechanisms: PLCdelta (PLC δ 1, 3, and 4), PLCbeta (PLC β 1-4), PLCgamma (PLC γ 1 and 2), PLCepsilon (PLC ϵ), PLCzeta (PLC ζ), and PLCeta (PLC η 1 and 2) (Rhee, 2001; Hwang *et al.*, 2005; Nakahara *et al.*, 2005; Zhou *et al.*, 2005; Fukami *et al.*, 2010). PLC isozymes may contain catalytic X and Y domains as well as various regulatory domains, including the C2 domain, EF-hand motif, and pleckstrin homology (PH) domain, in various conformations, depending on the isozyme. The specific functions of these domains are discussed at length later in this thesis. Some isozymes also consist of subtype-specific domains, thought to contribute towards specific regulatory mechanisms. These include the Src homology (SH) domain in PLC- γ (Rhee *et al.*, 2001) and the Ras-associating and Ras-GTPase exchange factor-like domains in PLC- ϵ (Kelley *et al.*, 2001; Song *et al.*, 2001).

Sperm from PLC δ 4 knockout (KO) mice induced activation in fewer oocytes following *in vitro* fertilisation, and failed to elicit Ca²⁺ oscillations indicating an essential role for sperm-borne PLC δ 4 in oocyte activation (Fukami *et al.*, 2001). Additionally, solubilised mouse ZP was able to induce the acrosome reaction (an essential step in fertilisation involving the digestion of the ZP) in sperm from normal mice, but could not do so in PLC- δ 4 KO sperm. Elevations of Ca²⁺ levels were thought to play a primary role in this process (Darszon *et al.*, 2001; Breitbart, 2002), and sperm from normal mice treated with ZP exhibited continuous elevations in Ca²⁺, while incubation of ZP with PLC δ 4 KO sperm induced only a small increase in Ca²⁺, suggesting a role for PLC δ 4 in the ZP-induced acrosome reaction (Nakamura & Fukami, 2009; Fukami *et al.*, 2003; 2010).

Evidence suggests that PLC γ -1, modulated by tyrosine phosphorylation, may be activated in mouse spermatozoa (Tomes *et al.*, 1996; Feng *et al.*, 1997; Roldan & Shi, 2007). Immunostaining indicated that PLC γ -1 was localised to the sperm head, and that capacitation induced a translocation of this localisation pattern (Tomes *et al.*, 1996). An increase in PLC γ -1 activity was observed following treatment with ZP. However, this elevation was prevented by suppressing ZP-induced acrosomal exocytosis (Leyton *et al.*, 1992; Roldan & Shi, 2007). While there is no direct evidence supporting a role for PLC β in sperm, both PLC β -1 and β -3 have been identified in acrosomal regions of mouse sperm, along with G α q/11 (Walensky & Snyder, 1995; Fukami *et al.*, 2001). Considering that PLC β is activated by pertussis toxin-insensitive GTP-binding proteins Gq and G11 in somatic cells, the observation that progesterone-stimulated DAG formation was not blocked by pertussin toxin may infer a possible role for PLC β in acrosomal exocytosis (Murase & Roldan, 1996; Roldan & Shi, 2007). Indeed, sperm from PLC β -1 KO mice exhibited lower acrosome reaction rates than their normal counterparts (Choi *et al.*, 2001). However, more in-depth studies are still required to elucidate the exact roles of these PLC isozymes during fertilisation (Roldan & Shi, 2007).

Several observations suggest that there may be a role for endogenous oocyte PLCs during oocyte activation (Saunders *et al.*, 2002). Oocytes contain significant levels of PLC isoforms, including β , γ , and δ , which may be regulated by the Ca²⁺ oscillations at fertilisation (Igarashi *et al.*, 2007). Reduced levels of oocyte PLC β 1 decreased the amplitude of Ca²⁺ oscillations at oocyte activation, but did not change their duration or frequency. Over-expression of PLC β 1 in oocytes prior to fertilisation did not result in spontaneous Ca²⁺ oscillations, but altered the Ca²⁺ oscillation profile following

fertilisation, indicating a role for oocyte derived PLCs in sperm-induced oocyte activation in mammals (Igarashi *et al.*, 2007). Furthermore PLC β 1 has recently been implicated in nuclear translocation following meiotic resumption in mouse oocytes, apparently to perichromatin and interchromatin granules, followed by a subsequent shift to the nucleoplasm (Avazeri *et al.*, 2000; Lefèvre *et al.*, 2007).

In starfish eggs, the Ca²⁺ rise associated with activation requires an egg Src family kinase (SFK) that activates PLC γ via an SH2 domain-mediated mechanism involving the endoplasmic reticulum (ER) (Tokmakov *et al.*, 2002; Runft *et al.*, 2004). The extent of the presence and role of PLC δ , ϵ , or ζ isoforms in echinoderm eggs is not yet known. However PLC β may be activated by heterotrimeric G protein-coupled receptors, while PLC γ may be activated by receptor and non-receptor protein tyrosine kinases (PTK), or via translocation to the plasma membrane (Runft *et al.*, 2004; Yin & Eckberg, 2009). However, whether G protein-PLC β or PTK-PLC γ plays a role during egg activation in other invertebrates, or whether these pathways function synergistically remains controversial (Yin & Eckberg, 2009).

Coward *et al.* (2007) recently identified a novel PLC δ isoform in sea urchin gametes, termed PLC δ su, although its precise role during fertilisation and early embryogenesis remains unknown. While a green fluorescent protein tagged PLC δ su PH domain was observed to localise to the plasma membrane of eggs, increasing in intensity at fertilisation, recombinant PLC δ su failed to elicit Ca²⁺ signals characteristic of fertilisation when injected into mouse oocytes and sea urchin eggs, suggesting that PLC δ su may not be

directly involved in egg activation, but may play a role in further downstream extracellular signals. Intriguingly, the *in vivo* expression of PLC δ su cRNA did not result in Ca²⁺ transients in either mouse oocytes or sea urchin eggs. This observation is consistent with the behaviour of recombinant PLC β 1, PLC γ 1, PLC γ 2, PLC δ 1, PLC δ 3 and PLC δ 4 protein and cRNA, none of which cause Ca²⁺ release in mouse oocytes.

However, the specific PLC isozyme responsible for oocyte activation in mammals remained a mystery until Saunders *et al.* (2002), using mouse express sequence tag (EST) databases, identified a novel, and testis-specific PLC, termed PLCzeta (PLC ζ), a ~74kDa protein which was proven to play a key role in oocyte activation. Subsequent studies have also identified further mammalian orthologues of PLC ζ in human, hamster, monkey, and horse sperm (Cox *et al.*, 2002; Saunders *et al.*, 2002; Yoneda *et al.*, 2006; Young *et al.*, 2009; Cooney *et al.*, 2010; Bedford-Guaus *et al.*, 2011).

1.3 PLC ζ : the mammalian oocyte activation factor

Expression studies in mice concluded that PLC ζ mRNA was first detectable in spermatids (Saunders *et al.*, 2002), while more systematic studies of PLC ζ mRNA expression during porcine spermatogenesis observed PLC ζ mRNA translation in elongating spermatids (Yoneda *et al.*, 2006). Northern blot analyses of testes from post-natal hamsters showed that PLC ζ mRNA was present as early as day 17 (Young *et al.*, 2009). Currently, however, developmental analysis of the first stages of PLC ζ expression within testes requires further work, most particularly in human beings.

Numerous studies support the identity of the mammalian oocyte activation factor as being PLC ζ . The injection of recombinant PLC ζ RNA into mouse oocytes resulted in the initiation of Ca²⁺ oscillations and supported embryonic development to the blastocyst stage (Saunders *et al.*, 2002; Kouchi *et al.*, 2005). Immuno-depletion of PLC ζ from sperm extracts suppressed their ability to release Ca²⁺ (Saunders *et al.*, 2002), while sperm fractionation studies correlated the presence of PLC ζ in sperm to their ability to induce Ca²⁺ oscillations (Fujimoto *et al.*, 2004; Kurokawa *et al.*, 2005). Furthermore, RNA interference (RNAi) experiments yielded transgenic mice exhibiting disrupted PLC ζ expression in the testis, while sperm from these mice induced Ca²⁺ oscillations that ended prematurely, with a clear reduction in litter size (Knott *et al.*, 2005). Data also strongly suggest that PLC ζ may be a universal feature of vertebrate oocyte activation. For example, sperm extracts and PLC ζ cRNA from one species are able to elicit Ca²⁺ release upon microinjection into oocytes from another species (Cox *et al.*, 2002; Beford-Gaus *et al.*, 2008). Furthermore, non-mammalian testis-specific PLC ζ homologues have been identified in the chicken (Coward *et al.*, 2005), medaka fish (Ito *et al.*, 2008), and quail (Mizushima *et al.*, 2008).

Interestingly however, Coward *et al.* (2011) recently identified ovarian and brain forms of PLC ζ in puffer-fish (*Takifugu rubripes*). Injection of cRNA corresponding to the ovarian isoform into mouse oocytes did not result in Ca²⁺ oscillations. While it is possible that puffer-fish PLC ζ is physiologically inactive, a comparison of puffer-fish PLC ζ amino acid sequences with those of PLC ζ orthologues from other species demonstrated that amino acid residues exhibited to be important for enzymatic catalysis and substrate/Ca²⁺ binding in other PLCs were all present within puffer-fish PLC ζ (Saunders *et al.*, 2002;

Kuroda *et al.*, 2006; Coward *et al.*, 2011). It therefore remains possible that puffer-fish PLC ζ requires further oocyte- or sperm-based factors for Ca²⁺ inducing activity. A surprising feature of the pattern of expression of puffer-fish PLC ζ is its expression within the brain, particularly considering that PLC ζ is generally thought to be testis-specific (Coward *et al.*, 2011). While, Yoshida *et al.* (2007) found expression of PLC ζ mRNA in the brain of both male and female mice, this directly contradicts the findings of Saunders *et al.* (2002), who found no such expression in the brain. While it would be of interest to investigate the physiological relevance of brain populations of PLC ζ , it would first be necessary to confirm these earlier findings using more rigorous methodology (Coward *et al.*, 2011).

Although it is possible that egg-based PLC ζ in puffer-fish is an evolutionary adaption related to reproductive strategy of the species, this discovery is particularly interesting given the debate regarding the mechanism responsible for oocyte activation (Evans & Kopf, 1998; Miyazaki & Ito, 2006; Parrington *et al.*, 2007; Coward *et al.*, 2011). The discovery of this oocyte-specific PLC ζ orthologue raises queries of how a sperm stimulus may activate the oocyte. While the majority of scientific opinion agrees that PLC ζ is the endogenous sperm factor, recent studies have demonstrated possible candidates for sperm factors apart from PLC ζ , which are able to induce meiotic progression or the typical pattern of Ca²⁺ oscillations in a variety of species. Harada *et al.* (2007) identified a new 45kDa protein, termed citrate synthase, as the major component responsible for egg activation in the newt *Cynops pyrrhogaster*. Wu *et al.* (2007) also reported another possible candidate for the sperm factor, which resides in the post-acrosomal sheath region of the perinuclear theca, termed post-acrosomal sheathWW domain-binding protein

(PAWP), in bovine sperm and other mammalian species. However, the molecular mechanisms underlying the precise function of both PAWP and citrate synthase are currently unknown (Wu *et al.*, 2007; Aarabi *et al.*, 2010). It is, of course, possible that oocyte activation involves the collective action of PLC ζ and other sperm factors. However, these theories remain to be established (Kashir *et al.*, 2010). Furthermore, it is possible that oocyte based factors, similar to puffer-fish PLC ζ may be contributing towards overall oocyte activation.

1.4 PLC ζ structure and function

Despite much targeted research, many questions remain concerning PLC ζ mechanism of action, and intracellular localisation within both sperm and oocytes. The current understanding is described in Fig 1.5. PLC ζ exhibits typical PLC domain structure (Saunders *et al.*, 2002) and has closest homology with PLC δ isoforms (Katan, 1998; Rebecchi & Pentyala, 2000), particularly with PLC δ 1 (Swann *et al.*, 2006). PLC ζ possesses characteristic X and Y catalytic domains which form the active site common to all PLCs (Rebecchi & Pentyala, 2000; Swann *et al.*, 2006), a C2 domain, and a set of EF hands. One major difference is the absence of a pleckstrin homology (PH) domain in PLC ζ , which is also the smallest known mammalian PLC (Rebecchi & Pentyala, 2000; Saunders *et al.*, 2002) (Figure 1.6). All PLC ζ active site residues are conserved, or conservatively replaced, mutagenesis of which leads to the loss of Ca²⁺ induction ability, confirming that as with PLC δ 1, the active site of PLC ζ is responsible for targeting PIP₂ to cause IP₃ mediated Ca²⁺ release (Swann *et al.*, 2006).

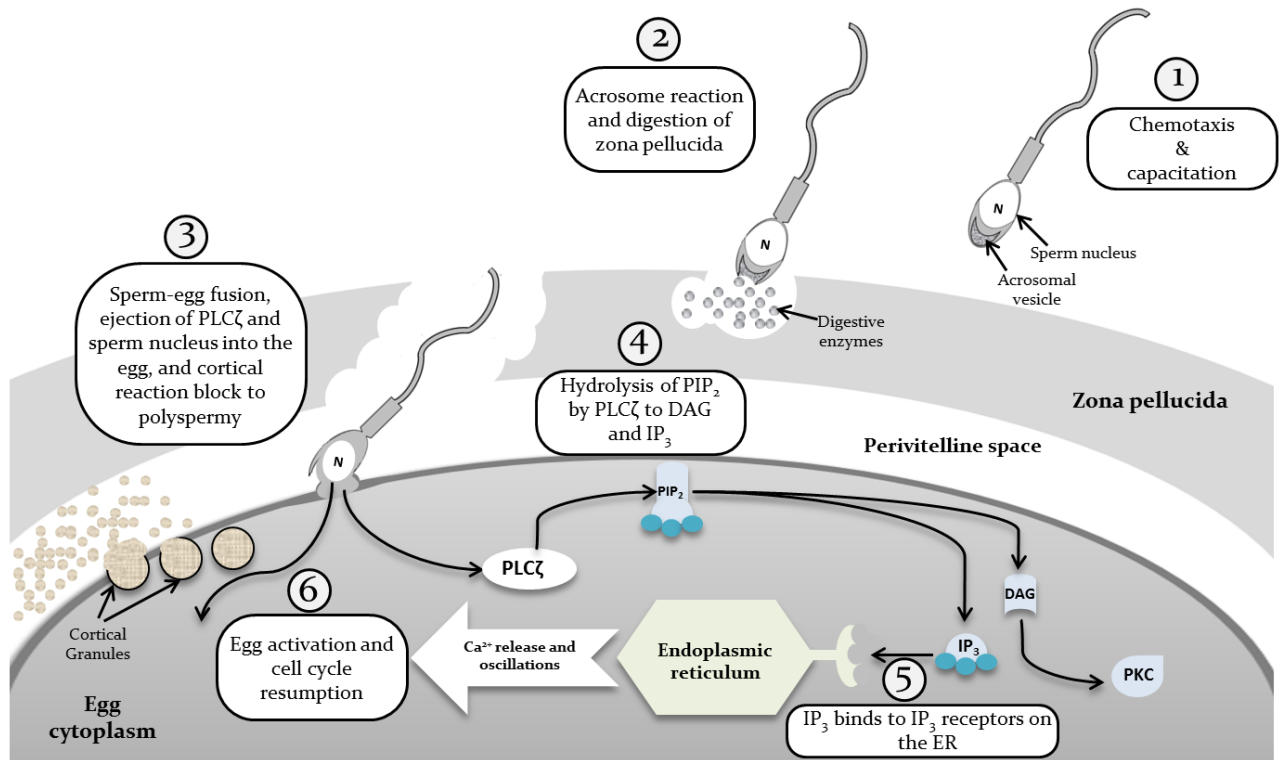


Figure 1.5: Schematic representation of the current understanding of PLCζ mechanism of action. Following sperm/oocyte fusion, PLCζ is released into the cytoplasm of the oocyte, where it facilitates the hydrolysis of membrane-bound PIP₂ to DAG and IP₃, triggering Ca²⁺ release from intracellular Ca²⁺ stores, leading to Ca²⁺ induced Ca²⁺ release and oocyte activation. N: Nucleus; PIP₂: Phosphatidylinositol 4,5-bisphosphate; IP₃: inositol trisphosphate; DAG: diacylglycerol; PKC: Protein kinase C; ER: Endoplasmic reticulum. Figure created by JK.

PLCζ is also distinct from other PLC isoforms in its high sensitivity to Ca²⁺ (Rebecchi & Pentyala, 2000; Kouchi *et al.*, 2004), which may explain why PLCζ is much more effective than other PLCs at causing IP₃ production and Ca²⁺ release in the oocyte cytoplasm (Swann *et al.*, 2006). Mammalian PLCζ isoforms have four EF-hand motifs at their N-termini, which appear to play an important structural role in PIP₂ targeting. Ca²⁺ sensitivity, and enzymatic activity (Fujimoto *et al.*, 2004; Kouchi *et al.*, 2004; Yoda *et al.*, 2004; Kurokawa *et al.*, 2005; Nomikos *et al.*, 2005; Swann *et al.*, 2006; Nakanishi *et al.*, 2008).

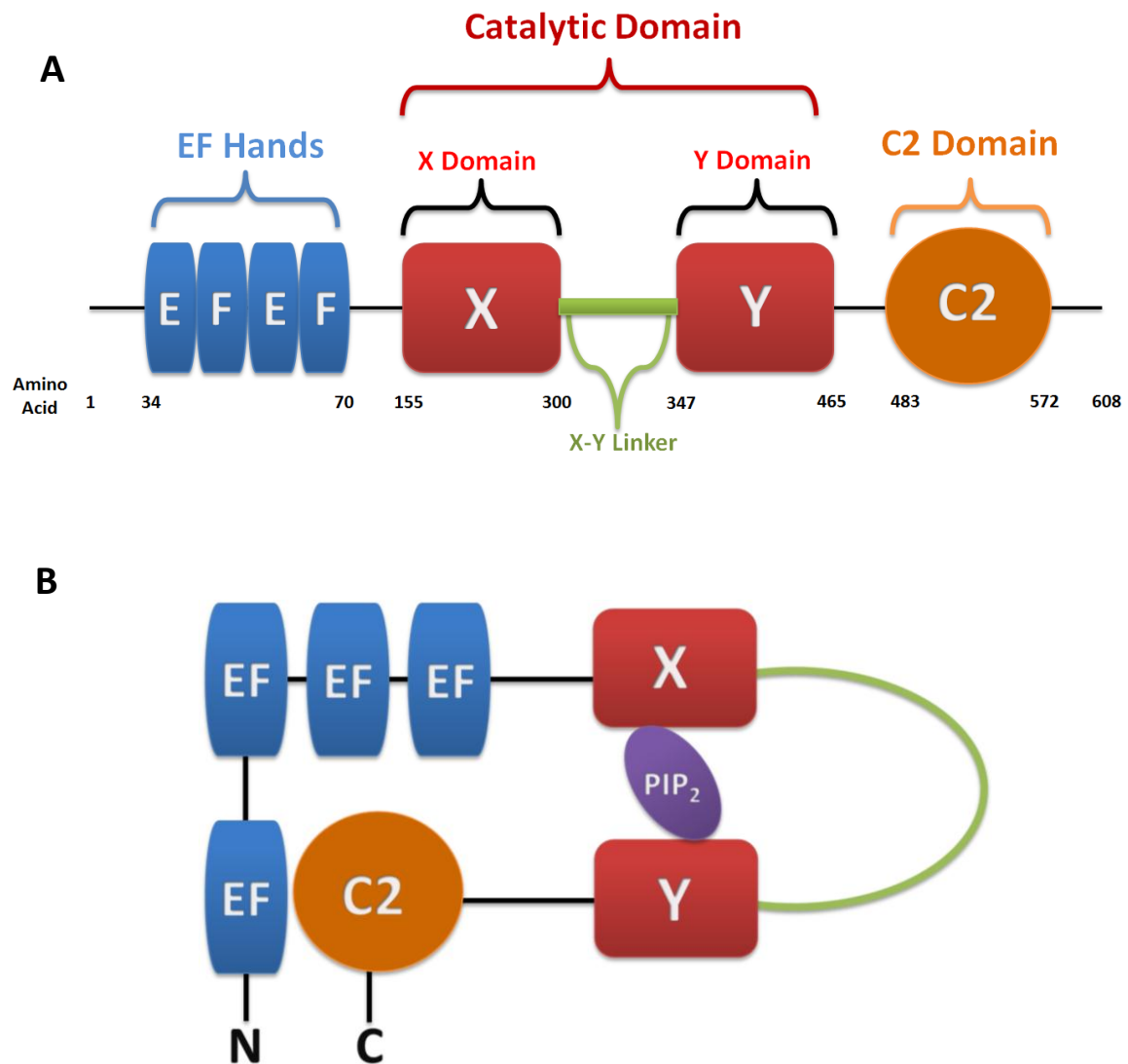


Figure 1.6: Schematics of **A**) PLC ζ domain distribution with domains highlighted in the indicated colours, and **B**) the predicted folding of PLC ζ required to maintain Ca^{2+} oscillatory activity. 6B adapted from Kuroda *et al.* (2006). N: N-terminus; C: C-terminus; PIP₂: phosphatidylinositol 4,5-bisphosphate

Without a PH domain, it currently remains unclear how PLC ζ targets its membrane-bound substrate PIP₂, since in other PLCs the PH domain serves to anchor the enzyme to either specific plasma membrane proteins, as is the case for the β , γ , and ϵ isoforms of PLCs, or directly to PIP₂ as is the case in PLC δ 1 (Rebecchi & Pentyala, 2000;

Rhee, 2001). Intriguingly, Lee *et al.* (2004) suggested that the PH domain is not integral to the membrane localising ability of PLC δ 4. Considered a regulatory domain, the C2 domain may also aid PLC ζ in targeting membrane bound PIP₂ (Swann *et al.*, 2006). There is also evidence to suggest that the C2 domain is required for the ability of PLC ζ to initiate oocyte activation (Nomikos *et al.*, 2005; Swann *et al.*, 2006).

The other non-catalytic domain of PLC ζ that may regulate its activity is the segment between the X and Y catalytic domains, the X-Y linker (Swann *et al.*, 2006; Nomikos *et al.*, 2007). The proximity of this apparently unstructured cluster of residues to the active site indicates some potential involvement in regulating catalytic activity, or PIP₂ binding (Swann *et al.*, 2006; Nomikos *et al.*, 2007). Evidence suggests that PLC ζ remains functional following proteolytic cleavage at the X-Y linker, although data indicates that these fragments can reform complexes to retain activity (Kurokawa *et al.*, 2007). Intriguingly, Hicks *et al.* (2008) showed that the X-Y linker in most, if not all PLC isoforms plays an auto-inhibitory role, the deletion of which results in elevated activity. Indeed, proteolytic cleavage at the X-Y linker is thought to be required in order for PLC ζ to be able to bind to the membrane and act upon PIP₂ (Saunders *et al.*, 2007), raising questions as to the involvement of the X-Y linker in PIP₂ binding. However, the X-Y linker has been shown to be a basic requirement for PLC ζ binding to PIP₂, seemingly also playing a role in eliciting Ca²⁺ oscillations (Nomikos *et al.*, 2011b; 2011c).

Following fertilisation in mouse oocytes, Ca^{2+} oscillations cease at the time of pronuclei formation (Marangos *et al.*, 2003) with subsequent oscillations observed in mouse zygotes during mitosis (Carroll, 2001; Marangos *et al.*, 2003). An explanation of such cell-cycle dependant termination and resumption, is that PLC ζ localises to the pronuclei during interphase due to a nuclear localisation signal (NLS), resulting in the cessation of Ca^{2+} oscillations which resume following pronuclear envelope breakdown during the fertilised oocyte's entry into mitosis (Deguchi *et al.*, 2000; Larman *et al.*, 2004; Yoda *et al.*, 2004; Sone *et al.*, 2005; Kuroda *et al.*, 2006; Swann *et al.*, 2006; Ito *et al.*, 2008). While it remains possible that there is a role for mouse PLC ζ within the nucleus itself, human PLC ζ does not translocate to the pronucleus when injected into mouse oocytes (Ito *et al.*, 2008; Kashir *et al.*, 2010), the relevance of which remains less clear for human fertilisation.

Currently, queries remain as to how PLC ζ is able to bind to its substrate PIP₂. Following injection of fluorescently tagged PLC ζ cRNA, activating oocytes demonstrated that PLC ζ universally diffuses to the oocyte cytoplasm, suggesting that its substrate PIP₂ may be targeted at intracellular membranes (Rice *et al.*, 2000; Yoda *et al.*, 2004; Sone *et al.*, 2005; Kuroda *et al.*, 2006; Ito *et al.*, 2008; Yu *et al.*, 2008a; 2008b). While general consensus agrees that the majority of cellular PIP₂ is located at the plasma membrane, this may not be the same for oocytes, considering that oocytes contain larger numbers of intracellular organelles such as ER clusters and yolk granules (Saunders *et al.*, 2007). However, questions still remain as to whether PLC ζ requires interaction with other molecular players such as regulators or modulators. Furthermore, the biochemical status of PLC ζ within sperm is not yet clearly understood, particularly in terms of how it can be

maintained in an inactive state within such gametes, or whether PLC ζ needs to be ‘activated’ in some way prior to fertilisation to allow it to play its role as an oocyte activation factor.

Proteolytic processing and different functional isoforms of PLC ζ have been proposed to be involved at oocyte activation. Kurokawa *et al.* (2005) demonstrated the presence of a full-length 72 kDa isoform of PLC ζ protein in an inactive porcine sperm extract, and conversely, its absence in several extracts that were capable of inducing oscillations. Fractions lacking the full-length protein exhibited proteolytic fragments, that were presumably cleaved PLC ζ isoforms. Kurokawa *et al.* (2007) further indicated that PLC ζ remains functional following proteolytic cleavage of the X-Y linker domain, and that N-terminal (33 and 37 kDa) and C-terminal (27 kDa) fragments were present in both fresh mouse and porcine sperm and sperm extracts, and were found to associate as functional complexes (Kurokawa *et al.*, 2007), possibly indicating a role for such truncated isoforms within oocyte activation.

1.5 Localisation of PLC ζ in mammalian sperm

While there is considerable variance in sperm morphology between mammalian species, a functional sperm universally consists of three main regions; the head, the midpiece, and the tail. The mammalian sperm head is completely engulfed within a plasma membrane, and is bordered from the sperm tail by a characteristic structure termed the posterior ring, where the plasma membrane is anchored to the nuclear envelope (Toshimori & Ito, 2003; Romalho-Santos *et al.*, 2007). The sperm head is divided into two major

regions; the anterior acrosomal region, and the posterior post-acrosomal region. The sperm nucleus is the major component present within the sperm head, in which sperm-specific DNA binding proteins termed protamines, have replaced histones, allowing for tighter chromatin packaging. A large Golgi-derived secretory vesicle, the acrosome covers the anterior part of the sperm, the area covered varying between species studied, and contains hydrolytic enzymes thought to aid in sperm penetration through the ZP, the release of which is dubbed the acrosome reaction (Toshimori & Ito, 2003; Sutovsky & Manandhar, 2006; Romalho-Santos *et al.*, 2007).

Excluding the acrosome, the space between the nucleus and the overlying plasma membrane is referred to as the perinuclear theca. A condensed cytosolic protein layer, the perinuclear theca can be divided both structurally and compositionally into subacrosomal and postacrosomal regions, which are thought to contain somatic and conventionally nuclear proteins involved in fertilisation (Oko & Sutovsky, 2009). Another major structural feature is the equatorial segment present in most mammalian sperm, which consists of a folded-over complex of the perinuclear theca and the inner and outer acrosomal membranes (Klaus & Hunnicutt, 2006; Sutovsky & Manandhar, 2006). The exact function of this structural feature remains to be elucidated, but considering that this region of the sperm head is reported to be the first to fuse with the oocyte membrane during gamete fusion, it is thought to play a role in sperm-oocyte interactions, harbouring key proteins associated with such events (Klaus & Hunnicutt, 2006; Sutovsky & Manandhar, 2006).

There appear to be varying amounts and solubility of the PLC ζ present depending on species studied, as well as varying potencies of PLC activity (Swann *et al.*, 2006; Cooney *et al.*, 2010). Data indicate that over a hundred-fold less human PLC ζ cRNA is required to cause Ca²⁺ oscillations within mouse oocytes as compared to mouse PLC ζ cRNA (Cox *et al.*, 2002). It would therefore seem that different species deliver ‘fine-tuned’ doses of PLC ζ , which have varying potencies adjusted via evolutionary processes to match the size and sensitivity of the egg/oocyte being activated (Swann *et al.*, 2006; Cooney *et al.*, 2010). For example, Ca²⁺ oscillations are initiated within 10 seconds of hamster sperm-oocyte fusion, but occur following a 1-3 minute delay in mice (Lawrence *et al.*, 1997).

Initial immunofluorescence studies in rodents suggested that PLC ζ protein was located at the perinuclear theca of the sperm head as well as in the acrosome (Fujimoto *et al.*, 2004), the expected location for the oocyte activation factor (Kimura *et al.*, 1998; Perry *et al.*, 1999; Sutovsky *et al.*, 2003; Swann *et al.*, 2006). However, subsequent studies indicated that PLC ζ was localised to post-acrosomal and equatorial regions in non-capacitated mouse and bull sperm, respectively (Yoon & Fissore, 2007). In contrast to these findings, a study of PLC ζ localisation in mouse and hamster sperm during capacitation and the acrosome reaction indicated that PLC ζ was most prominent in the acrosomal region in uncapacitated sperm of these species (Figure 1.7A), with a dominant post-acrosomal localisation being revealed following capacitation (Young *et al.*, 2009).

A possible role for acrosomal populations of PLC ζ in the acrosome reaction has been suggested by Grasa *et al.* (2008) and Young *et al.* (2009), and follows the identification of 55-60 kDa immunoreactive fragments of PLC ζ in fractions from rodent sperm containing acrosomal contents (Young *et al.*, 2009). Further to this, Bi *et al.* (2009) identified a potential new isoform of PLC ζ , a truncated 58 kDa fragment lacking the N-terminus EF domain. This was proposed to act as an intrinsic decapacitation factor, detaching from the acrosome during capacitation and playing a role in the acrosome reaction. However, this may be equivalent to one of the cleaved PLC ζ fragments previously identified in other studies, and currently remains the source of widespread speculation.

Recently, Nakai *et al.* (2011) demonstrated PLC ζ localisation to the sperm tail as well as within acrosomal and post-acrosomal regions of porcine sperm. A potential role for this population of PLC ζ within the sperm tail was postulated, but not directly involving oocyte activation. However, while supporting the hypothesis that PLC ζ may be involved in multiple events associated with oocyte activation (either directly or indirectly), the sperm tail population has not been identified by other immunofluorescence studies on mouse and human sperm (Grasa *et al.*, 2008; Young *et al.*, 2009). Further investigation is required to elucidate the physiological relevance of multiple localisation patterns of PLC ζ in mammalian sperm, and their respective roles on enzymatic activity and fertility.

In non-capacitated human sperm, PLC ζ was predominantly localised to the equatorial region of the sperm head (Grasa *et al.*, 2008), and this pattern of localisation was maintained during capacitation and the acrosome reaction. This location would be an ideal one for an oocyte activation factor, since this region would be one the first to be exposed to the oocyte cytoplasm following the acrosome reaction and gamete fusion, thus facilitating easy passage of the protein into the oocyte. However, as well as this predominantly equatorial pattern of localisation, PLC ζ was also detected to a lesser extent in acrosomal and post-acrosomal regions of human sperm (Figure 1.7B).

The fact that such variability in PLC ζ localisation is present in an ejaculate from a single fertile male, as well as between fertile males, raises the possibility that there may be differences in oocyte activation capabilities between individuals or within an ejaculate, even in the fertile male population (Grasa *et al.*, 2008), a point which has yet to be investigated. Recent studies suggest that full length PLC ζ RNA transcripts are present within human sperm (Platts *et al.*, 2007; Lalancette *et al.*, 2008). It has been suggested that such transcripts could be transcribed during fertilisation and sustain a 'long-lived' Ca²⁺ response (Lalancette *et al.*, 2008). However, considering that numerous studies indicate that the majority of protein transcription and translation occurs within meiotic stages of spermatogenesis, levels of which start to decline upon passage into the spermiogenesis stage (Bettegowda & Wilkinson, 2010), the functional significance of such RNA transcripts in human sperm remains unclear.

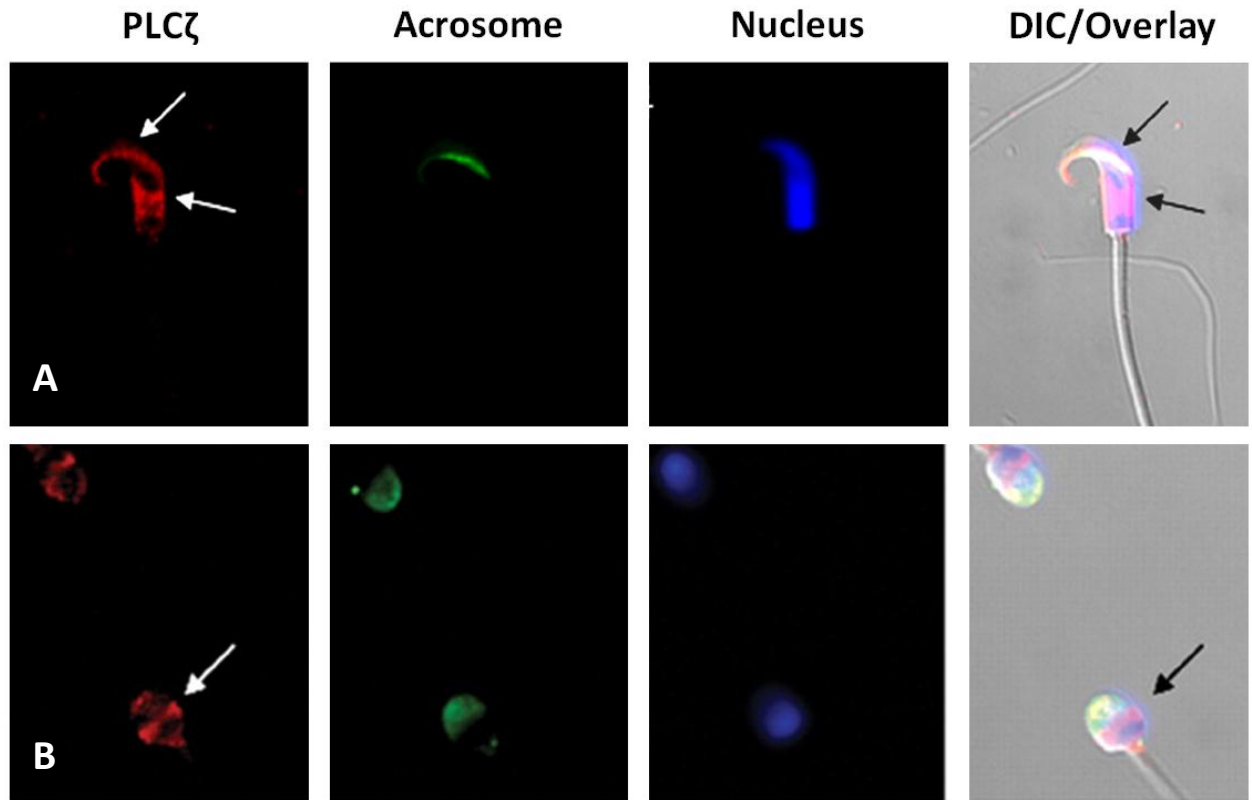


Figure 1.7: Immunofluorescent localisation of PLC ζ (red) in **(A)** hamster and **(B)** human sperm. Fluorescein isothiocyanate-conjugated peanut agglutinin (FITC-PNA)-lectin staining (green) identifies the acrosome, and Hoescht-33342 staining (blue) identifies the nuclei. Arrows indicate the immunolocalisation of PLC ζ within the acrosome and equatorial segment of hamster and equatorial segment of human sperm. DIC: differential interference contrast. Reproduced from Young *et al.* (2008) and Grasa *et al.* (2008) with permission.

1.6 Links between PLC ζ and human male factor infertility

Considering the proposed fundamental role of PLC ζ in oocyte activation, it is possible that abnormal forms or aberrant function of PLC ζ may be the underlying cause of certain forms of human male factor infertility and oocyte activation failure. Recent studies have demonstrated that the sperm of infertile men which consistently fail routine clinical procedures such as *in-vitro* fertilisation (IVF) and ICSI are unable to produce Ca²⁺ oscillations upon injection into mouse oocytes, or those which do are uncharacteristic of

oscillations observed from fertile men, being reduced in both frequency and amplitude (Yoon *et al.*, 2008; Heytens *et al.*, 2009).

Furthermore, immunofluorescence and immunoblot analysis revealed that infertile patients whose sperm failed ICSI and were unable to produce Ca^{2+} oscillations, exhibited reduced/absent levels of PLC ζ within the sperm head (Figure 1.8; 1.9A) (Yoon *et al.*, 2008; Heytens *et al.*, 2009). Moreover, the activating ability of human sperm that failed ICSI could be rescued upon co-injection with mouse PLC ζ mRNA (Figure 1.9B, C, D) (Yoon *et al.*, 2008), providing significant support to the potential use of recombinant PLC ζ as a therapeutic agent. Many of the patients in these studies were diagnosed with globozoospermia, a morphological condition resulting in round headed, acrosome-less sperm, a condition affecting 0.1% of infertile men (Dam *et al.*, 2007). Although analysis of the PLC ζ gene revealed no major genetic abnormalities in globozoospermic men, sperm from such patients consistently exhibited reduced levels or even complete absence of PLC ζ protein (Yoon *et al.*, 2008; Heytens *et al.*, 2009). Interestingly, following the use of ICSI along with a Ca^{2+} ionophore (to artificially induce Ca^{2+} release within the oocyte), high rates of fertilisation were observed along with a successful birth using globozoospermic sperm that exhibited total absence of PLC ζ expression (Taylor *et al.*, 2009; personal communication - Professor Rafael A. Fissore).

Yoon *et al.* (2008) reported the absence of PLC ζ in both normal and round-headed sperm from a partial globozoospermic patient (mixed populations of morphologically normal and round-headed sperm). This was in line with the inability of partial

globozoospermic sperm cell types to activate mouse oocytes reported by Heindryckx *et al.* (2008). Numerous reports studying familial cases have suggested that globozoospermia is a genetic syndrome (Kullander & Rausing, 1975; Flörke-Gerloff *et al.*, 1984; Dale *et al.*, 1994; Kilani *et al.*, 2004, Heindryckx *et al.*, 2005). However, the specific mode of inheritance remains unclear (Dam *et al.*, 2007).

Importantly, Heytens *et al.* (2009) provided the first genetic link between PLC ζ defects and infertility by identifying a heterozygous substitution mutation in the coding sequence of PLC ζ in a non-globozoospermic infertile male. This occurred within the Y domain at position 398 of the amino acid sequence resulting in histidine (H398) being changed to proline (Figure 1.10A) (Heytens *et al.*, 2009). Multiple sequence alignments confirmed histidine to be conserved in this position across all mammalian PLC ζ s (Cox *et al.*, 2002; Saunders *et al.*, 2002; Yoneda *et al.*, 2006; Young *et al.*, 2009), chicken PLC ζ (Coward *et al.*, 2005), medaka PLC ζ (Ito *et al.*, 2008), as well as all PLC δ isoforms (Ellis *et al.*, 1998; Saunders *et al.*, 2002), indicating that this residue may play an important role within the protein (Heytens *et al.*, 2009). It is possible that the H398P mutation could cause important changes in PLC ζ function as proline is a non-polar amino acid with a bulky planar ring side group, and a known disruptor of secondary structure and protein stability (Bajaj *et al.*, 2007). Indeed, modelling studies of human PLC ζ predicted that the mutation could disrupt an alpha helix structure in the catalytic region and affect interactions with neighbouring amino acids (Figure 1.10B) (Heytens *et al.*, 2009). In line with such predictions, injection of mutant human PLC ζ cRNA into mouse oocytes resulted in highly abnormal Ca²⁺ transients, which were insufficient for oocyte activation, in contrast to oocytes injected with control wild type PLC ζ which produced a series of Ca²⁺

oscillations characteristic of normal oocyte activation (Figure 1.10C) (Heytens *et al.*, 2009).

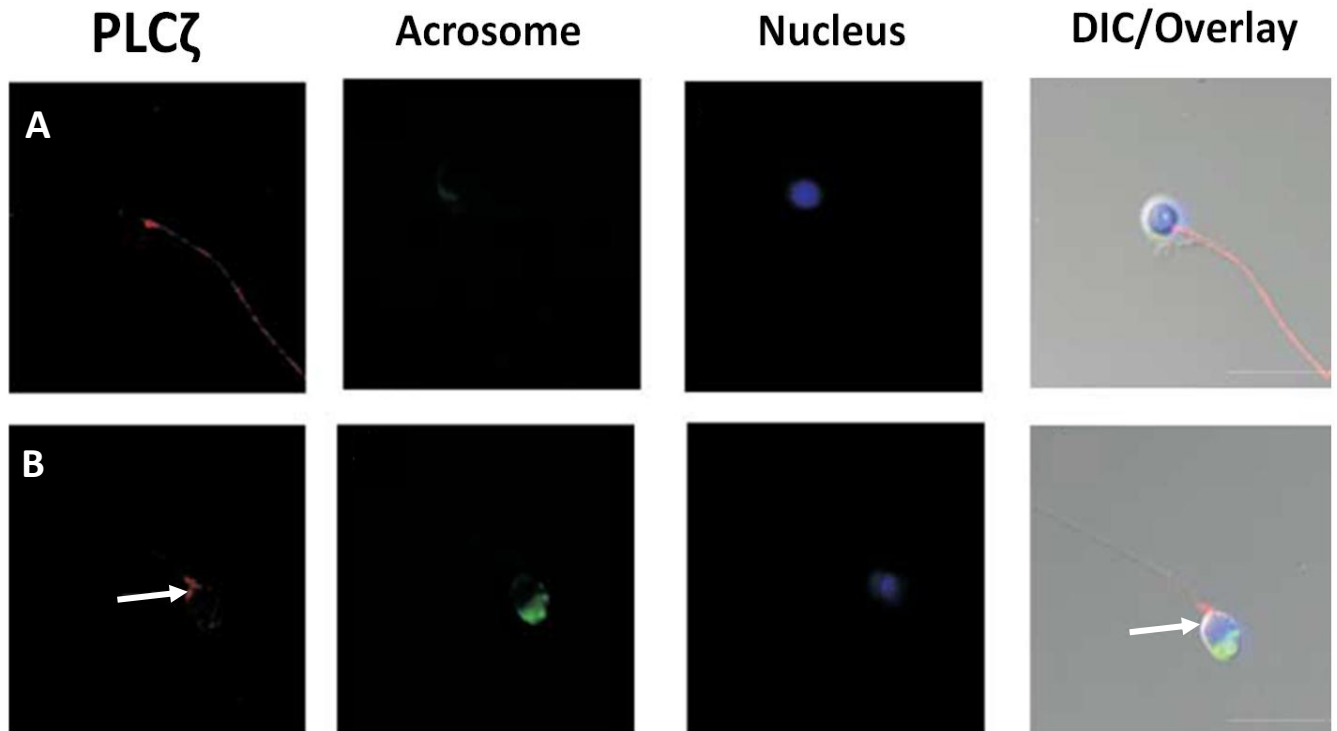


Figure 1.8: Reduced PLC ζ immunostaining (red) in sperm from infertile ICSI failed patients exhibiting **A)** globozoospermia, and **B)** normal morphology. Fluorescein isothiocyanate-conjugated peanut agglutinin (FITC-PNA)-lectin staining (green) identifies the acrosome, and Hoescht-33342 staining (blue) identifies the nuclei. Arrow indicates reduced PLC ζ immunolocalisation within the equatorial segment. Scale bars indicate 5 μ m. DIC: differential interference contrast. Reproduced from Heytens *et al.* (2009) with permission.

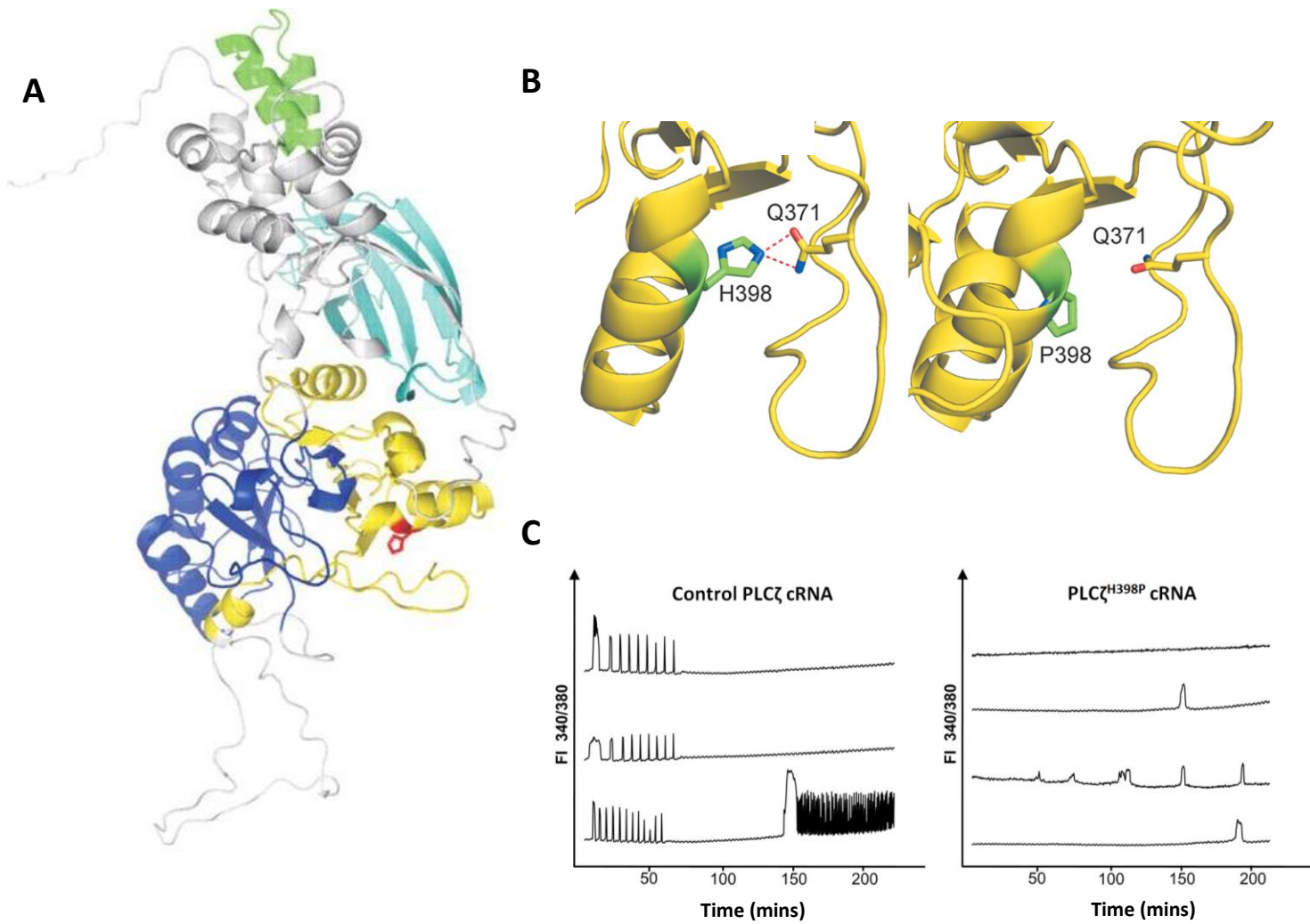


Figure 1.10: Histidine>Proline point mutation (PLC ζ^{H398P}) identified by Heytens *et al.* (2009) in an infertile male patient diagnosed with oocyte activation deficiency. **A)** Model of human PLC ζ functional domains (green – EF hands, blue – X catalytic domain, yellow – Y catalytic domain and cyan – C2 domain). Histidine398 (H398) is shown in red. **B)** Close-up of H398 showing side-chain-side-chain hydrogen bonds (left panel), alongside a close-up of P398 in mutant PLC ζ showing no side-chain-side-chain hydrogen bonds (right panel). **C)** Microinjection of wild type and mutant PLC ζ into mouse oocytes and resulting calcium release patterns. FI 340/380: ratio of fluorescence at wavelengths of 340nm and 380nm. Reproduced from Heytens *et al.* (2009) with permission.

Considering that mutations in PLC ζ have now been implicated in a form of male infertility where oocyte activation is deficient, it is still unclear as to whether this is the case for such patients in general, and its implications on wider society. It should be noted that the only mutation that has been found thus far has been from a single patient, and it is thus increasingly important to expand screening of the PLC ζ gene to a larger patient population from a variety of biogeographical regions to ascertain fully the specific role played by PLC ζ mutation on oocyte activation deficiency. Furthermore, further analytical studies need to be undertaken to explore the precise effects of the H398P mutation upon PLC ζ structure and function, and to establish whether this is a common, rare, or even unique molecular event.

1.7 Male factor infertility and assisted oocyte activation (AOA)

The findings of Yoon *et al.* (2008) and Heytens *et al.* (2009) strongly implicate a link between defective forms or absent levels of PLC ζ in cases of male infertility where oocyte activation is insufficient. Infertility affects both genders in very different ways (Evens, 2004). Although it is difficult to compile accurate data for the incidence of global infertility, it is believed that infertility affects 70 million couples worldwide, the majority of whom reside in developing countries (Ombelet *et al.*, 2008). The incidence of global infertility is now estimated to affect 1 in 7 couples (Evens, 2004; Ombelet *et al.*, 2008; Ledger, 2008; McVeigh *et al.*, 2008).

Assisted reproductive technology (ART) now accounts for 7% of all births in some developed countries (Nasr-Esfahani *et al.*, 2009). Worldwide, approximately 1 million ART treatments are performed each year and over eight million ART babies have been

born worldwide (ICMART, 2009). The most widely known example of ART is *in-vitro* fertilisation (IVF). Initially developed to treat women with tubal infertility, IVF is now an established treatment for a wide range of infertilities (McVeigh *et al.*, 2008). Over thirty years after the birth of the world's first IVF baby, more than 12000 births in the UK arise each year as a result of IVF, accounting for 1.4% of all births in the UK (HFEA register, 2007).

Current ART methods facilitate the treatment of most forms of infertility (Figure 1.11A). For example, infertile females suffering from abnormal ovulation can be treated by *in-vitro* maturation (IVM), a method used to mature oocytes *in-vitro* in preparation for IVF (Cohen *et al.*, 2005). Similarly, ICSI can rectify a number of male factor problems, such as oligospermia (low sperm count), transcending the need for normal sperm count/motility (Mangoli *et al.*, 2008; McVeigh *et al.*, 2008). However, although ART plays a critical role in reducing infertility, there are still groups of infertile couples for which ART has not yet proven successful. Of particular outstanding concern are infertile males whose sperm are unable to activate oocytes, even following ICSI, or males exhibiting unexplained (idiopathic) male factor infertility (Mangoli *et al.*, 2008; Nasr-Esfahani *et al.*, 2009).

Current data suggest that the incidence of reduced fertility, or 'sub-fertility', affects 1 in 15 men, with sperm defects being the most common cause (Publicover *et al.*, 2007). However, there is much debate surrounding the specific causative factors of unexplained male infertility, most particularly in cases of ICSI failure. It is estimated that male factor infertility contributes to 35-40% of infertility in couples (Forti & Krausz, 1998; Kumtepe

et al., 2009), depending upon the biogeographical region studied. Although male factor infertility can arise in a number of ways (Figure 1.11B) (Elliot & Cooke, 1997; Evens *et al.*, 2004; Meacham *et al.*, 2007; McVeigh *et al.*, 2008; Kumtepe *et al.*, 2009), it is widely accepted that genetic causes are of most concern, and that abnormalities in testicular gene expression may underlie many instances of idiopathic male infertility (Coward *et al.*, 2007).

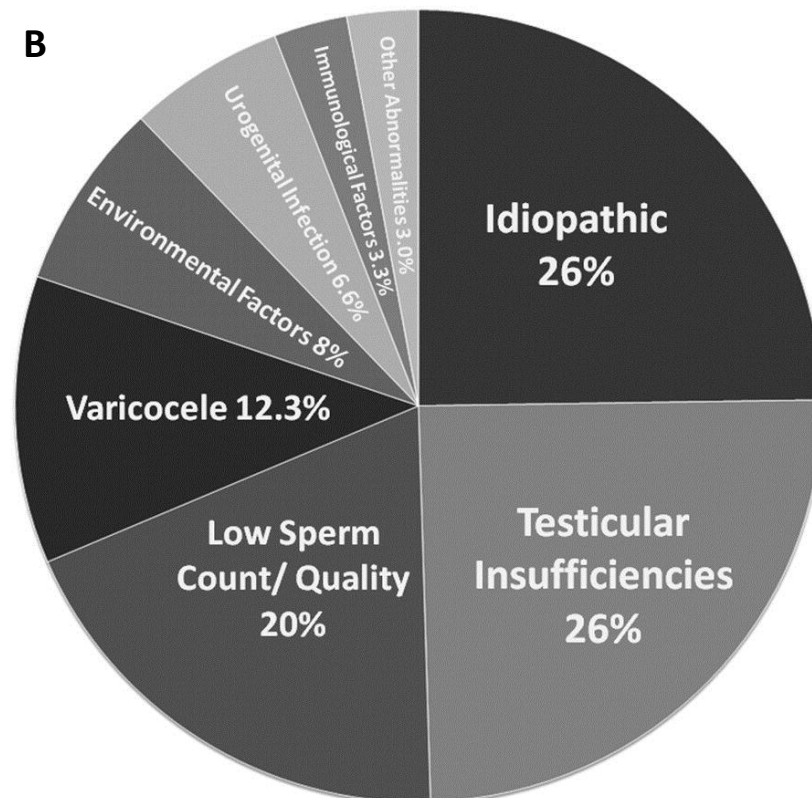
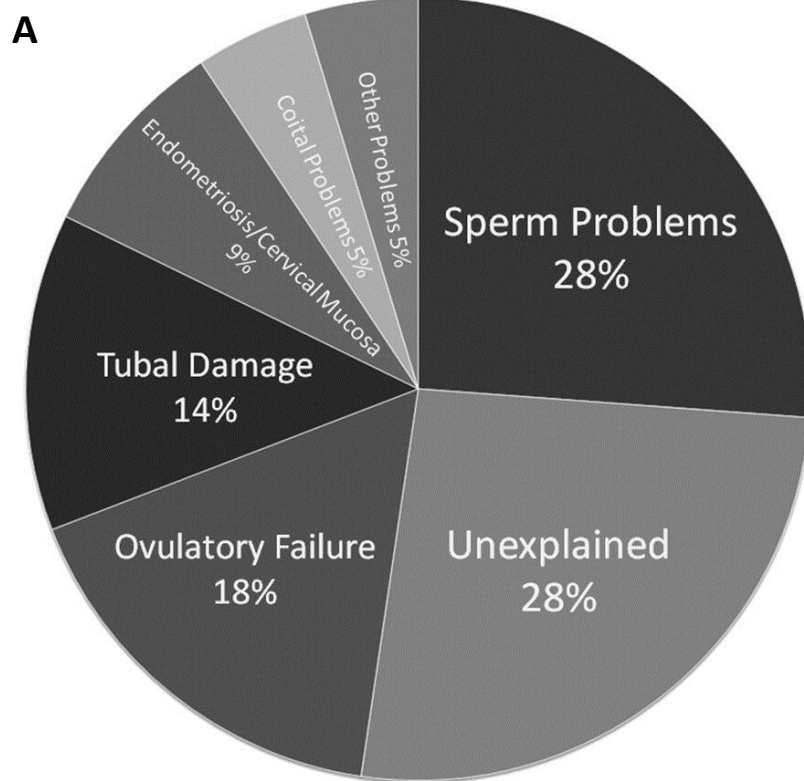


Figure 1.11: **A)** Primary causes of infertility. **B)** Primary causes of male factor infertility. Reproduced from Kashir *et al.* (2010) with permission.

Although ICSI results in average fertilisation rates of 70% (Heindryckx *et al.*, 2005), complete or virtually complete fertilisation failure still occurs in 1-5% of ICSI cycles (Flaherty *et al.*, 1998; Mahutte & Arici, 2003; Yanagida, 2008), corresponding to approximately 1000 cases per year in the UK alone. The incorrect injection of sperm, expulsion of injected spermatozoon from the oocyte, and the failure of sperm head decondensation are not considered to contribute substantially to fertilisation failure after ICSI (Yanagida, 2008). A deficiency in the mechanism of oocyte activation, which can recur in several cycle attempts, is regarded as the principal cause of fertilisation failure, or abnormally low fertilisation after ICSI (Sousa & Tesarik, 1994; Heindryckx *et al.*, 2008), and can currently only be resolved using assisted oocyte activation (AOA).

AOA can be induced by a wide range of chemical, mechanical, or physical stimuli, which mostly elicit only one Ca^{2+} transient in the oocyte (Alberio *et al.*, 2001). The most popular artificial activating agents for human oocytes include Ca^{2+} ionophores such as ethanol, ionomycin and IA23187, electrical pulses, and combinations with protein synthesis or kinase inhibitors such as 6-dimethylaminopurine (6-DMAP), or puromycin that block the re-synthesis of cyclin B or CDK1 activity (Heindryckx *et al.*, 2009). These agents result in a single prolonged Ca^{2+} rise, but fail to elicit normal Ca^{2+} patterns (Figure 1.12). Other activating agents have been shown to cause multiple transients, such as strontium chloride in mice (Kline & Kline, 1992; Kishikawa *et al.*, 1999), phorbol esters (Cuthbertson & Cobbold, 1985) or thimerosal (Fissore *et al.*, 1995). These, however, have only been reported to be efficient in a limited number of species, are less efficient than ionophores in most species, or can cause meiotic spindle disruption (Alberio *et al.*, 2001).

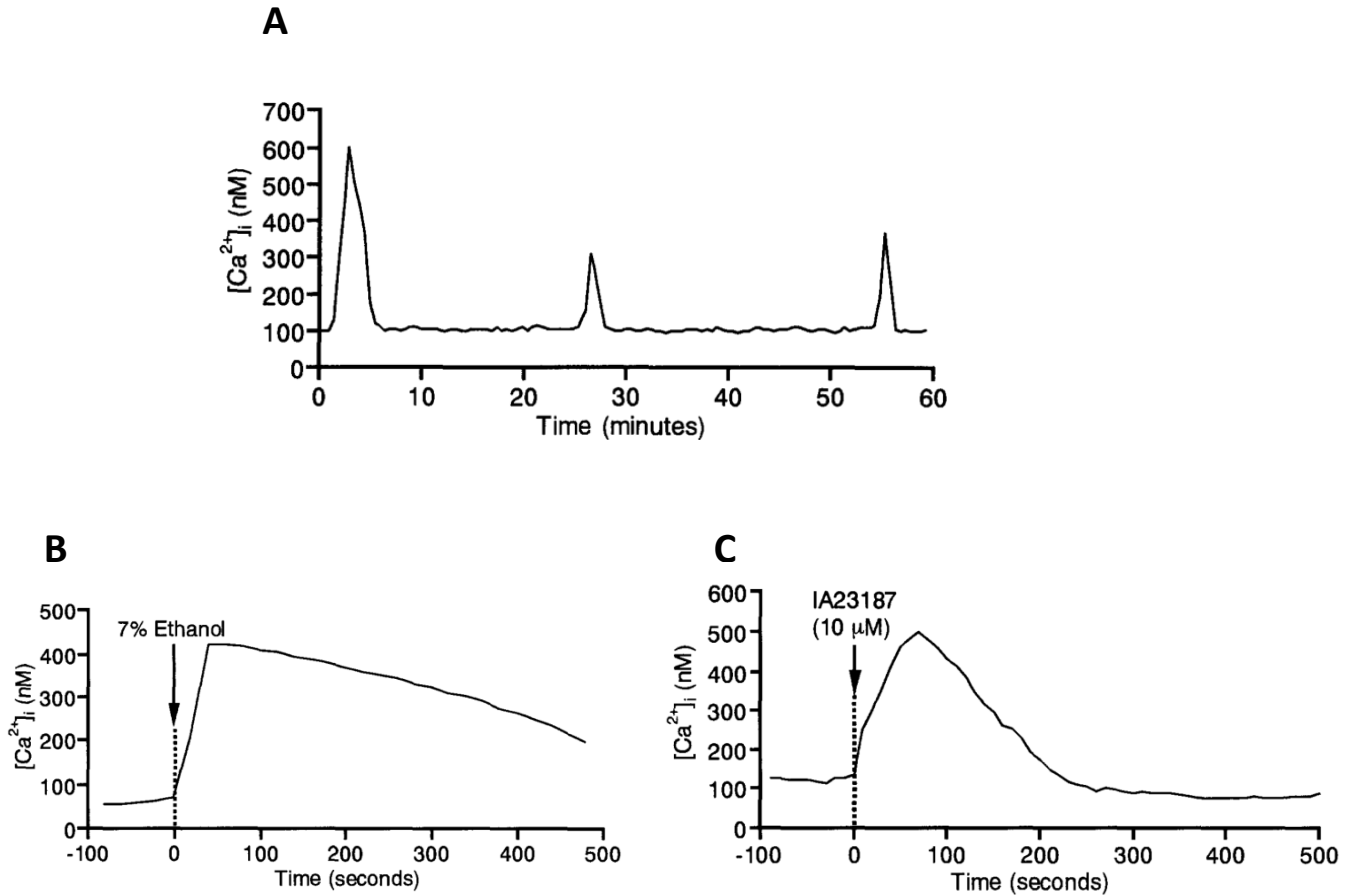


Figure 1.12: Representative Ca^{2+} responses of bovine oocytes treated with **A**) sperm (normal fertilisation), **B**) 7% ethanol, and **C**) Ca^{2+} ionophore IA23187. Reproduced from Nakada & Mizuno (1998) with permission.

Indeed, the advent of technologies such as ICSI combined with AOA has given much hope for the treatment of cases with recurrent oocyte activation deficiency. Numerous cases have demonstrated that ICSI combined with AOA greatly increased the success rate of fertilisation and subsequent pregnancy (Rybouchkin *et al.*, 1997; Kim *et al.*, 2001; Tesarik *et al.*, 2002; Heindryckx *et al.*, 2005; 2008; Dirican *et al.*, 2008; Tejera *et al.*, 2008; Kyono *et al.*, 2009; Taylor *et al.*, 2009). However, several studies in animals have demonstrated that the number and amplitude of Ca^{2+} transients not only affects activation efficiency, but has a profound influence upon subsequent embryonic

development (Ducibella *et al.*, 2002; Ozil *et al.*, 2006; Kim *et al.*, 2011), blastocyst quality (Bos-Mikich *et al.*, 1997), the implantation potential of rabbit parthenogenotes (Ozil & Huneau, 2001) and mouse zygotes (Ozil *et al.*, 2006), and results in altered embryonic gene expression (Ozil *et al.*, 2006). As such, there is significant concern over the clinical use of such agents.

However, there are artificial stimuli which induce Ca^{2+} oscillations similar to those observed at normal fertilisation. The most efficient of these is Strontium (Sr^{2+}), which was found to elicit Ca^{2+} oscillations through the synergistic action of PLC activation and IP_3 mediation through the IP_3 receptors (Zhang *et al.*, 2005), the use of which resulted in successful pregnancy of normozoospermic patients diagnosed with oocyte activation deficiency (Chen *et al.*, 2010). However, Sr^{2+} treatment varies in terms of oocyte activation efficacy depending on the species in which it is used (Yanagida, 2008) with success rates being relatively low in humans (Chen *et al.*, 2005). Furthermore, little is known as to the downstream effects of Sr^{2+} treatment on signalling molecules such as PKC and DAG. It would, therefore, be prudent to examine methods of AOA which elicit Ca^{2+} release in an endogenous manner, to circumvent these concerns for clinical scenarios. An ideal example of which would be a purified recombinant version of human $\text{PLC}\zeta$ protein, the principle of which has been previously demonstrated by Yoon *et al.* (2008) using mouse cRNA.

1.8 Therapeutic and diagnostic applications of PLC ζ

While Yoon *et al.* (2008) and Heytens *et al.* (2009) provided the first clinical link between defects in PLC ζ and human male infertility, this area of research is still very much in its infancy. Further analytical studies need to be undertaken to explore the precise effects of PLC ζ^{H398P} upon PLC ζ structure and function. Furthermore, there is a clear need to screen further patients diagnosed with oocyte activation deficiency for other mutations in PLC ζ . Studies investigating larger cohorts of patients, from a range of biogeographical regions, targeting specific male factor conditions, and utilising high-throughput genetic screening techniques may be invaluable in discovering the prevalence of such mutations in human infertility.

For clinical purposes, a combination of genetic analysis selectively targeting the PLC ζ coding region, along with immunofluorescent analysis of sperm samples to investigate PLC ζ localisation patterns, may prove useful diagnostic tests for ART clinics. Currently, only heterologous ICSI models are available for testing the activation capacity of human sperm, such as the mouse oocyte activation test (MOAT) (Rybouchkin *et al.*, 1996; Heindryckx *et al.*, 2008). Due to ethical and legal restrictions, inconvenience, and technical difficulty of mouse ICSI, it would be far more beneficial to develop simpler cell-free and enzymatic tests for PLC ζ activity as clinical diagnostic tests for oocyte activation deficiency. The identification of men with oocyte activation defects also raises the possibility of using recombinant PLC ζ protein as a potential therapeutic.

The principle of using PLC ζ therapeutically has already been demonstrated by Yoon *et al.* (2008), who ‘rescued’ the oocyte activating capability of oocyte activation deficient sperm by the co-injection of PLC ζ cRNA into mouse oocytes, although the pre- and post-implantation developmental potential could not be determined using this heterologous ICSI model. Rogers *et al.* (2004) also showed that PLC ζ cRNA could activate aged human oocytes which had previously failed to fertilise. However, for clinical purposes it would be necessary to use a recombinant protein form of PLC ζ , considering that the precise pattern of Ca²⁺ oscillations at fertilisation may influence both the efficiency and quality of embryo development, and most critically, gene expression. Furthermore, there are technical difficulties associated with cRNA production, partly due to potential problems relating to limiting Ca²⁺ oscillation inducing capacity during embryo development. Furthermore, studies suggest that mammalian embryos may contain an endogenous reverse transcriptase activity (Spadafora, 2004) that could potentially convert introduced cRNAs into PLC ζ cDNA that might then become incorporated into the genome.

1.9 The effects of clinician-induced (iatrogenic) damage upon sperm proteins

Whilst advances in ART have revolutionised infertility treatment, success rates vary. Pregnancy and delivery rates for IVF and ICSI still require improvement, with success rates (defined as live births per cycle started) rarely exceeding 30% and 23% respectively (ICMART 2009; de Mouzon *et al.*, 2010). It is, therefore, critical that ART protocols are constantly modified in line with scientific advances, so that clinics can consistently provide patients with the best chances of conception. Moreover, there seems to be some correlation between routine clinical techniques, such as sperm cryopreservation, in the

subsequent reduction of fertilisation success rates. Borges *et al.* (2007) demonstrated that fertilisation rates following ICSI were higher using fresh sperm (73.8%) compared to cryopreserved sperm (68.7%), and that sperm from patients diagnosed with asthenozoospermia (reduced motility) or oligoasthenozoospermia (reduced sperm count and motility), may be more susceptible to freeze/thaw damage, resulting in lower fertilisation rates.

Along with the well-documented effects of cryopreservation on fertilisation capacity, motility, morphology and viability of human spermatozoa (Nallella *et al.*, 2004; Thomson *et al.*, 2009b), cryopreservation is thought to induce sperm DNA fragmentation in not only fertile sperm, but exacerbate such conditions in sperm from patients seeking fertility treatment (de Paula *et al.*, 2006; Thomson *et al.*, 2009a; 2009b; Zribi *et al.*, 2010) via the increased presence of reactive oxygen species and oxidative stress (Thomson *et al.*, 2009b). While sperm DNA fragmentation has been shown to exert a negative effect on the chances of conception and achieving pregnancy either naturally or through ART (Borini *et al.*, 2006; Benchaib *et al.*, 2007; Thomson *et al.*, 2009b), the possible consequences of a pregnancy achieved using sperm exhibiting DNA damage remains of great concern, since this may result in defective embryo quality (Lewis & Aitken, 2005; Niu *et al.*, 2011).

Within the majority of cases in ART, sperm are prepared by density gradient centrifugation (DGW) or a swim-up protocol in order to isolate the most motile and morphologically normal sperm, with DGW being more efficient than most sperm preparation techniques (Allamaneni *et al.* 2005; Niu *et al.*, 2011). However, several studies indicate that sperm separation methods may also isolate sperm with nicked (damaged) DNA and poorly condensed chromatin. However, further studies will be required to

establish fully the relative importance of these observations due to conflicting results within the published literature (Gandini *et al.*, 2004; Bungum *et al.*, 2008; Chiamchanya *et al.*, 2010; Matsuura *et al.*, 2010; Niu *et al.*, 2011).

Further to this, little is currently known with regards to the potential effects of iatrogenic damage upon human sperm proteins and resultant pregnancy success rates in ART. Whilst cryopreservation can reduce levels of some sperm proteins such as glutathione (Gadea *et al.*, 2010) in human sperm, little is known of its potential effects upon PLC ζ , which may represent a powerful tool with which to investigate sperm quality. Further to this, the effects of DGW on levels of PLC ζ within human sperm have yet to be investigated, and may shed light as to the potential usefulness of PLC ζ as a biomarker of male fertility.

1.10 Aims of this thesis

In summary, there are many outstanding queries with regards to the precise mechanisms of PLC ζ action, the regulatory pathways involved, and the relative roles of the various localisation patterns reported in the literature. Little is known as to the mechanism employed by PLC ζ in PIP₂ targeting, the potential roles of post-translational modification and multiple PLC ζ isoforms at fertilisation and oocyte activation, as well as the identity or importance of PLC ζ interactors required to modulate activity. Systematic analyses of these issues are required for the translation of PLC ζ into a possible therapeutic agent for the clinic, as well as a useful biomarker to determine sperm quality and viability in ART.

This study had four predominant aims, and utilised a multifaceted approach with which to address the numerous concerns and potential regarding the mechanism and mode of action of PLC ζ , along with the potential diagnostic and therapeutic applicability for PLC ζ within the clinic. These aims were:

- 1) To examine the prevalence of loss-of-activity mutations in OAD patients, and to examine the potential viability of utilising PLC ζ as a diagnostic test of sperm and male fertility.** This project utilised a polymerase chain reaction (PCR) based approach to screen exons coding for the active site open reading frame (ORF; exons 5-12) of the PLC ζ gene from multiple infertile patients diagnosed with oocyte activation deficiency from various bio-geographical regions. These data were correlated with PLC ζ immunofluorescence within the sperm head using fluorescence and confocal microscopy.
- 2) To utilise the previously identified PLC ζ^{H398P} , and novel mutations identified by this project, to help characterise PLC ζ mechanism and mode of action within mammalian somatic cells and mouse oocytes.** The project utilised a combination of fluorescence and confocal microscopy, live cell imaging, and oocyte microinjection techniques to accomplish this aim.
- 3) To prepare a purified, active, recombinant human PLC ζ protein in order to examine the potential therapeutic viability of such a compound, and to investigate the functional properties of PLC ζ .** The project utilised bacterial and mammalian expression systems, and oocyte microinjection to accomplish this aim. Using a similar approach for mutant isoforms of PLC ζ , this thesis also aimed investigate the functional properties of PLC ζ in terms of potential post-translational modifications.

4) To utilise PLC ζ as a marker of sperm fertility with which to investigate the potential efficacy of routine ART methods to cause iatrogenic damage to sperm proteins implicated in sperm fertility. This project investigated the effects of density gradient washing (a method used to obtain the best quality sperm for IVF and ICSI), and cryopreservation upon total levels of, and proportions of sperm exhibiting, PLC ζ immunofluorescence.



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CHAPTER 2

General materials and methods

This Chapter describes methodology that was most commonly used throughout the research described herein. Specific modifications, or Chapter-specific methodology, are described where appropriate in subsequent Chapters.

2.1 Ethics and patient selection

Animal handling and procedures involving mouse oocyte collection, injection, and Ca^{2+} imaging were approved by the University of Massachusetts IACUC committee granted to Professor Rafael Fissore. Work with genetically modified (GM) organisms within this thesis was approved by the local University of Oxford GM committee (NDOG-19).

The use of all patient and control sperm, and genomic DNA samples, were ethically approved by appropriate regulatory bodies. All patients agreed to participate by providing informed written consent. Patient sperm and DNA samples obtained from Belgium were approved by the Ethical Committee of Ghent University Hospital, along with local ethical permission granted to Dr. Kevin Coward by the Oxford Tropical Research Ethics Committee (OXTREC; 31/09). Use of sperm and DNA samples from fertile controls obtained from local volunteers were approved by ethical permission granted to Dr. John Parrington by the National Ethics Research Service (NRES) Research Ethics Committee (07/H0605/111). Patient and control DNA and sperm samples obtained from the Oxford Fertility Unit (OFU) were approved for research by the NRES Oxford Research Ethics Committee (10/H0606/65). Screening of 100 fertile individuals by Reprogenetics UK was ethically approved by the Western Institutional Review Board (WIRB). Fertile controls were classified as males with proven fertility, whose sperm complied with world health organisation (WHO, 2010) guidelines for normal sperm morphology and semen quality

(Table 2.1), while ICSI-failed patients from Belgium were defined as patients whose sperm had failed multiple ICSI attempts. Patients from the OFU were defined as males whose sperm failed to fertilise ≥ 8 oocytes following ICSI, or exhibiting abnormal sperm morphology. Specific patient characteristics are detailed where appropriate per Chapter.

Table 2.1: Summary of the World Health Organisation (WHO) 2010 parameters for normal sperm.

Volume (ml)	Liquefaction time (minutes)	pH	Sperm concentration ($\times 10^6/\text{ml}$)	Total sperm number ($\times 10^6/\text{ejaculate}$)	Motility (%)	Vitality (%)
≥ 2.0	≤ 60	≥ 7.2	≥ 20	≥ 40	≥ 50 (grades a and b)	≥ 75

For genomic DNA screening, control (n=2) and patient (n=12) saliva samples, obtained from individuals recruited from the UK, Belgium, and Iran, were used. PLC ζ protein screening was performed in sperm obtained from controls (n=7) and infertile patients (n=3) recruited from the Oxford Fertility Unit to screen PLC ζ protein by quantitative immunofluorescence, and to perform proportional analysis of PLC ζ localisation patterns. Specific patient selection criteria are detailed per chapter where appropriate.

2.2 Genomic DNA extraction from buccal cells

DNA was obtained from saliva samples donated from patients and controls, and subsequent extraction performed using the Oragene DNA Self-Collection Kit (DNAgenotek, USA) following the manufacturer's protocol. Briefly, following collection of saliva and preservation in the collection kit, 500 μl of the saliva/preservative mix were incubated for 1 hour at 50°C in a micro-centrifuge tube, following which 20 μl of Oragene

Purifier was added, and the solution mixed by inversion. Following incubation on ice for 10 minutes, the mix was centrifuged at 18,000g for 5 minutes, after which the supernatant was transferred to a fresh tube and the pellet discarded. 100% ethanol was added to the supernatant, mixed by inversion, and incubated at room temperature for 10 minutes to allow DNA precipitation. Following centrifugation at 18,000g for 2 minutes, the supernatant was discarded, and the DNA pellet washed by incubation with 75% ethanol for 1 minute. The ethanol was then completely removed, and the pellet air-dried to ensure evaporation of all ethanol. The DNA pellet was finally resuspended in nuclease-free water (Ambion, UK), depending on pellet size, and DNA concentration determined by spectrophotometry.

2.3 Polymerase chain reaction (PCR) and direct DNA sequencing

Amplicons were generated with previously optimised cycle conditions (Table 2.2) using the High Fidelity PCR Master Kit (Roche, Germany). PCR reactions were performed using a G-storm Thermal Cycler (Model GS4, GSI, UK). Primer sets and templates are described where appropriate in the corresponding materials and methods sections of each Chapter. Amplicons were sequenced by Geneservice Ltd. (Department of Biochemistry, Oxford), and results compared with published amplicon and ORF sequences using NCBI BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Direct sequencing utilised various primer sets depending on the amplicon/construct being sequenced. Sequencing primer sets are described where appropriate in the corresponding materials and methods sections of each Chapter.

Table 2.2: PCR cycles utilised to amplify exons coding for the PLC ζ active site (5-12), as well as to amplify appropriate PLC ζ isoform sequences during cloning of molecular constructs.

Step	Temperature (°C)	Time (minutes . seconds)
Heated Lid	110	
Denaturation	94	4.00
Start Cycle 1 (x10)		
Denaturation	94	0.10
Annealing	58	1.10
Elongation	72	2.00
End Cycle 1		
Start Cycle 2 (x20)		
Denaturation	94	0.15
Annealing	58	0.30
Elongation	72	2.00
End Cycle 2		
Elongation	72	7.00
Store	8	Infinity

2.4 Determination of DNA and RNA concentration by spectrophotometry

DNA concentration was determined by measuring the absorbance of DNA (diluted 1:100) with nuclease-free water, using nuclease-free water as a blank. The blank and DNA dilutions were transferred to clear plastic cuvettes (Eppendorf, UK), and the absorbance at a wavelength of 260nm determined using a spectrophotometer (Eppendorf, UK). The absorbance was then multiplied by the dilution factor used (100), and the optical density of 1 unit of double-stranded DNA (50ng/ μ l) to give the DNA concentration in ng/ μ l. RNA concentration was determined by multiplying the absorbance of RNA (diluted 1:100) obtained at 260nm, with the optical density of 1 unit of single stranded RNA (40ng/ μ l) and the dilution factor (100) to obtain RNA concentration in ng/ μ l.

2.5 Agarose gel electrophoresis

Low-melting point, analysis grade Agarose (Promega, Spain) was dissolved in 1xTris-Acetate-Ethylenediaminetetraacetic acid (TAE; Applied Biosystems, UK) according to the percentage of gel to be made (i.e. a 1% agarose gel required 1g agarose in 100ml of 1xTAE). Agarose/TAE solution was heated in a microwave until the agarose powder had completely dissolved, and the solution was completely clear. This was then left to cool at room-temperature until 'hand-hot'. At this stage, following the addition of ethidium bromide (0.5µg/ml), the gel was poured into a gel cartridge, and allowed to set at room-temperature. Following loading of DNA samples, gels were run at 90 volts for 1% agarose gels, and 100-110 volts for $\geq 2\%$ agarose gels, until the DNA bands had migrated three quarters of the gel. DNA bands were visualised using a GelDoc Imaging System (UVP, UK) and molecular weights were deduced by comparison with a 1kb or 50bp DNA Ladder (New England Biolabs, UK), depending on the amplicon size being analysed.

2.6 Luria Bertani (LB)-Agar plates, LB-Broth, and phosphate buffered solution (PBS)

1M solutions of LB-Agar (Sigma-Aldrich, UK; 10g/L tryptone, 5g/L yeast extract, 5g/L NaCl, 15g/L Agar) and LB-Broth (Sigma-Aldrich, UK; 10g/L tryptone, 5g/L yeast extract, 5g/L NaCl) were prepared using double distilled water, and sterilised in an autoclave. After the solutions had cooled, and before the LB-Agar began to set, ampicillin was added under sterile conditions to both LB-Agar and LB-Broth to give a final concentration of 100µg/ml. Before it could set, the LB-Agar was poured onto petri dishes (~2/3 of the height of the dish) and allowed to set at room temperature. To obtain 1M PBS, 10 PBS tablets (Oxoid, UK; 8M NaCl, 0.2M KCl, 1.15M Na₂HPO₄, 0.2M KH₂PO₄) were added per liter of double-distilled water, and sterilised by autoclaving.

2.7 Cloning of molecular constructs

Amplicons were prepared utilising primers and template sets described where appropriate per Chapter using the PCR cycle described in Table 2.2 unless mentioned otherwise, and subject to PCR cleanup using the QIAquick PCR purification kit following the manufacturer's protocol (QIAGEN, UK). Five volumes of binding buffer (PB) were added to 1 volume of PCR reaction mix, and pH adjusted by adding 10 μ l of 3M sodium acetate. This was then added to a QIAquick spin column and centrifuged for 1 minute at 15,000g and the flow-through discarded. The column was then washed with buffer PE (wash buffer containing ethanol) by centrifuging at 15,000g for 1 minute, discarding the flow-through, and centrifuging once more for 1 minute at 15,000g. The column was then transferred to a micro-centrifuge tube, and nuclease-free water added directly to the column membrane. Following incubation at room temperature for 1 minute, the column was centrifuged at 15,000g for 1 minute to elute DNA from the column. DNA concentration was then determined using spectrophotometry as described in Section 2.4.

Amplified and purified DNA was subsequently digested with appropriate restriction enzymes for two hours at 37°C as described per Chapter, following the recommended guidelines for each restriction enzyme. Unless otherwise stated, all restriction enzymes were obtained from New England Biolabs (UK). Concurrently, the appropriate vector was also incubated with the same restriction enzymes for 1.5 hours at 37°C. Following this the digested vector was then dephosphorylated using the rAPid Alkaline Phosphatase kit (Roche Biosciences, UK) in accordance with manufacturer's instructions to prevent self-annealing. Briefly, restriction enzymes were first inactivated by incubation of the digestion mix at 60°C for 10 minutes. rAPID alkaline phosphatase buffer and rAPID alkaline phosphatase enzyme were then added to the digestion mix and incubated at 37°C for 15

minutes. Following this, the rAPID alkaline phosphatase enzyme was inactivated by incubating the dephosphorylation reaction mix for 5 minutes at 75°C.

Digested vectors and amplicons were analysed by 1% agarose gel electrophoresis. Briefly, digested amplicons and vectors were mixed with Orange Loading Dye (New England Biolabs, UK), and loaded onto the gel. Bands corresponding to the correct digestion profiles were extracted using the QIAquick Gel Extraction kit (QIAGEN, UK) in accordance with the manufacturer's instructions. Briefly, excised bands were deposited into a micro-centrifuge tube, and the weight of the excised band determined. Following this, 3 volumes of buffer QG (solubilisation buffer) was added to 1 volume of gel, and this was incubated at 50°C with sporadic vortexing to mix, until the gel had been completely dissolved in buffer QG, and the pH adjusted as described previously. For DNA fragments <500bp and >4kb, 1 gel volume of 100% isopropanol was added to the mix. This was then transferred to a QIAquick spin column, and centrifuged at 18,000g for 1 min, and the flow-through subsequently discarded. The column was washed by addition of buffer QG, and centrifuged at 15,000g for 1 minute, following which the flow-through was discarded. Following this, buffer PE (wash buffer containing ethanol) was added to the column, which was centrifuged at 18,000g for 1 minute. The flow-through was discarded, and the column centrifuged again for 1 minute at 18,000g. The column was then transferred to a clean micro-centrifuge tube, and 30µl nuclease-free water added to the column membrane, and left to stand at room temperature for 1 minute. DNA was then eluted by centrifuging the column at 18,000g for 1 minute, and its concentration determined by spectrophotometry as described in Section 2.4.

Digested amplicons and vectors were ligated using the Rapid DNA Ligation Kit (Roche Biosciences, UK) following the manufacturer's instructions. Briefly, appropriately digested amplicons and vectors were mixed to a molar ratio of 5:1. Following the addition of T4 DNA ligation buffer, DNA dilution buffer, T4 DNA ligase enzyme, the reaction mix was incubated at room temperature for 5 minutes. Following this, 1/10th of the ligation mix was transformed into TOP10 chemically competent *Escherichia coli* (*E. coli*; Invitrogen, UK) by heat shock. Briefly, the reaction mix was incubated on ice with TOP10 chemically competent *E. coli* for 30 minutes, following which the mix was transferred to a water bath at 42°C for 45 seconds, and transferred back to ice for 2 minutes. Following this, S.O.C medium (Invitrogen UK; 2% tryptone, 0.5% yeast extract, 10mM NaCl, 2.5mM KCl, 10mM MnCl, 10mM MnSO₄, 20mM glucose) was added to the transformation mix under sterile conditions, and incubated at 37°C for 1 hour whilst shaking at 225 rpm. Following this, the transformation reaction was transferred and spread onto LB Agar (Sigma-Aldrich, UK) plates containing 100µg/ml ampicillin, and cultured overnight at 37°C.

Following overnight culture at 37°C, three selected colonies for each construct were then grown in 4ml LB broth (Sigma-Aldrich, UK) containing 100µg/ml ampicillin overnight at 37°C. Plasmid DNA was isolated from transformed *E. coli* using the QIAprep Spin Miniprep kit (QIAGEN, UK) following the manufacturer's instructions. Briefly, cells were harvested by centrifuging the overnight cultures at 5,400g for 10 minutes at 4°C, and the supernatant discarded, the pellets resuspended in buffer P1 (resuspension buffer; containing RNase A) and transferred to a clean micro-centrifuge tube. Cells were then lysed by the addition of buffer P2 (lysis buffer), and the lysis reaction subsequently stopped by the addition of buffer N3 (neutralisation buffer). The reaction mix was mixed by inversion following each addition. This was then centrifuged for 10 minutes at 18,000g,

the supernatant transferred to a QIAprep spin column, and the pellet discarded. The column was centrifuged for 1 minute at 18,000g and the flow-through discarded. The column was washed with buffer PB (to remove endogenous bacterial nuclease activity) by centrifugation at 18,000g for 1 minute, and the flow-through discarded. Buffer PE (wash buffer containing ethanol) was added to the column and centrifuged for 1 minute at 18,000g. The flow-through was discarded, and the column centrifuged for a further minute at 18,000g. Nuclease-free water was added to the column membrane, and incubated at room temperature for 1 minute, followed by elution of plasmid DNA by centrifugation at 18,000g for 1 minute. DNA concentration was determined by spectrophotometry as described in Section 2.4. Successful ligation of plasmid DNA was verified by digestion with the restriction enzymes used to create them and agarose gel electrophoresis as described in Section 2.5. Positive colonies were subject to direct sequencing using the appropriate primer sets and sequencing strategies as described per Chapter. Successfully positive colonies were re-cultured overnight at 37°C in LB-broth, and added to glycerol (Sigma-Aldrich, UK) at a ratio of 4:1 under sterile conditions. These were stored at -80°C until required.

2.8 Site-directed mutagenesis

Site-directed mutagenesis was performed to introduce point mutations within the appropriate templates to obtain vectors containing mutant isoforms of PLC ζ , using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, USA) and following the manufacturer's instructions. The templates and primers used are detailed within each individual Chapter where appropriate. Primers were designed to be between 25-45bp in length, with a melting temperature $\geq 78^\circ\text{C}$. The desired mutation was located in the middle of the primer with ~10–15 bases of correct sequence on either side, and the

guanine/cytosine (GC) content of the primers was kept at ideally ~40%, terminating in one or more C or G bases.

The site-directed mutagenesis reaction mix consisted of 10x reaction buffer (100mM KCl, 100mM (NH₄)₂SO₄, 20mM Tris-HCl pH 8.8, 20mM Mg SO₄, 1% Triton X-100, 1mg/ml nuclease-free BSA, 50ng of template vector DNA, 125ng each of forward and reverse primer, dNTP mix, double distilled water, and *PfuTurbo* DNA polymerase (2.5 Units/μl) provided with the kit. The reaction mix was subjected to PCR as detailed in Table 2.3. Template DNA was digested by incubation with *Dpn I* restriction enzyme (10 Units/μl) for 1 hour at 37°C. The reactions were transformed into TOP10 *E. coli*, cultured and sequenced as described in Section 2.3. It is important to note that the LB-Agar/ampicillin plates used for this section were also coated with 800ng/μl X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) solution (Fermentas, UK), to allow for blue/white colony selection. White colonies exhibiting successful transformation were selected for sequencing.

Table 2.3: PCR cycles utilised for site directed mutagenesis utilising the QuikChange Site-Directed Mutagenesis Kit (Stratagene, USA)

Temperature (°C)	Time (minutes . seconds)
110	(Heated lid step)
95	0.30
Start Cycle (x12)	
95	0.30
55	1.00
68	2.00/kb of plasmid length
End Cycle 1	
8	Infinity

2.9 Plasmid DNA isolation for mammalian cell transfection

In order to provide sufficient quality for mammalian cell transfection, plasmid DNA was purified using the Hispeed Plasmid Midi kit (QIAGEN, UK), following the manufacturer's protocol, from appropriately transformed TOP10 *E. coli* glycerol stocks prepared previously. Briefly, 5ml of LB-broth containing 100µg/ml ampicillin was inoculated under sterile conditions with the appropriate glycerol stock, and cultured at 37°C for 8 hours whilst shaking at 225 rpm. This incubation was used to inoculate 100ml of LB-broth containing 100µg/ml ampicillin under sterile conditions, and cultured overnight at 37°C whilst shaking at 225 rpm.

Following this, cells were harvested as previously described in Section 2.7, resuspended with buffer P1 (resuspension buffer; 50mM Tris-Cl pH 8.0, 10mM EDTA, 100µg/ml RNase A), and homogenised by vortexing. Cells were lysed by adding buffer P2 (lysis buffer; 200mM NaOH, 1% SDS), and the lysis reaction stopped by addition of buffer P3 (neutralisation buffer; 3M potassium acetate pH 5.5). All suspensions were mixed by inversion. Cell debris was removed using a filtered syringe, and the clear lysate added to a Hispeed Midi Tip previously calibrated by addition of buffer QT (calibration buffer; 750mM NaCl, 50mM MOPS pH 7.0, 15% isopropanol, 0.15% Triton X-100). The lysate was allowed to flow through the column resin by gravity, and the flow-through discarded. The resin was washed with buffer QC (wash buffer; 1.0M NaCl, 50mM MOPS pH 7.0, 15% isopropanol), and DNA eluted with buffer QF (elution buffer; 1.25M NaCl, 50mM Tris-Cl pH 8.5, 15% isopropanol). DNA was precipitated from the elution mix by the addition of (0.7 volumes) 100% isopropanol and incubated at room temperature for 5 minutes. Precipitated DNA was purified using a syringe and the QIAprecipitator from the kit, and washed in the QIAprecipitator using 70% ethanol. The DNA in the QIAprecipitator was eluted using double-distilled nuclease-free water, and DNA yield maximised by repeating this step with the same water. DNA concentration was determined using a spectrophotometer as described in Section 2.4.

2.10 Mammalian cell culture and transfection using the jetPEI kit

Human embryonic kidney transformed (HEK293T) cells were cultured under standard conditions and transfected using an established calcium phosphate precipitation protocol (described in the materials and methods of Chapter 6), and the jetPEI transfection (Poly-plus transfection, USA). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with high glucose, and supplemented with 10% fetal bovine serum

(FBS), 4mM glutamine, 100U/ml penicillin, and 0.1mg/ml streptomycin. Cells were incubated at 37°C with 5% CO₂ in a humidified chamber. Cells were then transfected at a confluency of ~60-70%.

To transfect using the jetPEI transfection reagent, the manufacturer's protocol was followed (Poly-plus transfection, USA) under sterile conditions. Briefly, to transfect cells in 4-well plates, 1µg of the appropriate plasmid DNA was diluted to 50µl with 150mM NaCl provided with the kit. Concurrently, 2µl of the jetPEI transfection reagent was also diluted with 150mM NaCl to a final volume of 50µl. These were thoroughly mixed by vortexing, followed by brief centrifugation to collect the mix at the bottom of the tube. Diluted jetPEI transfection reagent was added to the diluted DNA, mixed by vortexing, and briefly centrifuged to collect the mix at the bottom of the tube. The mix was then incubated at room temperature under sterile conditions for 30 minutes to allow DNA transfection complexes to form. Following incubation, the entire mix (100µl) was added per well, and the 4-well plate gently swirled to ensure mixing. To transfect 175cm² flasks, the same protocol was followed, but the amounts of DNA and transfection reagent used were scaled up according to the manufacturer's protocol (Poly-plus transfection, USA).

2.11 Preparation of HEK293T cell lysates

Fifty six hours post-transfection of HEK293T cells, culture media was removed and cells lysed by freezing at -80°C overnight followed by resuspension in Ca²⁺ free intracellular medium (120mM KCl/ 20mM HEPES pH=7.4) containing protease inhibitors (Roche biosciences, UK). Cells were then repeatedly manually aspirated with a blunt 20 gauge needle and syringe to disrupt the plasma membrane, and centrifuged at 18,000g for

20 minutes at 4°C. Pellets were discarded, and supernatants divided into single-use aliquots for storage at -80°C until required.

2.12 cRNA synthesis

cRNA was transcribed for each PLC ζ isoform using either a T7 or SP6 mMESSAGE mMACHINE kit (Ambion, USA; depending on which promoter was to be used: T7 or SP6), where appropriate, in accordance with the manufacturer's instructions. Briefly, the appropriate plasmid DNA (adjusted to concentration of 1 μ g/ μ l in the linearisation reaction) was linearised using either *SpeI* (New England Biolabs; T7 Promoter) or *NotI* (New England Biolabs; SP6 promoter). The linearisation reaction was terminated by addition of 0.5M EDTA, followed by addition of 3M sodium acetate. Linearised DNA was precipitated by incubation at -20°C for 30 minutes, and centrifuged at 18,000g for 15 minutes to pellet the precipitated DNA. The supernatant was removed, and the pellet re-suspended in an appropriate volume of nuclease-free water (adjusted to a concentration of 1 μ g/ μ l).

cRNA synthesis and purification were performed in an RNase free environment, using RNaseZap (Ambion, UK). 1 μ g of linearised plasmid DNA was added to a reaction mix containing nuclease-free water, 10X reaction buffer, 2X NTP/CAP, and enzyme mix (all included in the kit; Ambion, UK), and mixed by gentle pipetting. The reaction mix was then incubated at 37°C for 1 hour, and the template DNA subsequently removed by incubation with TURBO DNase (Ambion, UK) for 15 minutes at 37°C. Following synthesis, cRNA was purified using the RNeasy Mini kit (QIAGEN, UK), following the manufacturer's instructions. Briefly, the cRNA reaction mix was adjusted to 100 μ l with RNase-free water (Ambion, UK), followed by addition of 100% ethanol and mixing by

gentle pipetting. The mix was then transferred to an RNeasy mini spin column, and centrifuged at 8,000g for 15 seconds. The flow-through was discarded, and the column washed by addition of buffer RPE (wash buffer containing ethanol) to the column and centrifugation at 8,000g for 15 seconds. After discarding the flow-through, the column was washed again with buffer RPE by centrifugation at 8,000g for 2 minutes. Following this, the column was placed in a fresh collection tube, and centrifuged at 18,000g for 1 minute. The column was then placed in a clean RNase-free micro-centrifuge tube, RNase-free water added to the column membrane, and the column centrifuged for 1 minute at 8,000g. The eluted RNA was then divided into single-use aliquots and stored at -80°C. RNA concentration was determined using spectrophotometry as previously described in Section 2.4.

2.13 RNA agarose denaturing-gel electrophoresis

cRNA integrity and purity were assessed utilising 1% agarose gel electrophoresis. Briefly, 2g agarose was dissolved in 176ml RNase-free, Diethylpyrocarbonate (DEPC)-treated water (Ambion, UK) by microwaving until the agarose had fully dissolved. After the solution had cooled, 4ml formaldehyde (Sigma-Aldrich, UK) and 20ml 10x 3-N(Morpholino) Propane Sulfonic Acid (MOPS) solution were added, and mixed by gentle swirling in a fume hood. The solution was poured into a gel-cassette and left to set at room temperature. After the gel had set, it was immersed in a gel tank (treated with 3% hydrogen peroxide, ethanol, and RNase zap to remove RNase) filled with RNA gel running buffer (1xMOPS solution; 100ml 10xMOPS, 20ml formaldehyde, 880ml DEPC-treated water).

Prior to loading cRNA samples onto the gel, samples were mixed with 5µl 10x Orange loading dye (New England Biolabs, UK), and 2µl of ethidium bromide (200µg/ml). 3µl of the 0.28kb-6.6kb Transcript RNA marker (Sigma-Aldrich, UK) was also added to 2µl ethidium bromide (200µg/ml) and 3µl 10x Orange loading dye (New England Biolabs, UK). Samples were incubated at 65°C for 10 minutes, and concurrently, the gel was 'pre-run' for 10 minutes at 70 volts. Samples were loaded onto the gel and run at 90 volts until the dye bands had migrated approximately three quarters the length of the gel and visualised using a GelDoc Imaging System (UVP, UK). For a representative RNA agarose gel, refer to Supplementary Appendix 1.

2.14 Density gradient washing (DGW) of human sperm samples

All semen samples were subject to DGW prior to analysis. 40% PureSperm gradient media was overlayed on top of 80% media (PureSperm 40/80, Nidacon International AB). Media were allowed to equilibrate to room temperature (RT) for 1 hour and liquefied fresh semen was layered on top of the gradient and centrifuged at 300 g for 20 minutes at RT. The supernatant was discarded leaving 0.5ml above the pellet containing the most motile sperm. This was aspirated into tubes containing PureSperm Wash (Nidacon International AB) and centrifuged at 500 g for 5 minutes at RT. Pellets were resuspended in PureSperm Wash media and centrifuged at 500 g for 5 minutes at RT. Pellets were finally resuspended in a suitable quantity of PureSperm Wash according to pellet size. All fractions were fixed with an equal volume of 4% paraformaldehyde in phosphate-buffered saline (PBS) for 10 minutes at RT.

2.15 Determination of PLC ζ immunofluorescence in human sperm

PLC ζ was detected by immunofluorescent staining with an anti-PLC ζ antibody as previously described by Grasa *et al.* (2008). This was an affinity-purified anti-human-PLC ζ primary polyclonal antibody (previously designed in Dr. Parrington's laboratory and donated to the Coward laboratory), used in previously published studies (Grasa *et al.*, 2008; Yoon *et al.*, 2008; Heytens *et al.*, 2009). Binding specificity of the antibody to PLC ζ was determined by Grasa *et al.* (2008) following peptide blocking of target residues. All these studies identified the localisation patterns in sperm detailed within this thesis. Antibody binding was observed within the sperm tail. However, this was confirmed by Grasa *et al.* (2008) to be non-specific, and due to the polyclonal nature of the antibody. Similar non-specific binding using this antibody was also observed in immunoblots (Grasa *et al.*, 2008; Heytens *et al.*, 2009).

In brief, samples were added to PAP moulds (Immedge Pen, Vector Laboratories, USA) drawn on glass slides previously coated with 0.01% poly-L-lysine (Sigma-Aldrich, UK), and placed in a humidifying chamber. Slides were left at RT for 45 minutes to allow sperm to settle, and the remaining suspension discarded. Samples were then permeabilised by overnight incubation with 0.5% Triton X-100/PBS (Sigma-Aldrich, UK). Following this, slides were rinsed three times with PBS, and non-specific binding sites blocked by incubation with 3% bovine serum albumin (BSA; Sigma-Aldrich, UK)/PBS at RT for 1 hour, after which slides were washed another three times with PBS.

The anti-human-PLC ζ primary polyclonal antibody was diluted to a final concentration of 25 μ g/ml in 0.05 % in BSA/PBS, and applied to the PAP moulds for an overnight incubation at 4°C. Antibody specificity was previously confirmed by pre-

incubation with an excess of immunogenic peptide, and cross-reactivity to other PLCs tested by Cova-Lab, UK (Grasa *et al.*, 2008). Slides were then washed three times in PBS, and incubated for 1 hour at RT in the dark, followed by the application of 5µg/ml of fluorescent secondary antibody (AlexaFluor 488 F(ab'')₂ goat anti-rabbit, Invitrogen, UK) in 0.05%BSA/PBS. Slides were finally washed three times, and mounted using Vectashield H-1000 mounting media for fluorescence containing 4'-6-Diamidino-2-phenylindole (DAPI) (Vector, UK) and 13 mm diameter glass cover slips. Clear nail varnish was used to seal the edge of each coverslip. PLCζ immunofluorescence was detected using a fluorescein isothiocyanate filter (FITC; fluorescence microscopy) at a wavelength of 488nm (confocal microscopy), while DAPI fluorescence was detected using a DAPI filter (fluorescence microscopy) and at a wavelength of 405nm (confocal microscopy).

2.16 Polyacrylamide gel electrophoresis and immunoblotting for protein analysis

Proteins were separated on pre-cast polyacrylamide gels (Invitrogen, UK). The concentration of gels used is described within individual Chapters where appropriate. Briefly, proteins were mixed with 1x loading dye (Invitrogen, UK) and 1X reducing agent (Invitrogen, UK), and incubated at 101°C for 5 minutes to denature protein structure. The denatured protein was loaded onto gels, which were run at 150 volts until all visible bands had reached the bottom of the gel. To visualise separated protein bands, gels were initially washed 3 times with double distilled water for 15 minutes each, incubated with coomassie G-250 stain (SimplyBlue SafeStain, Invitrogen, UK) for 1 hour, and destained with double-distilled water. The molecular weights of target protein bands were deduced by comparison with the Novex Sharp pre-stained protein standard (Invitrogen, UK).

For immunoblotting, separated proteins were transferred onto polyvinylidene difluoride (PVDF) membranes (GE Healthcare, UK) using a semi-dry transfer cell (Bio-Rad, UK) at 15V and 0.5 A for 1 hour. Membranes were blocked for 1 hour at room temperature with PBS containing 0.5% Tween-20 (PBS-T) and fat-free milk (5% w/v). Membranes were incubated with primary antibody in PBS-T/1% milk and incubated overnight at 4°C, then washed 3x15 minutes with PBS-T. Membranes were incubated with secondary antibody for 1 hour at room temperature, followed by a further 3x15 minutes wash with PBS-T. Specific primary and secondary antibodies used are detailed where appropriate per Chapter. Immunoreactivity was detected using an Enhanced Chemiluminescence Reaction kit (GE Healthcare, UK). Membranes were exposed to Amersham Hyperfilm ECL (GE Healthcare, UK) and developed using a Compact X2 automatic developer (X-Ograph, UK).

2.17 Mouse oocyte collection, culture and micro-injection

Metaphase II (MII) stage oocytes were collected from the oviducts of 4- to 6-week-old CD-1 female mice. Females were super-ovulated by injection of 5 injection units (IU) pregnant mares serum gonadotropin (PMSG; Sigma, St Louis, MO). 42-46 hours later, ovulation was induced by the injection of human chorionic gonadotropin (hCG). Oocytes were collected 14 hours post-hCG injection in a HEPES-buffered Tyrode-lactate solution (TL-HEPES) supplemented with 5% heat-treated fetal calf serum (Gibco, Grand Island, NY). Cumulus cells were removed by brief incubation in TL-HEPES supplemented with 0.1% bovine testes hyaluronidase (Sigma, USA) followed by repeated pipetting. Denuded oocytes were placed in 50µl drops of KSOM (Potassium Simplex Optimised Medium; Specialty Media, Phillipsburg, NJ) containing 0.1% polyvinyl alcohol (PVA; Sigma, USA) until injection. Oocytes were micro-injected as previously described by Kurokawa *et al.*

(2005). In brief, reagents were loaded into glass micropipettes and delivered by pneumatic pressure (PLI-100 picoinjector, Harvard Apparatus, Cambridge, MA). Each oocyte received 7-12pl (~1-3% of the total volume of the oocyte). Post-injection, oocytes were cultured in the same media at 37°C with 5% CO₂ in an air humidified chamber. Animal handling and procedures were approved by the University of Massachusetts IACUC committee. All procedures described in this section were performed by HC Lee and Professor RA Fissore (University of Massachusetts, USA).

2.18 Ca²⁺ imaging in mouse oocytes

Intracellular Ca²⁺ response within injected mouse oocytes was measured using the Ca²⁺ sensitive dye Fura-2AM (Molecular Probes, USA). Recording of Ca² oscillations was performed as previously described by Kurokawa *et al.* (2005). Oocytes were loaded with 1.25 μM Fura-2AM supplemented with 0.02% pluronic acid (Molecular Probes, USA) for 20 min at room temperature. Within 20 minutes of injection, oocytes were placed in drops of TL-HEPES under mineral oil and fluorescence ratios of 340/380 nm were obtained every 20 seconds. Ultra violet light was provided by a 75 W xenon arc lamp and modulated by neutral density filters. Emitted light above 510 nm was collected by a cooled Photometrics SenSys CCD camera (Roper Scientific, Tucson, USA). Ca²⁺ changes were registered, and filter wheel changes controlled by SimplePCI software (C-Imaging System, USA). All procedures described in this section were performed by HC Lee and Professor RA Fissore (University of Massachusetts, USA).

2.19 Microscopy and digital imaging of transfected HEK293T cells

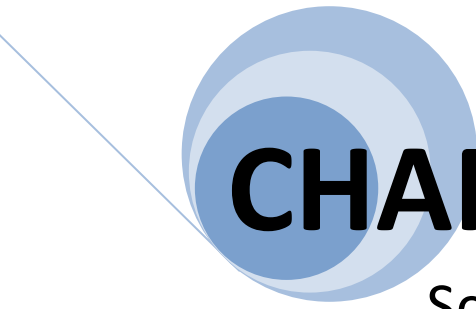
Throughout the course of this thesis, the predominant imaging techniques utilised were fluorescence microscopy and confocal microscopy. The specific fluorophores used are described more thoroughly in each individual Chapter where appropriate. In general, unless otherwise stated for fluorescence microscopy, a Nikon Eclipse 80i fluorescence microscope (10x, 20x, and 40x objectives) was used unless otherwise stated. Blue fluorescence was captured using a DAPI filter, green fluorescence using a fluorescein isothiocyanate (FITC) filter, and red fluorescence using a tetraethyl rhodamine isothiocyanate (TRITC) filter. Images were captured using NIS-Elements Basic Research 3.0 software (Nikon, UK). For confocal microscopy, a Zeiss LSM6 upright 510 META microscope (Department of Pathology, University of Oxford) was used, and blue fluorescence captured at a wavelength of 405nm, green fluorescence captured at a wavelength of 488nm, and red fluorescence captured at a wavelength of 560nm.

For fluorescence microscopy, HEK293T cells were grown on 25x75x1mm glass coverslips in 4-well plates (VWR, UK) and transfected at ~60% confluency with mCherry constructs (1µg) using the jetPEI kit (Poly-plus transfection, France), following manufacturer's instructions. At the appropriate time-points (discussed in each Chapter individually), media was removed, and coverslips washed three times with Hanks' buffered saline solution (HBSS; Gibco, UK), fixed with 10% formalin neutral buffered containing paraformaldehyde 4% w/v for 5 minutes, and washed a further three times with HBSS. Following any additional staining (as discussed in each individual Chapter where appropriate), coverslips were mounted with Vectashield mounting medium containing DAPI (Vector Laboratories, USA) and slides stored at 4°C. Fluorescence quantification of sperm PLC ζ immunofluorescence and transfected HEK293T cell fluorescence was

performed using ImageJ software (NIH, USA), using the regions of interest (ROI), ROI manager, and quantification tools, while confocal images were captured and analysed using the Zeiss LSM image browser software (Zeiss, Germany).

2.20 Statistical analyses

Mean values and the standard error of the mean (SEM) were predominantly subject to two statistical tests to determine statistical significance ; the Students *t*-test, and one-way or two-way analysis of variance (ANOVA) using Graphpad Prism software (Graphpad, USA). One-way ANOVA analysis was also followed by Bonferroni *post-hoc* analysis. Data within the results sections are presented as mean $\pm$ SEM, while data represented as proportions were ARCSIN transformed prior to statistical analysis to avoid truncation of data. A *p*-value ≤ 0.05 was considered to be statistically significant.



CHAPTER 3

Screening of the PLC ζ gene from genomic DNA, and immunofluorescent analysis of PLC ζ in sperm, from fertile controls and infertile patients

3.1 Introduction

Mounting evidence suggests that the agent responsible for inducing Ca^{2+} oscillations within activating oocytes at fertilisation is a sperm-specific phospholipase C, phospholipase C zeta (PLC ζ), located on chromosome 12 in humans, and consisting of 15 coding exons (Saunders *et al.*, 2002; Swann *et al.*, 2006; Parrington *et al.*, 2007; Saunders *et al.*, 2007; Kashir *et al.*, 2010). Given the apparent fundamental role of PLC ζ in activating the oocyte following gamete fusion, PLC ζ isoforms exhibiting abnormal expression profiles, or defective functional ability, may underlie certain types of human male factor infertility in which oocyte activation is deficient (OAD).

Yoon *et al.* (2008) provided the first crucial link between OAD and PLC ζ deficiency, demonstrating that sperm from patients repeatedly failing to activate oocytes via ICSI could not initiate Ca^{2+} oscillations when injected into mouse oocytes, and were associated with abnormal expression and localisation of PLC ζ . Heytens *et al.* (2009) provided the first genetic link to PLC ζ deficiency by identifying a substitution mutation in an infertile male diagnosed with OAD. Occurring within the Y domain of the active site at position 398 of the PLC ζ open reading frame (ORF), this mutation resulted in a histidine to proline substitution (PLC ζ^{H398P}). Injection of PLC ζ^{H398P} cRNA into mouse oocytes resulted in highly abnormal Ca^{2+} transients that were unable to activate oocytes (Heytens *et al.*, 2009). Moreover, a mouse equivalent of PLC ζ^{H398P} , resulting in a histidine to proline substitution at position 435 of the mouse PLC ζ ORF (PLC ζ^{H435P}), also lacked the ability to induce Ca^{2+} oscillations in oocytes, and was defective in PLC activity (Nomikos *et al.*, 2011a).

While the H398P mutation has been critical towards improving the understanding of PLC ζ mode and mechanism of action as discussed in subsequent Chapters of this thesis, this mutation remains the only published PLC ζ mutation identified thus far. Infertility is now thought to affect 1 in 7 couples worldwide, with ART now accounting for ~7% of births in some developed countries (Kashir *et al.*, 2010). While the H398P mutation illustrates the potential involvement of loss-of-activity mutations in PLC ζ within cases of male infertility where oocyte activation is deficient, PLC ζ also holds much promise as a marker of male fertility.

Several studies have attempted to define the precise localisation pattern of PLC ζ in sperm. Initial studies indicated that PLC ζ was predominantly localised to post-acrosomal and equatorial regions in non-capacitated mouse and bull sperm, respectively (Yoon & Fissore, 2007), while the post-acrosomal localisation was shown as being prevalent in capacitated mouse and hamster sperm (Young *et al.*, 2009), with human PLC ζ exhibiting a predominantly equatorial localisation in the sperm head. Recently, Nakai *et al.* (2011) demonstrated PLC ζ localisation to the sperm tail as well as within acrosomal and post-acrosomal regions in porcine sperm, indicating a potential role for this population of PLC ζ within the sperm tail, but not directly concerning oocyte activation. However, while supporting the hypothesis that PLC ζ may be involved in multiple events associated with oocyte activation (Grasa *et al.*, 2008; Young *et al.*, 2009; Kashir *et al.*, 2010), the sperm tail population has not been identified by similar immunofluorescence studies on sperm from other species. It currently remains to be established whether the multiple populations of PLC ζ identified in sperm by various studies have a functional purpose, and if so the extent to which they contribute towards oocyte activation.

A diagnostic test based on the oocyte activation ability of human sperm microinjected into mouse oocytes (known as the mouse oocyte activation test; MOAT) has previously been proposed (Kashir *et al.*, 2010). A heterologous ICSI model proposed to evaluate the activation capacity of human sperm by microinjection into mouse oocytes (Heindryckx *et al.*, 2005; 2008), the MOAT may represent a useful method with which to investigate cases of low ICSI success rates. Indeed, Yoon *et al.* (2008) utilised this test to illustrate the lack of oocyte activation of sperm from ICSI failed patients. However, considering that human PLC ζ is thought to be more potent in its activity compared to mouse PLC ζ (Swann *et al.*, 2006) and that oocytes from various species are thought to be ‘fine-tuned’ to specific levels and activity of PLC ζ from that species (Cooney *et al.*, 2010), it is possible that the MOAT may only be useful to detect extreme cases of OAD where PLC ζ is completely absent from sperm, and not cases where only a more subtle reduction in PLC ζ is observed in a clinical setting. Coupled with the requirement for animal facilities and specialised equipment and skills which may not be available to many assisted reproductive centres, the MOAT may not be considered a viable diagnostic for oocyte activation capability (Kashir *et al.*, 2010), and may represent more of a specialised research tool.

The utilisation of sperm-specific biomarkers linked to conditions of male infertility may represent powerful diagnostic tools. Indeed, levels of seminal factors such as β -microseminoprotein, one of the most abundant proteins in human seminal plasma thought to act as an immunoglobulin binding factor and/or tumor marker for prostate cancer, have been shown to be greatly abnormal compared to fertile controls (in this case present at higher levels compared to control seminal fluid; Kamada *et al.*, 1998; Sakai *et al.*, 1999; Anahi Franci *et al.*, 2008). Another such example is the correlation between sperm

protamine 1 and 2. The ratio between these two proteins directly correlated with DNA fragmentation in human sperm, with the ratio differing significantly between fertile and infertile individuals (García-Peiró *et al.*, 2011). Indeed, the use of immunofluorescence to determine accurately levels of sperm protein to evaluate semen quality has been shown recently by Odhiambo *et al.* (2011), who used flow cytometry to demonstrate a positive correlation between abnormal bovine sperm morphology and levels of ubiquitin-induced fluorescence, and lectin *Arachis hypogaea* agglutinin (PNA) to assess the maturity of bovine sperm.

Considering that the only mutation that has thus far been identified by Heytens *et al.* (2009) occurred within the active site and was shown to affect PLC ζ activity, this Chapter aimed to investigate the prevalence of similar loss-of-activity mutations within infertile males who had been diagnosed with OAD. This thesis utilised a modified version of the PCR-based screen of patient genomic DNA, previously employed by Heytens *et al.* (2009), to screen the exons coding for the active site of the PLC ζ ORF (exons 5-12), to identify further novel loss-of-activity mutations in PLC ζ which may be affecting PLC ζ activity in males diagnosed with OAD attending clinics in the UK, Iran, and Belgium for fertility treatment. A secondary aim was to investigate and discuss the possibility of utilising quantitative PLC ζ immunofluorescence in sperm as a diagnostic test to predict oocyte activation capability, and thus male fertility, utilising sperm from fertile controls and ICSI failed patients from the Oxford Fertility Unit (OFU). Collectively, this information would not only increase understanding of the potential role of PLC ζ in human male factor infertility, but aid the translation of PLC ζ as a clinical therapeutic and prognostic tool with which to combat male infertility.

3.2 Materials and Methods

3.2.1 Patient selection and genomic analysis

The use of donated samples from fertile controls and OAD patients was ethically cleared as discussed in Chapter 2. For the genomic DNA screen, control (n=2) and patient (n=12) saliva samples, obtained from individuals recruited from the UK, Belgium, and Iran, were used. Genomic DNA was extracted from buccal cell samples obtained from saliva as previously described (Chapter 2, Section 2.2). In the second part of this study, sperm was obtained from controls (n=7) and infertile patients (n=3) recruited from the Oxford Fertility Unit to screen PLC ζ protein by quantitative immunofluorescence, and perform proportional analysis of PLC ζ localisation patterns. Patient selection criteria for genomic DNA (Table 3.1) and sperm (Table 3.2) analysis were dependent upon factors such as low fertilisation rates, a history of ICSI failure, or a low MOAT result. Controls which were selected for this study exhibited no previous indications of male infertility.

3.2.2 PCR and direct sequencing of PLC ζ active site coding exons

PLC ζ exons 5-12 were amplified from each sample with optimised cycle conditions as previously described (Table 2.2, Chapter 2). Primers were originally designed by Dr John Parrington (Department of Pharmacology, University of Oxford; Table 3.3) using Mutation Discovery software (Transgenomic Ltd, Glasgow, UK). PCR reactions were performed using a G-storm Thermal Cycler (Model GS4, GSI, UK). Amplicons (and Orange loading dye) were analysed using 2% agarose gel electrophoresis. A 50bp DNA ladder (New England Biolabs, UK) was used to calibrate the data. Amplicons were extracted and purified using the QIAquick Gel Extraction Kit (Qiagen, UK), following manufacturers' instructions, and eluted with double distilled water. Amplicons were

sequenced by Geneservice Ltd. (Department of Biochemistry, Oxford), and results compared with published amplicon and ORF sequences using NCBI BLAST.

Table 3.1: Table illustrating the criteria used for patient selection for the patient population recruited from Belgium, Iran, and the UK. N/A denotes fields where data was unavailable. MOAT % denotes the percentage of oocytes that successfully elicited Ca^{2+} oscillations following the microinjection of patient sperm into mouse oocytes. Controls and patients listed in this table were recruited from the UK, Belgium, and Iran only for genomic DNA screening

Globozoospermic: round-headed sperm lacking an acrosome.

Patient	Sperm status		
	Location	Morphology	MOAT %
Control 1	UK	Normal	N/A
Control 2	UK	Normal	N/A
Patient 1	Belgium	Globozoospermic	0
Patient 2	Belgium	Globozoospermic	4
Patient 3	Belgium	Globozoospermic	50
Patient 4	Belgium	Globozoospermic	11
Patient 5	UK	Globozoospermic	N/A
Patient 6	Belgium	Globozoospermic	4
Patient 7	Iran	N/A	N/A
Patient 8	Iran	Globozoospermic	N/A
Patient 9	Belgium	N/A	N/A
Patient 10	Belgium	Normal	N/A
Patient 11	Belgium	N/A	N/A
Patient H398P	Belgium	Normal	0

Table 3.2: Summary of sperm motility grades and motility count following DGW, and outcome of ART procedure used. Patients were recruited following previous ICSI failure of ≥ 8 oocytes, or presented with sperm exhibiting abnormal morphology. Controls and patients listed in this table were recruited from the OFU only for sperm PLC ζ protein screening.

NB: This data was not available for Controls 1 and 2 since they were not undergoing fertility treatment. Both these controls were males with no previous history or indication of male infertility.

DGW: Density gradient wash; ART: Assisted reproductive technology; IVF: *In vitro* fertilisation; ICSI: Intracytoplasmic sperm injection

	Morphology	Motility following DGW (%)				Motile count (x10 ⁶ /ml)	ART procedure	Oocytes fertilised/utilised (%)
		a	b	c	d			
Control 3	Normal	85	4	1	10	18.07	IVF	6/7 (85.7)
Control 4	Normal	88	6	0	6	12.0	IVF	5/6 (83.3)
Control 5	Normal	62	13	4	22	14.3	IVF	2/10 (20)
Control 6	Normal	70	16	7	7	7.4	IVF	2/2 (100)
Control 7	Normal	96	0	0	4	11.6	IVF	7/10 (70)
Patient 1	Normal	91	0	0	9	7.55	ICSI	6/8 (75)
Patient 2	Normal	66	13	0	20	5.6	ICSI	0/4 (0)

Table 3.3: Sequences of the PLC ζ amplicons amplified (exons 5-12), and the forward and reverse primers used to amplify them.

Exon	Primer Sequence
5	Forward: GAGTAAGTTTACAAGTGAGATGTAG
	Reverse: CCTACTGAGGTAGACTGCCA
6	Forward: TTTAATGCAAATTTCTAGAGACTCAAA
	Reverse: TATCATCTAAAGTAAGGCTAAGCA
7	Forward: AGGTACTAAACGTTAGAAGTAAGT
	Reverse: CACAGGCCAATATAACAGTGACA
8/9	Forward: GCTAATTGGAAATATAGTGAAACAC
	Reverse: AAGTAAATTGTAAATGATAGACTAGAG
10	Forward: TCAAATGGTGTGTTGAGGAGATAAT
	Reverse: GAGCAATCTTAACACCCTAGCAG
11	Forward: TTCCTGAATGATCAGTGACATA
	Reverse: TACTTCTGCAAACAACTCAATATC
12	Forward: TCAGTAAGTGGTTTGTGTTCCCTT
	Reverse: GAAATTGAAGCAAATCAGTGGAAT

3.2.3 Sub-cloning of PLC ζ amplicons into the pCRII-TOPO vector

Amplicons obtained for fertile controls and infertile patients were sub-cloned into the pCRII-TOPO vector (Invitrogen, UK; refer to Supplementary Appendix 2 for vector map), following the manufacturer's protocol, and heat-shock transformed into chemically competent TOP10 *E. coli* cells. Transformation reactions were then streaked on to agar plates containing 100 μ g/ml ampicillin and 800ng/ μ l of X-gal solution (to permit blue/white colony selection; Fermentas, UK). Following incubation, 10 white colonies (indicating successful transformation) were selected from each plate and cultured overnight in Luria broth containing 100 μ g/ml ampicillin at 37°C, while shaking at 225rpm. DNA constructs were purified from resultant bacterial cultures using the QIAprep Spin Miniprep Kit (QIAGEN, UK). Positive ligation was confirmed by vector digestion using the *EcoRI* restriction enzyme (New England Biolabs, UK). Ten positively transformed colonies were submitted for sequencing as previously described (Chapter 2, Section 2.3), to verify DNA sequence and orientation within the plasmid.

3.2.4 Sperm density gradient washing (DGW)

Fresh semen samples were obtained from fertile volunteers with no previous history of infertility (n=7) and patients diagnosed with OAD (n=3). Both control and patient populations utilised for the sperm analysis section of this Chapter were attending the Oxford Fertility Unit for treatment and provided informed written consent in accordance with the ethical approval previously outlined (Chapter 2, Section 2.1). Semen samples were subject to DGW as detailed in Chapter 2, Section 2.14. Since the patient from whom H398P was previously identified attends a Belgian clinic, the sample was cryopreserved following production in Belgium, transported frozen to the Oxford laboratory and stored in liquid nitrogen to await processing. This was then also subject to DGW upon thawing.

3.2.5 Sperm PLC ζ immunofluorescence

PLC ζ was detected by immunofluorescent staining with an anti-PLC ζ antibody as previously described by Grasa *et al.* (2008) (Chapter 2, Section 2.15). Slides were imaged at 40x magnification using a Nikon Eclipse 80i fluorescence microscope utilising a brightfield filter to image sperm cells, and a fluorescein isothiocyanate (FITC) filter for PLC ζ immunofluorescence. Approximately 100 cells were analysed per male. Analysis consisted of two separate investigations; 1) quantification of total PLC ζ immunofluorescence in sperm, and 2) proportional analysis of sperm exhibiting PLC ζ immunoreactivity. This was further expanded to examine the proportional level of sperm exhibiting the various discrete patterns of PLC ζ localisation identified previously by Grasa *et al.* (2008). Analysis of patient and control sperm cells was performed using Image J software (National Institute of Health, USA) utilising the regions of interest (ROI) tool. Representative images were also obtained by confocal microscopy.

3.2.6 Statistical analysis

Correlations between mean levels of total PLC ζ fluorescence between patient and control samples were determined, along with correlations between proportions of sperm exhibiting PLC ζ immunofluorescence and sperm devoid of PLC ζ immunofluorescence. The proportions of sperm exhibiting the various localisation patterns reported by Grasa *et al.* (2008) were also quantified and compared between controls and patients. All proportions (represented as %) were arcsine transformed prior to statistical analysis using Prism software 5.0 (Graph-Pad, USA). Statistical significance was determined by one-way analysis of variance (ANOVA) followed by the Bonferroni multiple comparisons *post-hoc* test. The Students' *t*-test was also utilised to determine statistical significance between fertile and infertile populations. Additionally, two-way ANOVA was used to investigate

for statistical significance of PLC ζ localisation patterns between fertile and infertile sperm. Sperm from patient H398P has previously been reported by Heytens *et al.* (2009) to contain an original punctate pattern of PLC ζ localisation as opposed to the characteristic patterns reported by Grasa *et al.* (2008), and was thus not included in the localisation proportion analysis.

3.3 Results

3.3.1 PCR screening of PLC ζ exons from fertile and infertile human males

This part of the investigation utilised genomic DNA extracted from controls and patients recruited in the UK, Belgium, and Iran. Sequencing of amplicons corresponding to exons 5-12 of the PLC ζ ORF (Figure 3.1) revealed the vast majority of exons to be normal when compared to ORF sequences (Table 3.4; Supplementary Appendix 3). Sequencing also yielded inconclusive results for several exons due to sequencing failure (Table 4; Figure 3.2), which persisted following multiple attempts. However, a previously unobserved base change was identified in exon 6 from patient H398P; an adenine (A) > thymine (T) change at position 698 of the PLC ζ ORF (within the X-Domain), resulting in a Histidine to Leucine substitution at position 233 (H233L) in the amino acid sequence of PLC ζ , confirmed by four independent repeats (Figure 3.3; described and characterised in more detail in Chapter 4).

Sub-cloning of PCR generated amplicons (which were unsuccessful following direct sequencing) into pCRII-TOPO resulted in successful sequencing following restriction digest confirmation of successfully cloned colonies (as indicated by their digestion profile; Figure 3.4). The example discussed in this section pertains to the sub-cloning of exon 8/9 from Control 1, where a band at the top of the gel (corresponding to digested vector) and a band at ~376bp (corresponding to the amplicon size for exon 8/9) indicated a successful cloning reaction. However, considering the nature of using genomic DNA as a template (maternal and paternal alleles are both amplified simultaneously), each successfully transformed colony would have contained pCRII-TOPO plasmid ligated with PCR product from only a single allele (Figure 3.5).

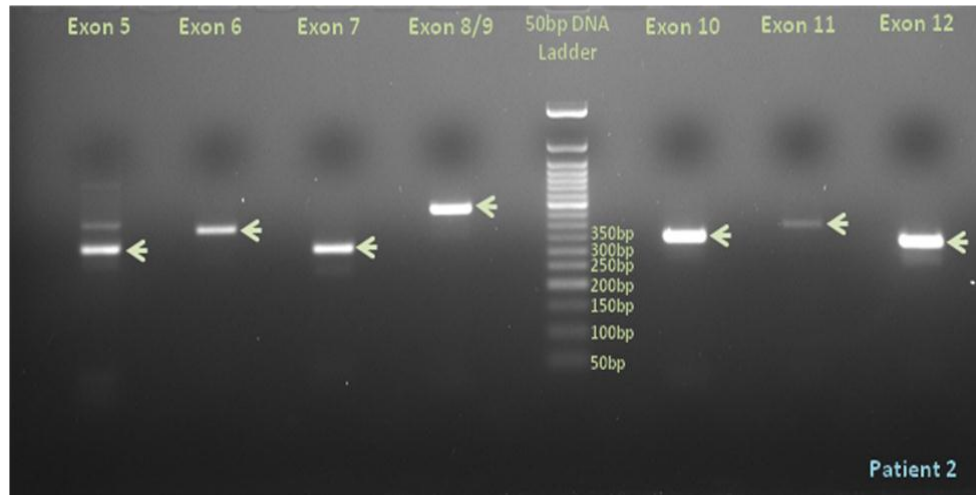


Figure 3.1: Representative 2% agarose gel showing PCR amplification of the PLC ζ active site. Arrows indicate excised bands which were extracted and submitted for sequencing.

Table 3.4: Table showing the results of the sequencing for exons 5-12 of all patients and controls studied thus far. ✓: exon sequence normal compared to PLC ζ ORF, amplicon sequence, and exon sequence. ✕: sequencing failure due to non-specific sequencing.

*: indicates exon where a mutation was discovered.

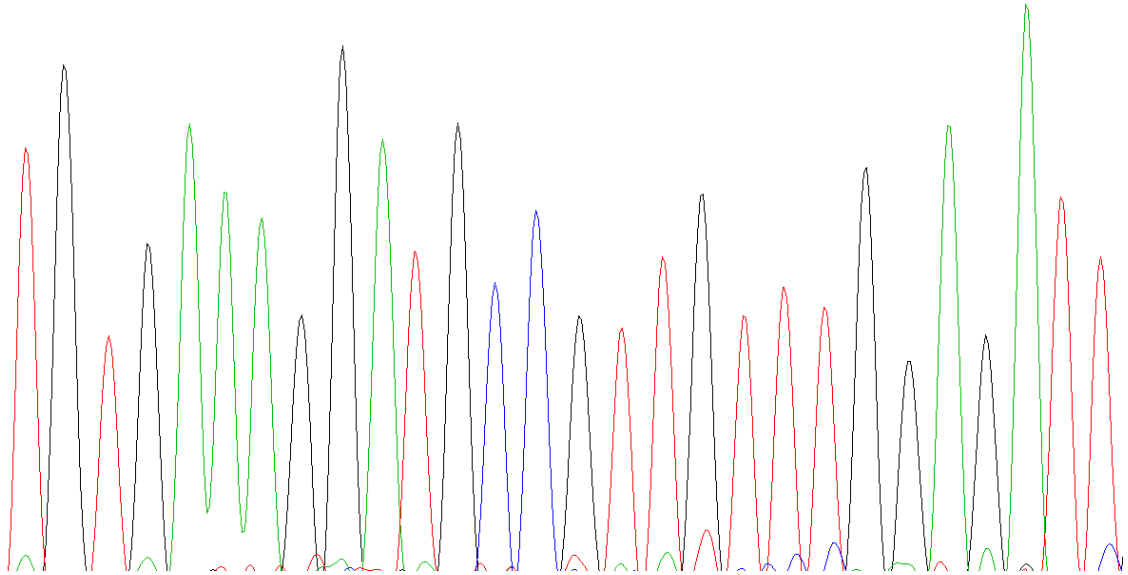
Note: Mutation indicated for exon 11 of Patient H398P indicates the H398P mutation previously published by Heytens *et al.* (2009). Novel data described herein revealed an additional Histidine to Leucine substitution in the X-domain (exon 6) of the same patient.

Patient	Exon amplicon sequencing result						
	5	6	7	8/9	10	11	12
Control 1	✓	✓	✓	✓	✓	✓	✓
Control 2	✓	✓	✓	✓	✓	✓	✓
Patient 1	✓	✓	✓	✓	✓	✓	✓
Patient 2	✓	✓	✓	✓	✓	✓	✓
Patient 3	✓	✓	✓	✓	✓	✓	✓
Patient 4	✕	✓	✕	✓	✕	✓	✕
Patient 5	✕	✓	✓	✕	✓	✓	✓
Patient 6	✓	✓	✓	✕	✓	✓	✓
Patient 7	✓	✕	✓	✓	✓	✕	✓
Patient 8	✓	✓	✓	✓	✓	✓	✓
Patient 9	✓	✕	✕	✓	✕	✕	✕
Patient 10	✓	✓	✓	✕	✓	✕	✓
Patient 11	✓	✓	✓	✕	✓	✓	✓
Patient H398P	✓	*	✓	✓	✓	*	✓

Legend

Adenine (A): ■ Cytosine (C): ■ Guanine (G): ■ Thymine (T): ■

A



B

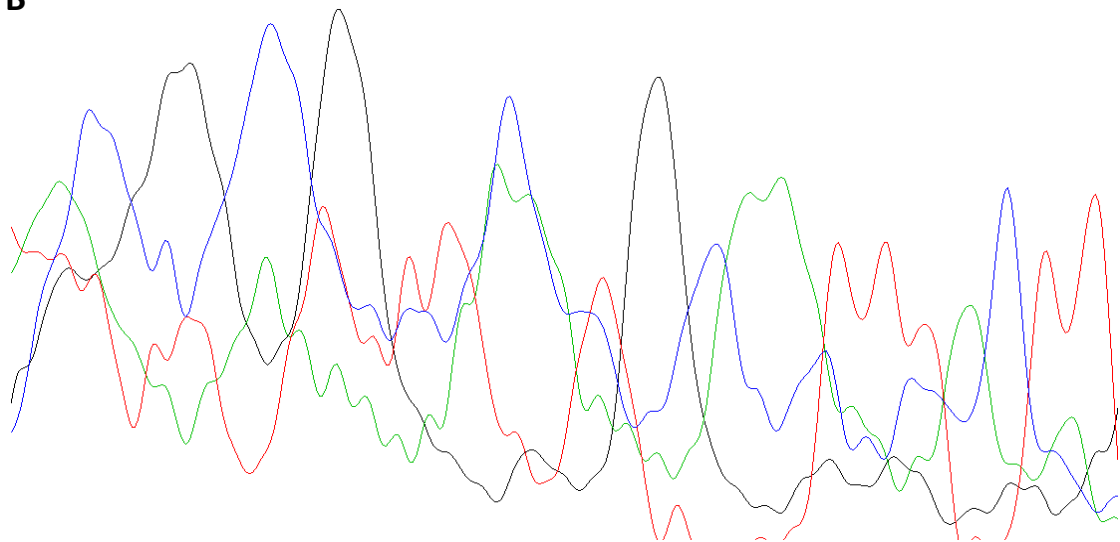


Figure 3.2: Representative sequencing peaks obtained from sequencing exon 6 (**A**) and exon 8/9 (**B**) from Control 1 using their respective forward primers. Sequencing from exon 6 indicates strong peaks, giving a confident readout, while sequencing from exon 8/9 does not give predominant peaks, indicating sequencing/amplification failure.


```

Score = 263 bits (142), Expect = 3e-75
Identities = 144/145 (99%), Gaps = 0/145 (0%)
Strand=Plus/Plus

Query 139  TGCCCTTGTGAAAGGATGCCGTTGTTGGAGATTGACTGCTGGGATGGAGCACAAAATGA 198
          |||
Sbjct 1    TGCCCTTGTGAAAGGATGCCGTTGTTGGAGATTGACTGCTGGGATGGAGCACAAAATGA 60

Query 199  ACCTGTTGTATATCATGGCTACACACTCACAAGCAAACCTTCTGTTTAAACTGTTATCCA 258
          |||
Sbjct 61   ACCTGTTGTATATCATGGCTACACACTCACAAGCAAACCTTCTGTTTAAACTGTTATCCA 120

Query 259  AGCTATACCAAGTATGCATTCATG 283
          |||
Sbjct 121  AGCTATACCAAGTATGCATTCATG 145

```

Figure 3.3: BLAST result of sequencing exon 6 from patient H398P using forward primer (Query) as compared with the exon 6 sequence (Sbjct). Red highlight shows location of A>T base change leading to the H233L mutation.



Figure 3.4: Representative 2% agarose gel showing restriction enzyme digested colonies following sub-cloning of a PCR generated amplicon for exon 8/9 of control 1. Following restriction digestion with *EcoRI*, a positive colony exhibited a band higher than 1kb corresponding to the digested vector, and a lower band at ~376bp corresponding to the expected amplicon size for exon 8/9. In this case, colonies 1, 3, 4, and 5 exhibit a positive match, whereas colony 2 does not, owing to the absence of a band at ~376bp.

A

```
Score = 211 bits (114), Expect = 3e-59
Identities = 116/117 (99%), Gaps = 0/117 (0%)
Strand=Plus/Minus

Query 95 CCATTGACAACCTATATTCCAAAATTCTTGGGGATTAAAATTAGAAGAGTCTGCTCTTG 154
          |||
Sbjct 117 CCATTGACAACCTATATTCCAAAATTCTTGGGGATTAAAATTAGAAGAGTCTGCTCTTG 58

Query 155 TTGCTTTGGGATATATTCTGGTAATGAACTTCCTGGTGGGAAAAATAAACTCATGGA 211
          |||
Sbjct 57 TTGCTTTGGGATATATTCTGGTAATGAACTTCCTGGTGTGAAAAATAAACTCATGGA 1
```

B

```
Score = 217 bits (117), Expect = 6e-61
Identities = 117/117 (100%), Gaps = 0/117 (0%)
Strand=Plus/Minus

Query 95 CCATTGACAACCTATATTCCAAAATTCTTGGGGATTAAAATTAGAAGAGTCTGCTCTTG 154
          |||
Sbjct 117 CCATTGACAACCTATATTCCAAAATTCTTGGGGATTAAAATTAGAAGAGTCTGCTCTTG 58

Query 155 TTGCTTTGGGATATATTCTGGTAATGAACTTCCTGGTGTGAAAAATAAACTCATGGA 211
          |||
Sbjct 57 TTGCTTTGGGATATATTCTGGTAATGAACTTCCTGGTGTGAAAAATAAACTCATGGA 1
```

Figure 3.5: Representative BLAST result of sequencing PCR generated amplicons sub-cloned into pCRII-TOPO. **A)** BLAST result of sequencing a colony transformed with a PCR generated amplicon for exon 6 utilising genomic DNA of patient H398P as a template. Yellow highlight shows location of mutation which is the reverse complement for the A>T mutation for H233L. **B)** BLAST result of sequencing a separate colony transformed with a PCR generated amplicon for exon 6 utilising genomic DNA of patient H398P as a template. This is a 100% match, indicating that a different allele has been sub-cloned into this particular colony.

3.3.2 Quantitative total PLC ζ immunofluorescence in fertile control and infertile patient sperm

This part of the study utilised sperm obtained from fertile controls and infertile patients recruited from the OFU. Quantitative immunofluorescence of PLC ζ indicated that total levels of PLC ζ immunofluorescence were significantly higher ($p \leq 0.001$; as indicated by the *t*-test) in fertile sperm (47.8 arbitrary units; a.u) compared to infertile OAD sperm (24.0 a.u; Figure 3.6). However, further analysis also indicated that sperm from different fertile controls exhibited a large degree of variability, while sperm from infertile patients exhibited severely reduced levels of PLC ζ immunofluorescence (Figure 3.7). Some control sperm also exhibited levels of PLC ζ immunofluorescence similar to those present in patient sperm.

One-way ANOVA indicated that levels of total PLC ζ immunofluorescence between sperm were significantly different from each other ($p \leq 0.001$). Bonferroni *post-hoc* multiple comparison tests demonstrated that control 1 exhibited significantly ($p \leq 0.05$) higher levels of PLC ζ immunofluorescence (mean fluorescence intensity of 57.4 a.u.) compared to control 3 (39.6 a.u), control 5 (26.4 a.u.), control 6 (24.6 a.u.), control 7 (15.4 a.u.), patient 1 (26.4 a.u.), patient 2 (27.7 a.u.), and patient H398P (18.2 a.u.). Control 2 (59.8 a.u.) also exhibited significantly higher levels of PLC ζ immunofluorescence compared to the other controls and patients. Control 3 exhibited significantly higher levels ($p \leq 0.05$) compared to controls 5-7, but was not significantly different from patient sperm apart from sperm from patient H398P. Control 4 (47.4 a.u.) only exhibited significantly higher levels of PLC ζ immunofluorescence compared to sperm from control 7 and patient H398P.

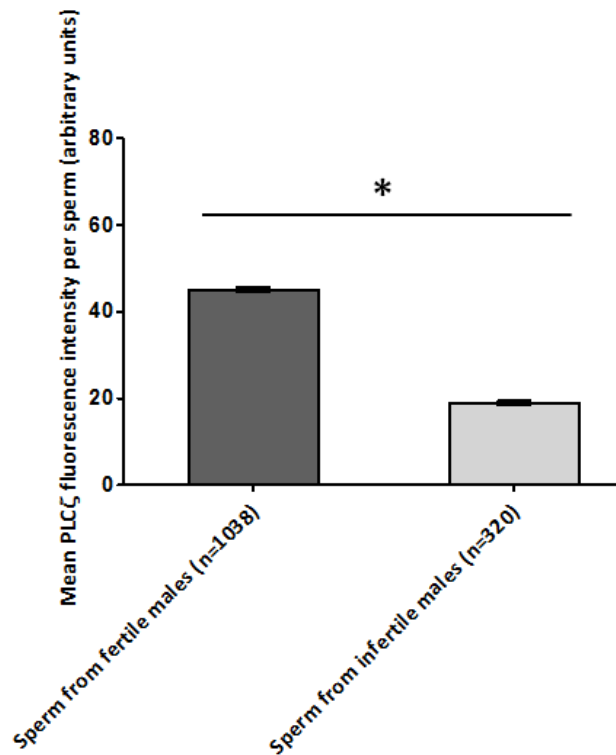


Figure 3.6: Mean relative total PLCζ fluorescence levels exhibited by fertile control and from infertile patient sperm. Fluorescence intensity was quantified in arbitrary units using ImageJ software. The asterisk (*) on the histogram denotes a statistically significant difference ($p < 0.001$). Data are shown as mean $\pm$ SEM.

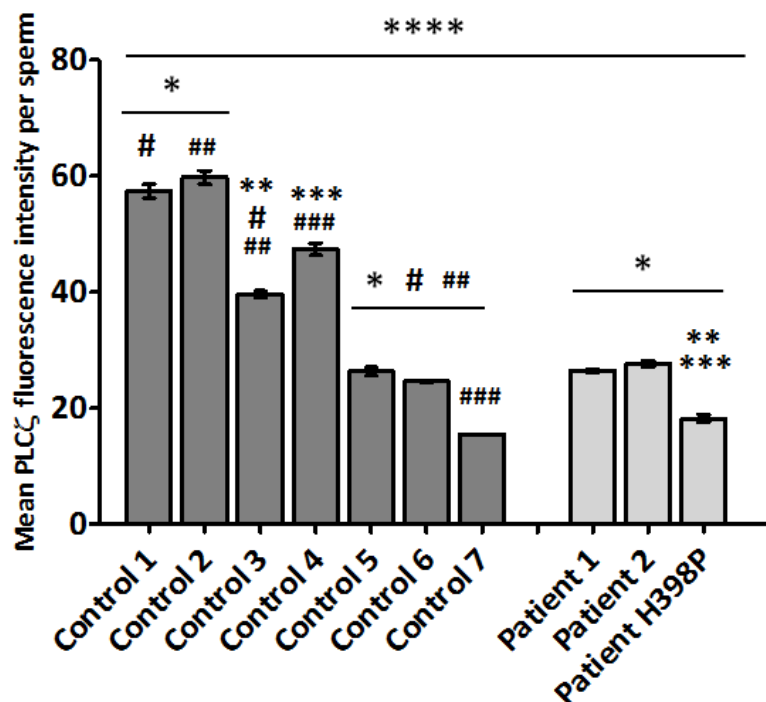


Figure 3.7: Mean relative total PLCζ fluorescence levels exhibited by individual fertile control sperm and from three patients. Fluorescence intensity was quantified in arbitrary units using ImageJ software. Asterisks (*; **; ***; ****), and hash (#; ##; ###), on the histogram denote statistically significant differences ($p < 0.05$). 100 sperm were analysed per individual. Data are shown as mean $\pm$ SEM.

3.3.3 Proportions of sperm exhibiting PLC ζ immunofluorescence

The proportions of sperm from fertile controls and infertile patients exhibiting patterns of PLC ζ immunofluorescence previously characterised by Grasa *et al.* (2008) (Figure 3.8) were also quantified. Proportional analysis of sperm exhibiting PLC ζ immunoreactivity indicated that a significantly larger proportion (69.7%) of control sperm ($p \leq 0.001$; as indicated by the *t*-test) exhibited PLC ζ immunoreactivity compared to sperm from infertile males (26.8%; Figure 3.9). This was further confirmed by comparison of individual controls and infertile patients (Figure 3.10). One-way ANOVA determined that all values were significantly different from each other ($p \leq 0.001$). Bonferroni *post-hoc* multiple comparisons tests indicated that controls 1-5 and control 7 exhibited a significantly larger ($p \leq 0.05$) proportion of sperm (63.2%, 70.2%, 85.4%, 88.7%, 69.3%, and 86.7% respectively) compared to the proportion of sperm exhibiting PLC ζ immunofluorescence from patient H398P (17.5%). Furthermore, controls 3 and 4 also demonstrated significantly higher ($p \leq 0.05$) proportions of sperm exhibiting PLC ζ immunofluorescence compared to sperm from patients 1 and 2 (33.3% and 31.9% respectively).

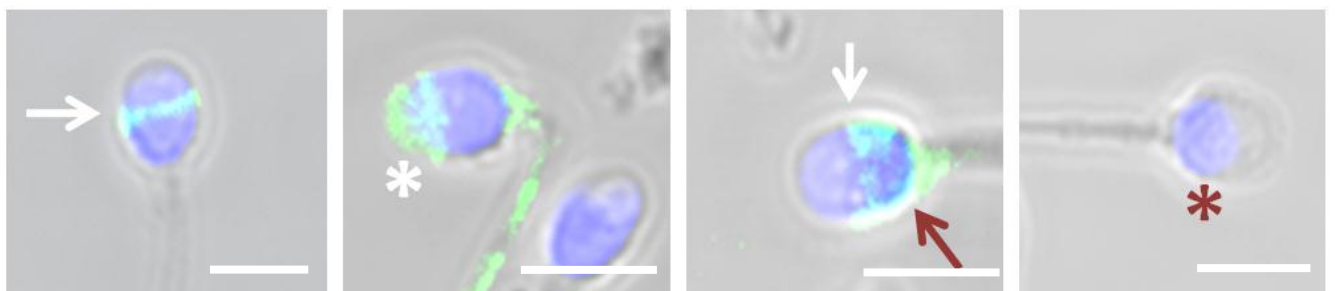


Figure 3.8: Representative confocal images of PLC ζ immunofluorescence in fertile human sperm showing characteristic localisation patterns previously identified by Grasa *et al.* (2008). Images were captured at 63x optical magnification, and represent overlays of nuclei (blue), PLC ζ (green), and differential interference contrast (DIC) images. White scale bars indicate 5 μ m. White arrow indicates equatorial localisation; white asterisk (*) indicates acrosomal localisation; red arrows indicate post-acrosomal localisation; and red asterisk indicates reduced/absent PLC ζ levels in the sperm head. Some binding of the PLC ζ antibody could be observed on the sperm tail, which was shown by Grasa *et al.* (2009) to be non-specific binding.

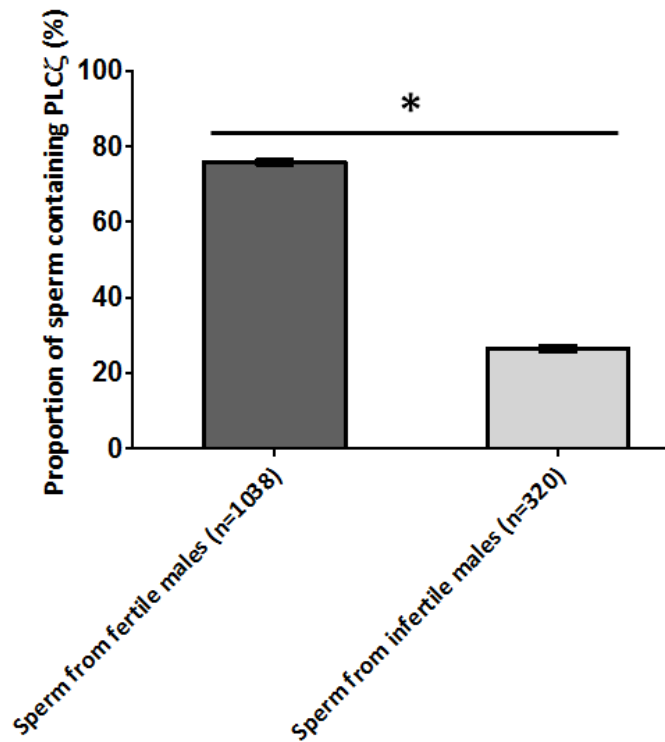


Figure 3.9: Mean proportions of sperm (percentages) exhibiting PLCζ immunofluorescence from fertile and infertile sperm. Proportions were determined using ImageJ software. The asterisks (*) on the histogram denotes a statistically significant difference ($p < 0.001$). Data are shown as mean $\pm$ SEM.

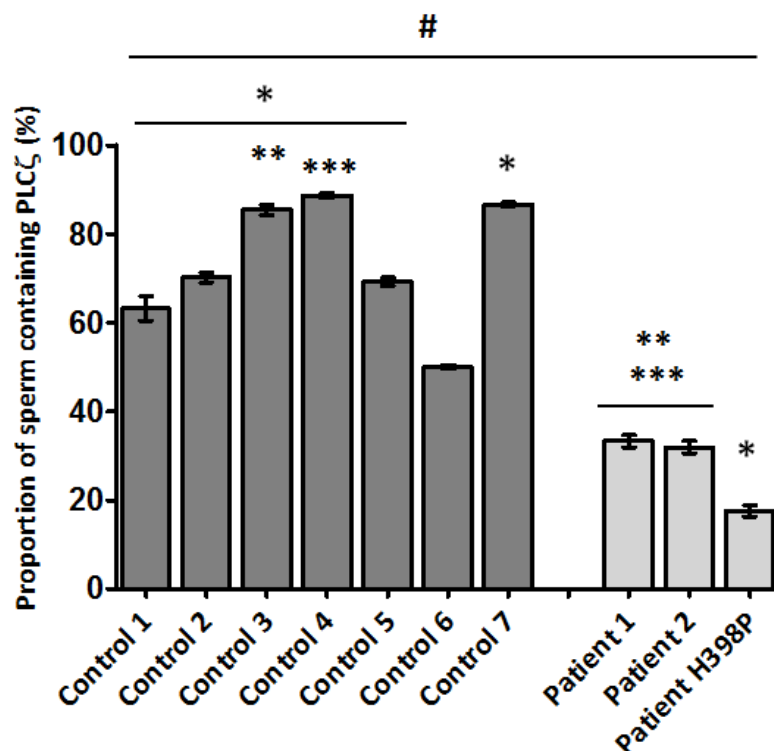


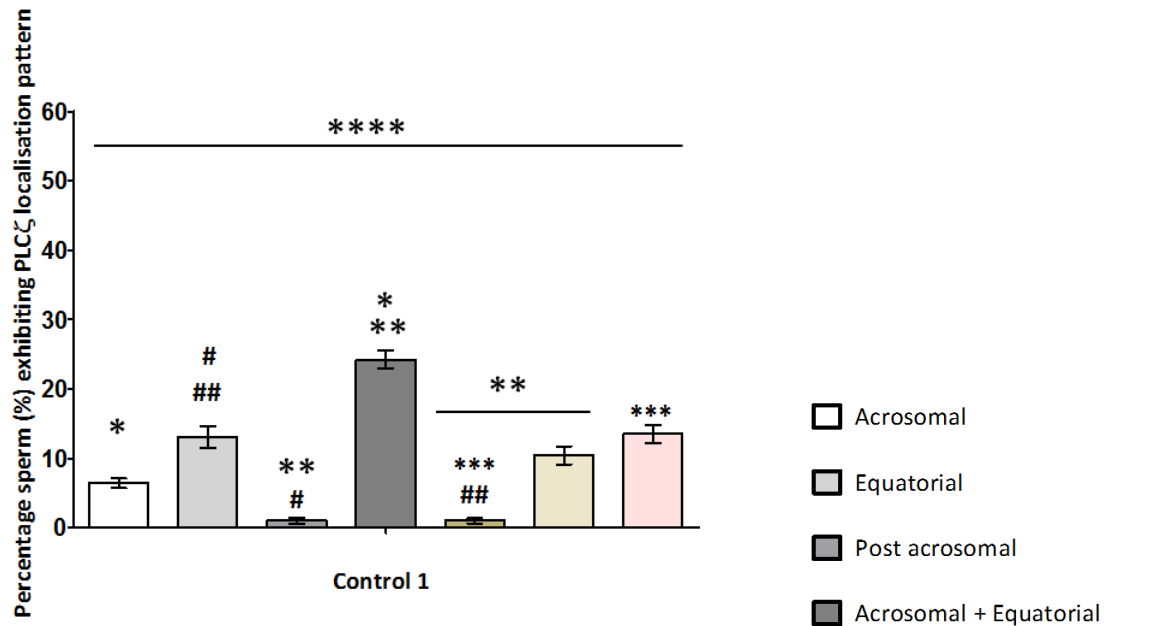
Figure 3.10: Mean proportions of sperm (percentages) exhibiting PLCζ immunofluorescence from fertile controls and from the three patients. Proportions were determined using ImageJ software. Asterisks (*, **, ***), and hash (#), on the histogram denote statistically significant differences ($p < 0.05$). 100 sperm were analysed per individual. Data are shown as mean $\pm$ SEM.

3.3.4 Proportional analysis of PLC ζ localisation patterns in control sperm

Analysis of the discrete populations of PLC ζ localisation patterns in sperm from individual controls revealed large variance in the proportions of localisation patterns previously identified by Grasa *et al.* (2008). The localisation patterns of PLC ζ observed were: acrosomal; equatorial; post-acrosomal; acrosomal + equatorial; acrosomal + post-acrosomal; equatorial + post-acrosomal; and all three localisation patterns together. Analysis indicated that sperm from only one control exhibited a predominantly equatorial pattern of PLC ζ localisation, with the rest exhibiting either an equatorial + post-acrosomal localisation pattern, or multiple predominant patterns.

One-way ANOVA indicated that all proportions were significantly different ($p \leq 0.001$) within each control. Sperm from control 1 exhibited the acrosomal + equatorial population (24.2%) as the predominant pattern of PLC ζ localisation, which exhibited significantly higher levels ($p \leq 0.05$) compared to the acrosomal (6.4%), post-acrosomal (1.1%), acrosomal + post-acrosomal (1.1%), and equatorial + post-acrosomal (10.4%) patterns of localisation (Figure 3.11A). However, following the Bonferroni post-hoc multiple comparisons test, only one fertile control (control 2) within the population studied exhibited a predominantly equatorial localisation (53%), which was significantly different from all other patterns ($p \leq 0.05$) (Figure 3.11B).

A



B

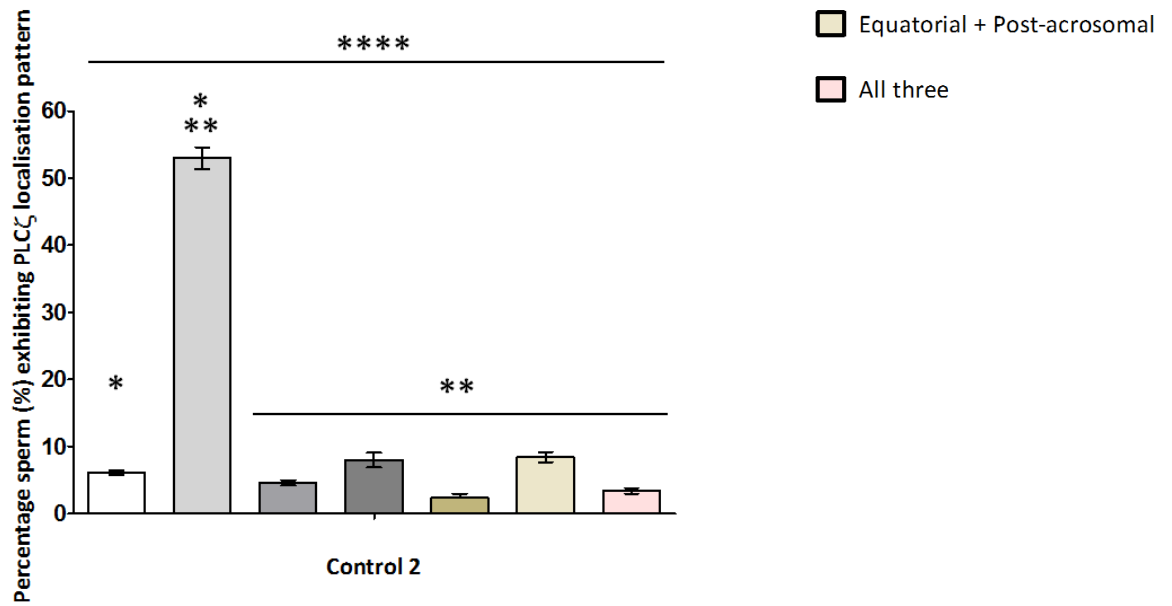


Figure 3.11: Mean proportions of sperm (percentages) exhibiting PLC ζ immunofluorescence patterns in sperm from (A) control 1 and (B) control 2. Proportions were determined using ImageJ software. Asterisks (*; **, ***; ****), and hash (#; ##), on the histograms denote statistically significant differences ($p < 0.05$). 100 sperm were analysed per individual. Data are shown as mean $\pm$ SEM.

However, sperm from control 3 appeared to exhibit two predominant patterns of PLC ζ localisation: post-acrosomal (38.8%) and equatorial + post-acrosomal (33.2%). The post-acrosomal population exhibited significantly higher levels ($p \leq 0.05$) compared to the acrosomal (0%), equatorial (4.7%), acrosomal + equatorial (1%), acrosomal + post-acrosomal (1.7%), and all three (9.2%) patterns of PLC ζ localisation. The equatorial + post-acrosomal pattern of localisation was also significantly higher ($p \leq 0.05$) compared to the other populations apart from the acrosomal population (Figure 3.12A). Sperm from control 4 also appeared to exhibit this trend, with the predominant populations seemingly being the post-acrosomal (31%) and equatorial + post-acrosomal (20.5%).

The post-acrosomal population exhibited significantly higher levels ($p \leq 0.05$) compared to the acrosomal (4.3%), acrosomal + equatorial (2.5%), and acrosomal + post-acrosomal (1.8%) patterns of PLC ζ localisation. However, the equatorial + post-acrosomal population was only found to be significantly higher ($p \leq 0.05$) compared to the acrosomal + equatorial and acrosomal + post-acrosomal populations (Figure 3.12B). Further to this, sperm from control 5 only seemed to exhibit the post-acrosomal pattern of localisation (35.2%) as the predominant population, which was present at significantly higher levels ($p \leq 0.05$) compared to the acrosomal (3.1%), equatorial (7.5%), acrosomal + equatorial (3.4%), acrosomal + post-acrosomal (4.4%), and all three (5.0%) patterns of PLC ζ localisation (Figure 3.12C).

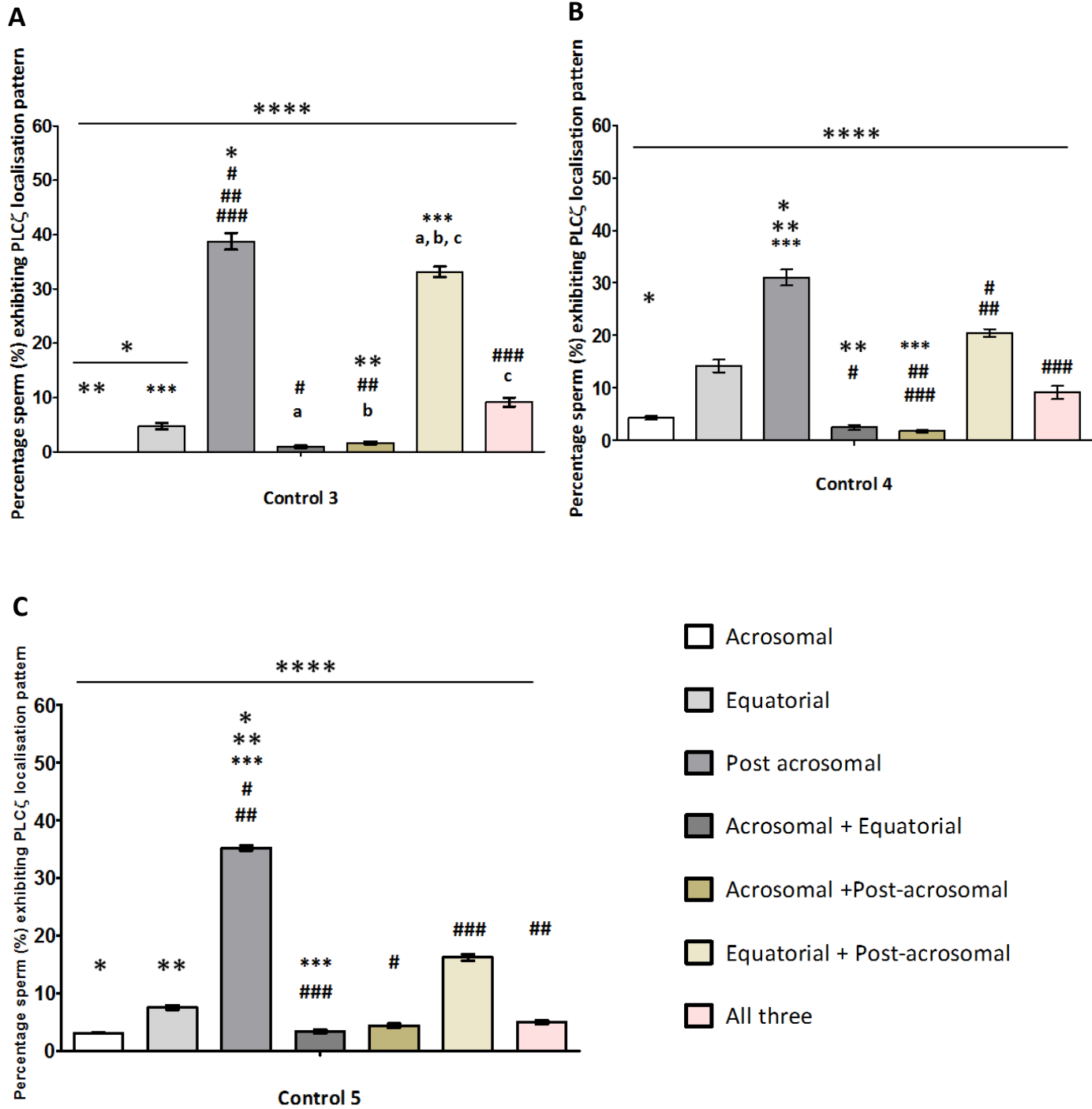


Figure 3.12: Mean proportions of sperm (percentages) exhibiting PLC ζ immunofluorescence patterns in sperm from (A) control 3, (B) control 4, and (C) Control 5. Proportions were determined using ImageJ software. Asterisks (*; **, ***, ****), hash (#; ##; ###), and letters (a; b; c) on the histograms denote statistically significant differences ($p < 0.05$). 100 sperm were analysed per individual. Data are shown as mean $\pm$ SEM.

Intriguingly, however, sperm from control 6 did not exhibit any pattern of PLC ζ localisation which was predominantly significant from the other populations (Figure 3.13A). Further to this, sperm from control 7 seemed to exhibit three predominant patterns of localisation: equatorial (17.4%), post-acrosomal (40.1%), and equatorial + post-acrosomal (19.7%). The post-acrosomal population was present at significantly higher levels ($p \leq 0.05$) compared to the acrosomal (3.8%), acrosomal + equatorial (3.5%), acrosomal + post-acrosomal (2%), and all three (0%) patterns of PLC ζ localisation. However, the equatorial pattern of PLC ζ localisation only differed significantly ($p \leq 0.05$) from the acrosomal + post-acrosomal and all three patterns of localisation, while the equatorial + post-acrosomal pattern only differed significantly ($p \leq 0.05$) from the acrosomal + equatorial and all three patterns of PLC ζ localisation (Figure 3.13B).

To summarise, sperm from only one control (control 2) exhibited a predominantly equatorial population of PLC ζ localisation as described by Grasa *et al.* (2008). Control 1 exhibited a predominantly acrosomal + equatorial pattern, controls 5 and 7 exhibited a predominantly post-acrosomal pattern, while controls 3 and 4 exhibited two predominant patterns of PLC ζ localisation; post-acrosomal and equatorial + post-acrosomal. Control 6 did not exhibit any predominant pattern of PLC ζ localisation.

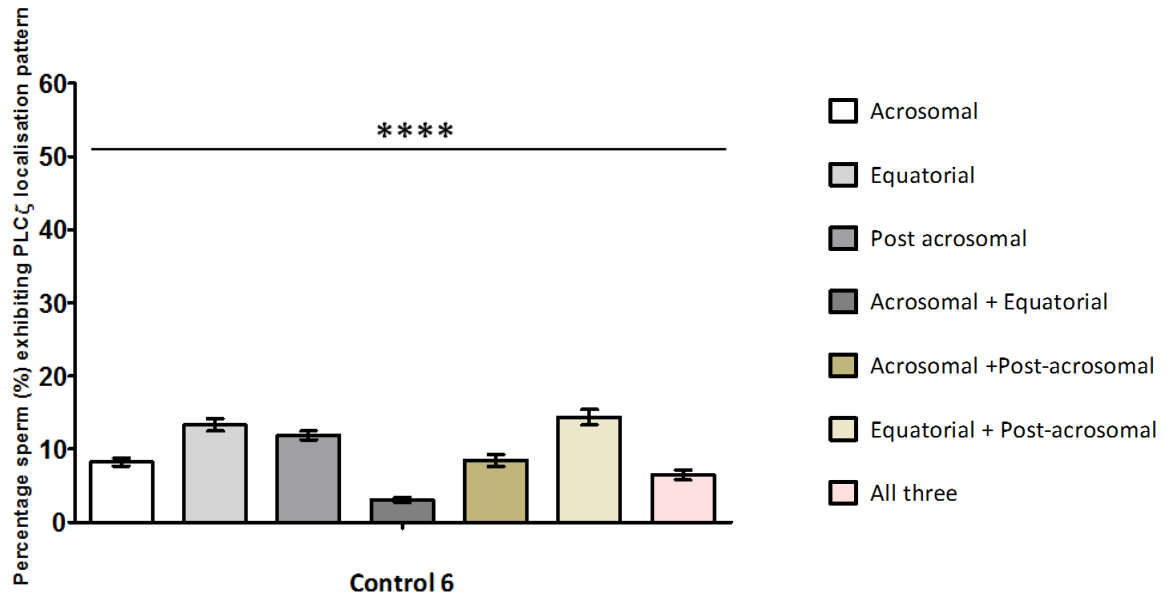
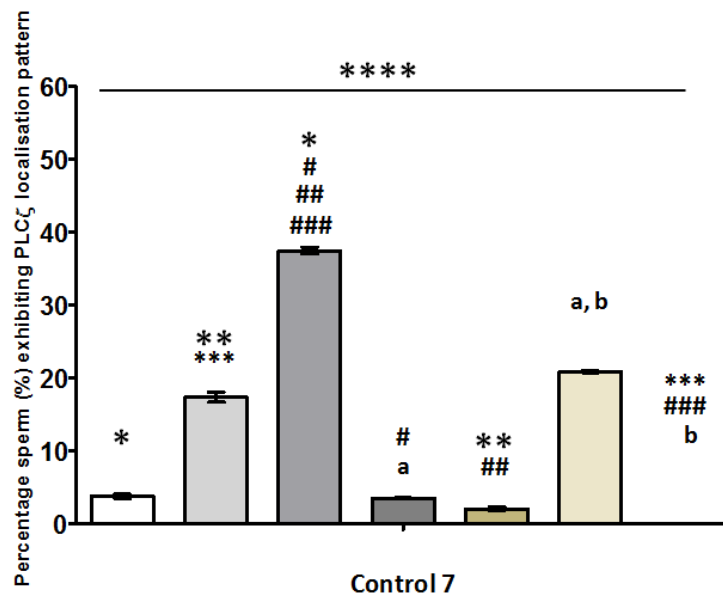
A**B**

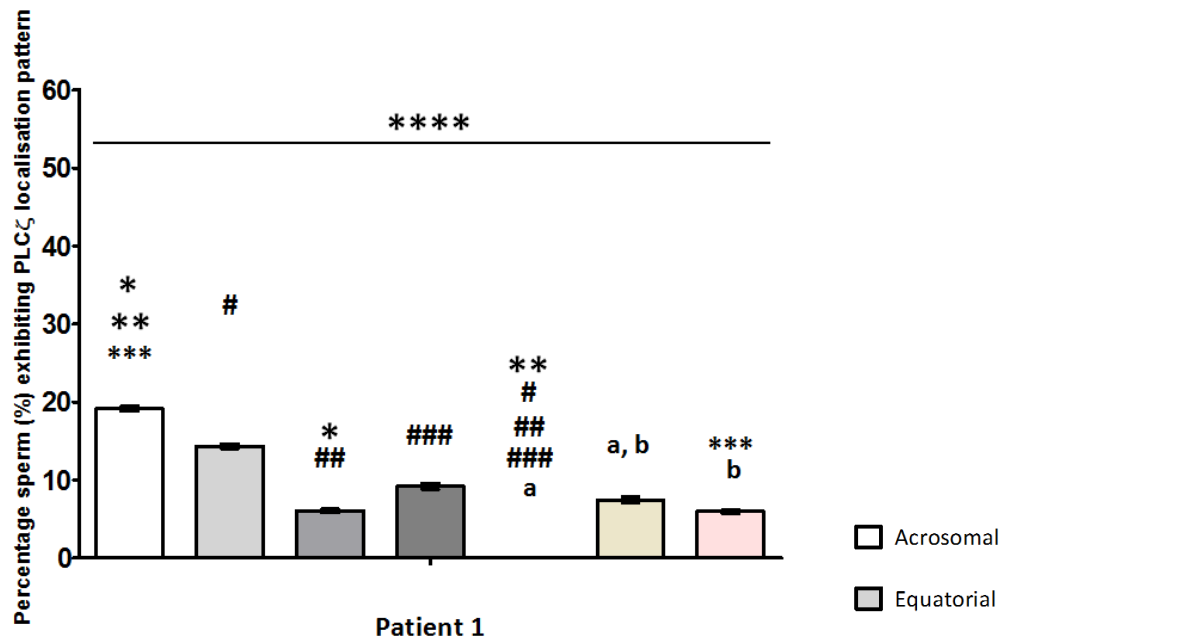
Figure 3.13: Mean proportions of sperm (percentages) exhibiting PLC ζ immunofluorescence patterns in sperm from (A) control 6 and (B) control 7. Proportions were determined using ImageJ software. Asterisks (*, **, ***, ****), and hash (#; ##; ###), and letters (a; b) on the histograms denote statistically significant differences ($p < 0.05$). 100 sperm were analysed per individual. Data are shown as mean $\pm$ SEM.

3.3.5 Proportional analysis of PLC ζ localisation patterns in sperm from infertile males

While some populations of PLC ζ localisation patterns were present at higher proportions compared to others, this was not as profound as observed in control sperm. Sperm from patient 1 exhibited the acrosomal population (19.2%) as the numerically predominant population, exhibiting significantly higher levels ($p \leq 0.05$) compared to the post-acrosomal (6.1%), acrosomal + post-acrosomal (0%), and all three (6.0%) patterns of PLC ζ localisation. The equatorial (14.3%), post-acrosomal, and acrosomal + equatorial (9.2%) populations exhibited significantly higher levels ($p \leq 0.05$) compared to the acrosomal + post-acrosomal pattern of PLC ζ localisation, but did not differ significantly from each other, nor did they differ significantly from the equatorial + post-acrosomal (7.5%) pattern of PLC ζ localisation, which itself was significantly higher ($p \leq 0.05$) compared to the populations exhibiting the acrosomal + post-acrosomal and all three patterns of PLC ζ localisation (Figure 3.14A).

Interestingly however, those sperm from patient 2 which exhibited PLC ζ immunofluorescence indicated that the post-acrosomal (17.1%) population of PLC ζ localisation was predominant (Figure 11B), exhibiting significantly higher levels ($p \leq 0.05$) compared to the equatorial (4.6%), acrosomal + equatorial (0.7%), acrosomal + post-acrosomal (1.9%), and all three (1.7%) patterns of PLC ζ localisation. Intriguingly, however, the acrosomal (7.9%) and equatorial + post-acrosomal (7.7%) populations did not differ significantly from all other populations, including the post-acrosomal population, indicating these may also be present at equal levels (Figure 3.14B). To summarise, while patient sperm exhibited populations of PLC ζ localisation patterns which were numerically predominant, this effect was not as profound as within control sperm.

A



B

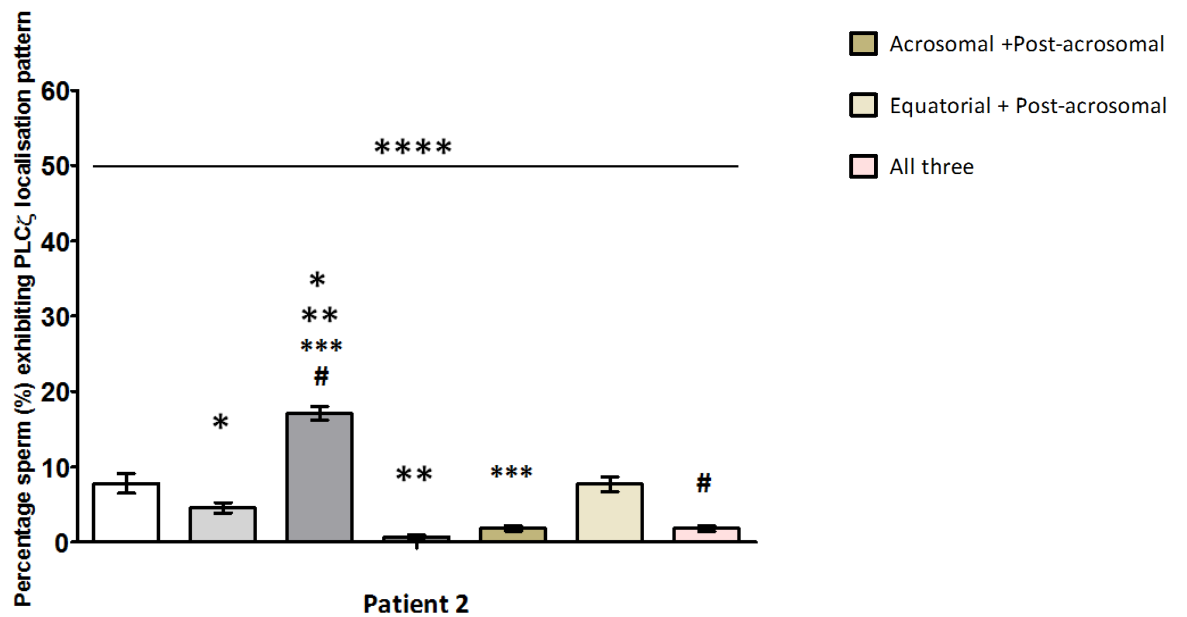


Figure 3.14: Mean proportions of sperm (percentages) exhibiting PLC ζ immunofluorescence patterns in sperm from (A) patient 1 and (B) patient 2. Proportions were determined using ImageJ software. Asterisks (*; **, ***; ****), and hash (#; ##; ###), and letters (a; b) on the histograms denote statistically significant differences ($p < 0.05$). 100 sperm were analysed per individual. Data are shown as mean $\pm$ SEM.

3.3.6 Comparative proportional analysis of PLC ζ localisation pattern populations within sperm from fertile and infertile males

While some populations exhibited significantly higher levels ($p \leq 0.05$) compared to others, proportional analysis of localisation pattern populations in total fertile sperm and total infertile sperm studied did not reveal any one predominant population exhibiting significantly higher levels in either fertile or infertile sperm (Figure 3.15). One-way ANOVA indicated that all proportions within fertile and infertile sperm were significantly different ($p \leq 0.001$) from each other. Two-way ANOVA did not identify any statistical significance in localisation proportions between fertile and infertile sperm.

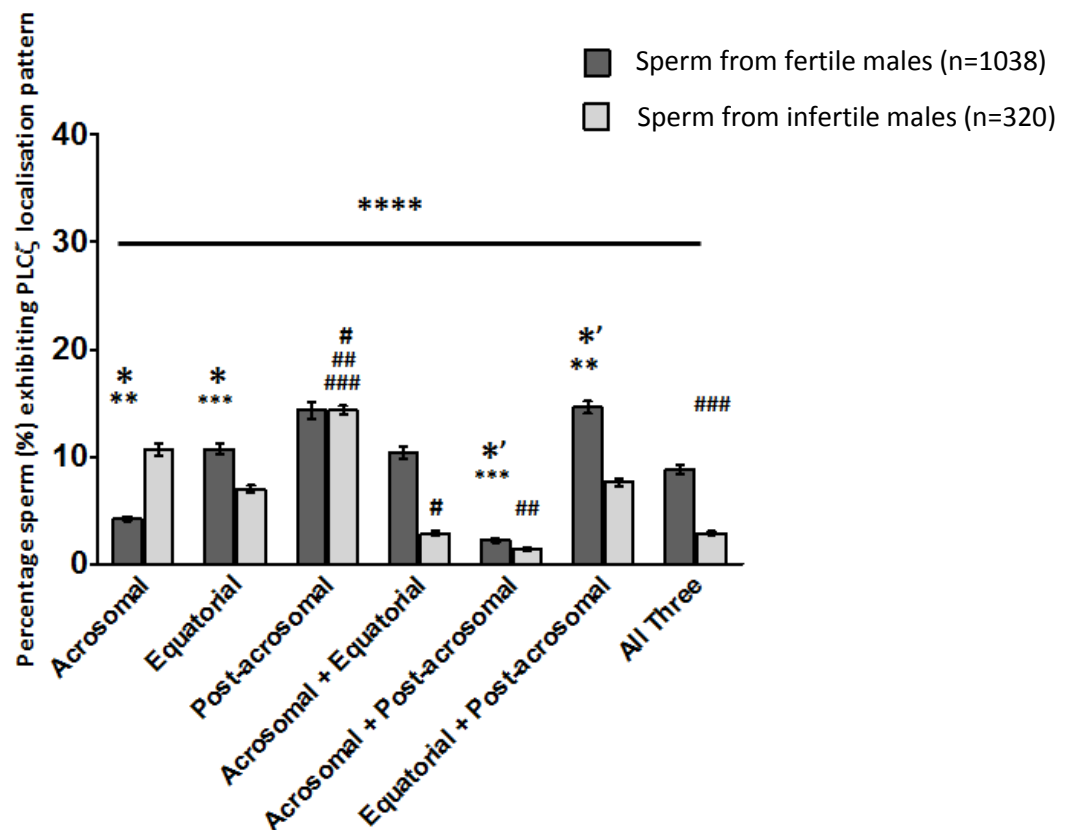


Figure 3.15: Mean proportions of sperm (percentages) exhibiting PLC ζ immunofluorescence patterns in total sperm from controls (n=634) and patients (n=580). Proportions were determined using ImageJ software. Asterisks (*, **, ***, ****), and hash (#, ##, ###) on the histograms denote statistically significant differences ($p < 0.05$). Data are shown as mean $\pm$ SEM.

In summary, results indicate that the predominant patterns of PLC ζ localisation within human sperm varies considerably, with some controls exhibiting two predominant patterns of localisation. Equatorial localisation of PLC ζ was only predominant in sperm from control 2 (52.96%; $p \leq 0.05$), with post-acrosomal PLC ζ localisation being predominant in sperm from controls 3, 4, 5, 7 and patient 2 (38.8%, 31%, 35.2%, 37.5%, and 17.1% respectively; $p \leq 0.05$). However, sperm from controls 3 and 4 also exhibited the equatorial + post-acrosomal population as a secondary predominant population (33.2%, 20.5% respectively; $p \leq 0.05$). Sperm from patient 1 exhibited the acrosomal population as the predominant one (19.1%; $p \leq 0.05$). However, there was no significantly predominant population of PLC ζ localisation within sperm from either fertile controls, or infertile patients combined

3.4 Discussion

Mounting evidence implies dysfunctional isoforms or significantly reduced levels of PLC ζ in sperm underlie certain types of male factor infertility (Yoon *et al.*, 2008; Heytens *et al.*, 2009; Taylor *et al.*, 2010). Collectively, these findings emphasise the clinical potential of PLC ζ , not only as a possible prognostic marker of sperm ability to induce oocyte activation, but as a therapeutic target for infertility treatment. One aim of this Chapter was to investigate the prevalence of loss-of-activity mutations similar to the H398P mutation reported by Heytens *et al.* (2009) in exons coding for the active site of the PLC ζ ORF from genomic DNA of patients diagnosed with OAD. A secondary aim was to investigate the viability of utilising quantitative immunofluorescence of PLC ζ in sperm from patients diagnosed with OAD as a diagnostic predictor of oocyte activation capability, and thus male fertility. Collectively, these experiments aimed to further understanding of the role that PLC ζ plays in human infertility, and to characterise the precise localisation of PLC ζ within sperm, aiding the translation of research to the ART clinic.

3.4.1 Loss-of-activity mutations within the PLC ζ active site may be rare occurrences

This study utilised a PCR-based approach to screen exons coding for the active site (exons 5-12) of the PLC ζ gene obtained from 2 control males, with no previous history of infertility, alongside 12 males diagnosed with OAD. No mutations were identified from any patient apart from a single novel mutation; an A>T change at position 698 of the PLC ζ ORF (within the X-Domain), resulting in a Histidine to Leucine substitution at position 233 (H233L) in the amino acid sequence of PLC ζ . While the novel H233L mutation is discussed in more detail in Chapter 4 of this thesis, the fact remains that only one novel

mutation was identified by this study, and this was detected in the same patient from whom H398P had previously been identified (Heytens *et al.*, 2009). Collectively, this study was more extensive than the screening described by Heytens *et al.* (2009), and suggests that loss-of-activity mutations within the active site of PLC ζ may be rare occurrences. However, it should be noted that some exons screened across a number of patients resulted in sequencing failure, a constant occurrence over multiple sequencing attempts for these patients. It is possible that this occurred due to sub-optimal primer design or low amplicon concentration, however, sequencing failure persisted following primer re-design for the most problematic of exons; exons 8/9 and 11. However, one should note that the problem here was that of direct sequencing, and not of amplicon generation, since amplicons were successfully generated, but failed all attempts to sequence. To attempt to solve this problem, we aimed to sub-clone PCR-generated amplicons into the pCRII-TOPO vector and submit them for sequencing, to try and rectify any potential issues with amplicon concentration. While this successfully solved the problem of sequencing failure, implying that the prior failure was that of primer incompatibility with the sequencing method, the sub-cloning method contained intrinsic difficulties of its own, most profoundly that of single allele sequencing.

Since genomic DNA was utilised as a template to generate PCR based amplicons, fragments corresponding to both the maternal and paternal alleles of the amplicon will, of course, be generated. However, sub-cloning would only involve the ligation of one copy, either maternal or paternal, to the vector, a conundrum confirmed by the sequencing of exon 11 generated from genomic DNA of patient H398P. Therefore, to try and circumvent this issue, at least 10 colonies were selected for each amplicon sub-cloning reaction to be submitted for sequencing. While ultimately successful, this method was by no means high-

throughput; an essential criterion to meet if the PLC ζ gene is to be screened from multiple patients diagnosed with OAD. To eliminate concerns such as low starting template yield (due to variances in buccal cell isolation from patient saliva), one possible modification to the current protocol may be to utilise whole genome amplification methodology prior to amplicon generation, which allows amplification of the entire genome in large portions to provide an increased starting template with which to generate amplicons for sequencing. However, this would in turn present its own difficulties such as that of allele dropout and/or artificial mutation induction during amplification.

Regardless, it is vital that high-throughput, and more cost-efficient, protocols be utilised such as temperature gradient capillary electrophoresis (TGCE), a method which can be used to distinguish heteroduplex from homoduplex DNA molecules and can thus be applied to the detection of various types of DNA polymorphisms. While TGCE can also be used in the absence of prior knowledge of the sequences of the underlying polymorphisms, it is a very high-throughput technique, able to detect a single polymorphism in amplicons of over 800 bp, whose efficiency can be increased following multiplexing of sequencing reactions (Hsia *et al.*, 2005). Furthermore, the utilisation of software enhancements such as the recently designed ‘GeneScreen’ to increase the rapidity of high-throughput protocols such as TGCE, by analysing capillary electropherograms and comparing their sequences with a known reference for identification of mutations (Carr *et al.*, 2011). One other possible alternative is to utilise matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS) to examine base-specific cleavage products (Honisch *et al.*, 2004), and to investigate the prevalence of mutations occurring due to a truncation/deletion of coding regions of the PLC ζ gene. Collectively, the use of either one or a combination of alternative next-generation sequencing technologies would allow for a

more rapid dissemination of sequencing data when dealing with multiple patients, which using the current protocol was difficult to do, and would need to be overcome if mutation screening of OAD patients is to be continued.

3.4.2 Screening of exons coding for other functional domains of PLC ζ

Considering that the H398P mutation results in a severe loss-of-activity and occurred within the active site loop of the PLC ζ structure (Heytens *et al.*, 2009), it was reasoned that the chances of finding mutations of similar severity would be maximised by screening exons coding for the active site of PLC ζ (exons 5-12). However, the PLC ζ gene consists of 15 coding exons, with exons 1-4 coding for the promoter region and the EF hands, and exons 13-15 coding for the C2 domain of the human PLC ζ gene (Saunders *et al.*, 2002). As discussed in more detail in Chapter 1, both the EF hands and C2 domain perform regulatory functions such as PIP₂ targeting and Ca²⁺ sensitivity of the PLC ζ enzyme. Indeed, Kuroda *et al.*, (2006) suggested that the functional quaternary structure of PLC ζ involves association of the EF hands with the C2 domain for full enzymatic activity to occur. Even slight disruption of this association results in reduced activity. It is therefore possible that further loss-of-activity mutations in exons coding for regulatory domains of PLC ζ may be present. Alternatively, intronic regions may be important in gene expression, and mutations could disrupt protein function due to exon skipping or transcriptional regulation (Kasowski *et al.*, 2010). Other mutations could possibly arise in promoter regions or in genes encoding proteins involved in transcriptional/translational control.

Thus far, while only one genetic link between PLC ζ deficiency and recurrent ICSI failure has been identified (Heytens *et al.*, 2009); reduced/absent levels of PLC ζ in sperm from men diagnosed with OAD have been demonstrated by multiple studies (Yoon *et al.*,

2008; Heytens *et al.*, 2009; Taylor *et al.*, 2010). It is therefore possible that the reduced/absent levels of PLC ζ in sperm from men diagnosed with OAD is due to a deficiency within the PLC ζ promoter in these men, or possibly due to deficiencies in promoting factors required for the successful transcription and translation of this gene. An interesting point to observe is that the majority of patients screened by Heytens *et al.* (2008) exhibited globozoospermic morphology (round-headed, acrosome-less sperm heads), and absent/reduced levels of PLC ζ in the sperm head.

Intriguingly, PLC ζ shares its promoter sequence with the promoter for the spermatid-specific capping protein (actin filament) muscle Z-line, alpha 3 (*CAPZA3*) gene, which is thought to contribute towards the filamentous actin (F-actin) network of the developing spermatid (Geyer *et al.*, 2009). It may be possible that the globozoospermic phenotype along with absent/reduced levels of PLC ζ in the sperm head may be due to deficiencies in their shared promoter. Intriguingly, Sermondade *et al.* (2011) recently reported a successful pregnancy and healthy childbirth in a case of total globozoospermia after intracytoplasmic morphologically selected sperm injection (IMSI) without assisted oocyte activation (AOA), using globozoospermic sperm which had an acrosomal bud structure (a morphology presented by 1% of sperm from the patient), indicating that the presence of an acrosomal, or acrosome-like, structure may be linked to the oocyte activation capacity of sperm.

However, while the case studied by Sermondade *et al.* (2011) presented a normal karyotype with no mutations in genes linked to globozoospermia such as the *SPATA16* and *DPY19L2* genes, numerous reports studying familial cases suggest that globozoospermia is a genetic syndrome possibly involving multiple genetic causes

(Kullander and Rausing, 1975; Flörke-Gerloff *et al.*, 1984; Dale *et al.*, 1994; Kilani *et al.*, 2004, Heindryckx *et al.*, 2005), the specific mode of inheritance of which remains unclear (Dam *et al.*, 2007). It is thus essential that regions such as the PLC ζ promoter region are included in future PLC ζ screens, alongside exons coding for non-catalytic domains of the PLC ζ ORF. It is vital that genomic screening of PLC ζ from infertile males is continued, and expanded to include patients from wider biogeographical regions, a vital step in assessing the potential value of therapeutic PLC ζ .

3.4.3 Quantitative immunofluorescence analysis indicates a large degree of variability in the levels of total PLC ζ fluorescence in control fertile sperm

Numerous studies have demonstrated a direct correlation between absent/reduced levels of PLC ζ in sperm from infertile patients who routinely fail ICSI (Yoon *et al.*, 2008; Heytens *et al.*, 2009; Taylor *et al.*, 2010). Thus, we investigated the applicability of quantitative immunofluorescent testing of PLC ζ in sperm from patients diagnosed with OAD, compared to fertile controls, as a possible diagnostic test of oocyte activation capability, and thus male fertility. Indeed, Kaewmala *et al.* (2011) indicated that higher levels of PLC ζ mRNA correlated to better sperm quality in the boar, while Aghajanzpour *et al.* (2011) indicated that reduced levels of PLC ζ mRNA transcripts within sperm from human males diagnosed with OAD directly correlated to low levels of IVF and/or ICSI success.

It should be noted that the analyses described in this thesis did not examine immunofluorescence for other markers (such as β -actin or nuclear proteins) within control and patient sperm owing to ethical restrictions. While it would be necessary for future studies to take this into account, to examine whether the methodology utilised remains

consistent for other markers, and whether similar variability is observed per sperm, the current analyses revealed some very interesting data.

While the current analysis indeed indicated that sperm from OAD patients exhibited a significantly reduced level of total PLC ζ immunofluorescence compared to sperm from fertile controls corroborating the findings of recent studies (Yoon *et al.*, 2008; Heytens *et al.*, 2009; Taylor *et al.*, 2010), further analysis indicated that utilising this as a diagnostic would not be as straightforward as previously thought. Large variance in levels of total PLC ζ fluorescence was identified in sperm from fertile controls studied. Furthermore, three controls (controls 5, 6, and 7) exhibited similar levels of PLC ζ immunofluorescence compared to the infertile patient population, while other controls (controls 1 and 2) exhibited significantly higher levels of total PLC ζ immunofluorescence compared to patient sperm. Additionally, controls 3 and 4 indicated mid-range levels of PLC ζ immunofluorescence, not significantly higher or lower than the other populations. These results suggest that a significant amount of variability is present in terms of total PLC ζ levels in human sperm amongst fertile men, and indeed, within samples from the same male. Of course, while not all sperm produced in a given sample would be capable of fertilisation owing to inherent heterogeneity, it is vital to note that all samples analysed in this study had been subjected to DGW, a method routinely used to select the most motile and morphologically normal sperm from an ejaculate for IVF or ICSI (Allamaneni *et al.*, 2005), which increased the concentration of sperm exhibiting PLC ζ (Kashir *et al.*, 2011; Chapter 5 of this thesis). Indeed, the proportional analyses indicated that the control population contained a higher proportion of sperm exhibiting PLC ζ immunofluorescence compared to the infertile male population.

While the proportion of sperm from Control 6 exhibiting PLC ζ immunofluorescence was higher than infertile patient sperm, this was the lowest value obtained for the control population. Considering that ART treatment using sperm from this patient resulted in normal fertilisation of 100% of oocytes, it is possible that the low values for total PLC ζ fluorescence relates to sub-fertility of this particular sample. Indeed, sperm from Patient 1 had failed fertilisation on a previous IVF attempt, but a high success rate of normal fertilisation following ICSI. However, this sperm also exhibited lower values of total PLC ζ fluorescence, as well as the total proportion of sperm exhibiting PLC ζ immunofluorescence. While this may indicate that ejaculates produced on separate occasions from the same male differs greatly with regards to the status of PLC ζ within the sperm, it is also possible that reduced proportions of sperm exhibiting PLC ζ immunofluorescence indicates cases where an ejaculate has a reduced fertilisation capacity, but still able to result in successful pregnancy. Indeed, sperm from Control 5, while exhibiting normal sperm morphology, only successfully fertilised 20% of oocytes following IVF treatment. However, quantitative immunofluorescence indicated a level of total PLC ζ similar to sperm from infertile patients. While it is possible that the low IVF success rate may have been due to other sperm factors than PLC ζ , such as sperm/oocyte binding, or poor oocyte health due to advanced age of the patients partner (personal communication – Mr. Tim Childs) it is also possible that the low levels of total PLC ζ in sperm from this control may indicate sub-fertility of the sperm sample produced from Control 5.

Mouse oocytes are thought to require a range of 20-50 fg of mouse PLC ζ to successfully undergo oocyte activation (Saunders *et al.*, 2002; Yu *et al.*, 2008). It is therefore possible that human PLC ζ may successfully activate human oocytes within a

particular range of PLC ζ protein concentration. It is worth noting however, that successful activation may not necessarily mean successful embryo development to the blastocyst stage. Yu *et al.* (2008) demonstrated that while a range of injected human PLC ζ cRNA concentrations were able to elicit Ca²⁺ oscillations in mouse oocytes leading to their activation, only an estimated 1fg of human PLC ζ protein generated from such injection of cRNA was required for successful development to the blastocyst stage. It is therefore possible, that the variance observed in levels of total PLC ζ in control sperm means that not all sperm isolated following DGW, and exhibiting PLC ζ immunofluorescence, are able to induce oocyte activation or initiate development to the blastocyst stage. An interesting point to note is that the amplitude and frequency of the Ca²⁺ oscillations observed at oocyte activation play a direct role in the rate of cell cycle progression and early embryo gene expression profiles (Ducibella *et al.*, 2002). It is therefore possible that the observed variance in PLC ζ levels in fertile sperm may lead to differential rates of cell cycle progression, as well as ensuring genetic and epigenetic variety in the resulting offspring. It is essential that future studies attempt to investigate the role of variable levels of PLC ζ in establishing and/or maintaining genetic diversity in the developing embryo.

While large variability in the amount of PLC ζ present in sperm from different individuals may have implications for the role of PLC ζ in early embryo development, it would seem that quantitative immunofluorescent analysis may not necessarily be a good indicator of oocyte activation capability, and would thus not provide a viable diagnostic method to be used in the clinic as proposed by previous studies (Grasa *et al.*, 2008; Heytens *et al.*, 2009; Kashir *et al.*, 2010; Aghajanzpour *et al.*, 2011). However, the present analysis also indicated that the control population seemed to exhibit a higher proportion of sperm that contained PLC ζ compared to the infertile population, which may indicate that

since the proportion of sperm containing PLC ζ from the fertile population is higher, and may lead to an increased chance of successful fertilisation. The reduced proportion of sperm exhibiting PLC ζ within the infertile population may reflect a reduced chance of successful conception. It is therefore possible that examining the proportion of sperm exhibiting PLC ζ immunofluorescence may serve as a good indicator of oocyte activation capability as opposed to the utilisation of quantitative immunofluorescent analysis.

3.4.4 The predominant localisation pattern of PLC ζ varies in control sperm, with no predominant pattern present in sperm from infertile males

Grasa *et al.* (2008) reported that the precise distribution of the pattern of PLC ζ localisation in the sperm head varied between individuals, with the predominant pattern of PLC ζ localisation occurring within the equatorial segment of the sperm head, also reporting subsidiary PLC ζ immunofluorescence within the acrosomal and post-acrosomal regions of the sperm head. The current study, however, indicated that the predominant pattern of PLC ζ localisation varies between fertile individuals, while it is also possible that this may vary between ejaculates from the same person. Additional localisation patterns were also identified, namely all three patterns of localisation, and acrosomal + post-acrosomal localisations were also found. Analysis of 7 fertile controls indicated that only control 2 exhibited a predominantly equatorial localisation of PLC ζ , while control 1 exhibited a more profuse distribution of localisation patterns, with the post-acrosomal localisation seemingly predominant. However, analysis of controls 3, 4, 5 and 7 indicated that two patterns of PLC ζ localisation seemed predominant; the post-acrosomal and equatorial + post-acrosomal patterns of localisation.

Analysis of infertile patient sperm revealed that only a very small proportion of sperm exhibited PLC ζ immunofluorescence, present at a much lower proportion compared to sperm from the fertile population. Of those which did, no clear predominant pattern of PLC ζ localisation was observed. Interestingly, sperm from control 6 also failed to exhibit any significant differences between localisation patterns. It is possible that this lack of predominance for a particular localisation pattern correlates to reduced oocyte activation capability of sperm, concurring with the findings that the proportion of sperm exhibiting PLC ζ immunofluorescence may serve as a prognostic indicator of male fertility. However, it is intriguing to note that Control 6 did not exhibit any statistically predominant pattern of localisation, compared to the other controls. Furthermore, sperm from this control also exhibited levels of total PLC ζ immunofluorescence which were similar to sperm from the patient population.

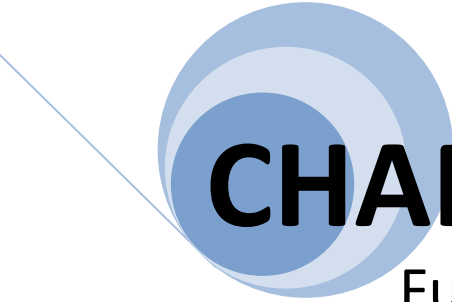
It remains to be seen if the levels of PLC ζ immunofluorescence observed in this study have any significant correlations with the distribution of PLC ζ localisation patterns in the sperm head. This is particularly important for future studies to investigate considering the implications of the involvement of varying levels of PLC ζ in early embryo development. The varying patterns of localisation have previously been proposed to correspond either to different isoforms of PLC ζ , or to be responsible for functions other than eliciting Ca²⁺ oscillations at fertilisation (Grasa *et al.*, 2008; Bi *et al.*, 2009). Indeed, Bi *et al.* (2009) proposed that the acrosomal population of PLC ζ may be involved in functions such as the acrosome reaction. However, such speculations have yet to be proven.

3.5 Conclusions and future work

In conclusion, this Chapter demonstrates that a PCR-based screening approach to identify novel loss-of-activity mutations in the PLC ζ ORF may not be sufficient when confronted with multiple patient samples within a clinic. Out of 12 patients and 2 fertile controls screened, only one novel mutation was identified which resulted in a change of a histidine to a leucine at position 233 of the PLC ζ amino acid ORF. It would seem that mutations similar in nature to H398P, occurring on exons coding for the active site, may indeed be rare. However, it is essential that future studies continue genomic screening while expanding both the size and bio-geographical range of patients examined, as well as expanding the screen to other exons, and possibly introns, utilising alternative, more efficient screening methods, to structurally and functionally characterise novel mutations discovered.

Quantitative immunofluorescent analysis indicated that while overall levels of total PLC ζ are significantly reduced in sperm from infertile patients compared to sperm from fertile controls, levels of total PLC ζ in sperm from fertile controls vary dramatically, with some fertile controls exhibiting similar levels of PLC ζ fluorescence as sperm from infertile patients diagnosed with OAD. While implying a contributory role for PLC ζ in ensuring genetic and epigenetic diversity, the current results indicate that quantitative immunofluorescence may not be a useful prognostic indicator of oocyte activation capability. However, analysis of the proportions of sperm within a sample exhibiting PLC ζ immunoreactivity may be indicative of male fertility. While these results agree with those of Grasa *et al.* (2008) in that the predominant pattern of PLC ζ localisation varies between individuals, the equatorial pattern of PLC ζ localisation was only predominant in one

control, with the rest exhibiting post-acrosomal and equatorial + post-acrosomal patterns of localisation. An interesting avenue of investigation would be to examine possible correlations between localisation pattern(s) and the Ca^{2+} oscillation profile induced in mouse oocytes following microinjection of specific sperm structures corresponding to a particular pattern of PLC ζ localisation (such as the acrosome or equatorial segment). It is necessary that future studies explore the identity of the PLC ζ populations inherent within human sperm (i.e. whether they correspond to different isoforms of PLC ζ) in order to examine their functional roles if PLC ζ immunofluorescence is to be used as a prognostic marker of male fertility within the ART clinic.



CHAPTER 4

Functional characterisation and modes of inheritance of a novel PLC ζ mutation (H233L) identified in an infertile male

4.1 Introduction

Male factor and idiopathic infertility are significant contributors to global infertility, with abnormalities in testicular gene expression considered to be a major cause (Coward *et al.*, 2007; Kashir *et al.*, 2010). Strong evidence indicates that certain types of human male infertility are caused by failure of the sperm to activate the oocyte successfully (oocyte activation deficiency; OAD). The global incidence of infertility is estimated to affect 1 in 7 couples (Evens, 2004; Boivin *et al.*, 2007, Ombelet *et al.*, 2008). Whilst techniques such as intracytoplasmic sperm injection (ICSI), where a single sperm is micro-injected directly into the oocyte, is able to rescue fertility in 35 - 40% of infertile cases (Kashir *et al.*, 2010), complete or virtually-complete fertilisation failure is known to occur in 1 - 5% of all ICSI cycles (Flaherty *et al.*, 1998; Mahutte and Arici, 2003; Yanagida, 2008).

The first link between OAD and PLC ζ deficiency was reported by Yoon *et al.* (2008), who found that sperm from patients repeatedly failing to activate oocytes via ICSI could not initiate Ca²⁺ oscillations when injected into mouse oocytes. Moreover, PLC ζ could not be detected in the sperm of males with a history of ICSI failure. Crucially, Heytens *et al.*, (2009) identified a substitution mutation in an infertile male diagnosed with OAD. This mutation occurred in exon 11 within the Y domain of the active site at position 398 of the PLC ζ open reading frame (ORF), resulting in a histidine to proline substitution (PLC ζ^{H398P}). Whilst Heytens *et al.* (2009) hypothesised that the infertile phenotype of this patient could have arisen via a dominant negative mechanism, subsequent experiments using a mouse model indicated that this was not the case (Nomikos *et al.*, 2011). Furthermore, Heytens *et al.* (2009) indicated only a very weak potential dominant negative effect of the human PLC ζ^{H398P} when coinjected with wild type human PLC ζ into mouse

oocytes, a finding mirrored by Nomikos *et al.* (2011a), although it should be noted that the mouse mutation may behave in a very different manner compared to the human one.

Genetic dysfunction represents the greatest cause for concern for the incidence of global infertility (Kashir *et al.*, 2010). Moreover, some cases of idiopathic male infertility may represent the result of multiple genetic defects that disrupt spermatogenesis and testicular gene expression, indicating that ART interventions such as ICSI could potentially transmit paternal fertility problems from father to son (Coward *et al.*, 2007). However, prior study of such genetic causes of infertility has mostly been limited to paternal modes of inheritance, with potential maternal involvement being largely ignored. Furthermore, considering that PLC ζ^{H398P} has been demonstrated by Nomikos *et al.* (2011a) not to function via a dominant negative manner, it remains a mystery as to how the patient exhibiting this mutation could present with complete infertility. This conundrum, however, may be made clearer by the discovery of a novel mutation from this patient; an A>T change at position 698 of the PLC ζ ORF (within the X-Domain; Exon 6 of the PLC ζ gene), resulting in a Histidine to Leucine substitution at position 233 (PLC ζ^{H233L}) in the amino acid sequence of PLC ζ , identified in Chapter 3 of this thesis.

It is evident that PLC ζ may represent an ideal marker of sperm fertility with which to study the genetic modes of inheritance underlying infertile phenotypes, for instance those linked to failure of the sperm to activate the oocyte. In response to concerns about the ability of PLC ζ^{H398P} to act in a dominant negative fashion, and thus be capable of causing infertility in a heterozygous manner, a more thorough re-examination of the PLC ζ gene was performed from the patient in which the H398P mutation was discovered, following which the novel H233L mutation was identified. Further to this therefore, the

aims of this chapter were to analyse the functional effects of the novel H233L mutation in comparison to wild type PLC ζ (PLC ζ^{WT}) and PLC ζ^{H398P} . Crucially, the genetic origin and specific mode of inheritance of the two PLC ζ mutations was investigated across three generations of the family of patient^{H398P}, in an attempt to gain further understanding of how such mutations may effect fertility, and the patterns of genetic inheritance involved.

4.2 Materials and Methods

4.2.1 Sample acquisition and processing

Genomic DNA was extracted from buccal cell samples obtained from 9 infertile males whose sperm lacked activation ability, including the patient from which PLC ζ ^{H398P} had been previously identified (patient^{H398P}; Heytens *et al.*, 2009). All patients had been previously diagnosed with recurrent ICSI failure or abnormal sperm morphology and were undergoing fertility treatment at the Department of Reproductive Medicine, Ghent University Hospital, Belgium. Additional buccal samples were obtained from four family members of patient^{H398P} (mother, father, halfbrother, and daughter – conceived by ICSI and assisted oocyte activation methodology). All samples were collected with informed written consent with ethical approval as previously described (Chapter 2, Section 2.1). Sperm from patient^{H398P} were also processed as previously described in Chapter 2, Section 2.14, for isolation and analysis. Genomic DNA was also extracted, with informed written consent, from the blood of 100 fertile individuals (50 male and 50 female) undergoing pre-implantation genetic diagnosis at Reprogenetics UK, Oxford (performed by Mr. Michalis Konstantinidis, NDOG, University of Oxford, UK).

4.2.2 Molecular analysis of the PLC ζ gene

Amplicons were generated for patient^{H398P}, four family members of patient^{H398P}, and eight further infertile males, by PCR using the High Fidelity Master Kit (Roche Biosciences, UK) and primer sequences/thermal cycling conditions previously described in Chapter 2, Table 2.2. Each exon of the PLC ζ gene was amplified from genomic DNA and their nucleotide sequence determined (Geneservice, UK). Open reading frame (ORF) sequences were identified and compared to the published wild type PLC ζ (PLC ζ ^{WT}) ORF

sequence (accession number NM_033123). In contrast to the analyses carried out by Heytens *et al.* (2009) and Chapter 3 of this thesis, this study aimed specifically to investigate whether sequence anomalies were homozygous or heterozygous in nature; a crucial consideration neglected by previous studies. In order to do this, sequencing peaks were additionally analysed using Chromas Lite freeware (Technelysium Ltd, Australia).

4.2.3 cRNA synthesis

PLC ζ^{WT} cRNA was synthesised as described previously (Chapter 2, Sections 2.12 and 2.13) following the same protocol and templates employed by Heytens *et al.* (2009). PLC ζ^{H233L} was created using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, USA) as previously described in Chapter 2, Section 2.8, using a molecular construct containing PLC ζ^{WT} as the template (Forward primer: 5'-TTATCCAAGCTATACTCAAGTATGCATTCAT-3'; Reverse primer: 5'-ATGAATGCATACTTGAGTATAGCTTGGATAA-3'). All molecular constructs were verified by DNA sequencing (Geneservice, Oxford) prior to downstream application. cRNA was transcribed for each PLC ζ isoform using the T7 promoter and a T7 mMESSAGE mMACHINE kit (Ambion, USA) in accordance with the manufacturer's instructions. cRNA was purified using the RNeasy Mini kit (QIAGEN, UK) as previously described in Chapter 2, Section 2.12, and stored immediately at -80°C in single-use aliquots.

4.2.4 Mouse oocyte collection, culture, micro-injection and Ca²⁺ imaging

Mouse oocytes were injected with cRNA corresponding to mutant and wild type isoforms of PLC ζ for a comparative analysis of the resultant Ca²⁺ traces. Oocyte collection, culture, microinjection and Ca²⁺ imaging were performed, as previously described (Chapter 2, Sections 2.17 and 2.18), by Mr. Hoi Chang Lee and Professor Rafael A Fissore (University of Massachusetts, USA). Animal handling and procedures were approved by the University of Massachusetts IACUC committee. cRNA corresponding to PLC ζ ^{WT} or PLC ζ ^{H233L} was injected at two separate concentration sets (344ng/ μ l and 352ng/ μ l; 172ng/ μ l and 176ng/ μ l, respectively). Statistical analysis was performed using Graphpad Prism software (GraphPad, USA). Correlations between the amplitude of the first Ca²⁺ peak, as well as the interval between the first and second Ca²⁺ peaks following microinjection of WT and H233L cRNA at both concentrations were determined using the *t*-test.

4.2.5 Direct mutation detection using individual sperm cells

A hemi-nested PCR strategy was used to amplify haploid DNA from individual patient sperm cells. Primer sequences (Table 1) were designed using Primer3 software (Rozen & Staletsky, 2000). Single sperm cells were lysed in alkaline lysis buffer (PCR grade water, 200mM NaOH, 250mM DTT) and incubated at 65°C for 20 minutes. Mutation detection involved first-round multiplex PCR, simultaneously amplifying the two regions of the PLC ζ gene containing the H233L and H398P mutations, followed by two hemi-nested inner reactions amplifying the two mutation regions individually, followed by mini-sequencing for direct mutation detection. Procedures were performed by Mr. M Konstantinidis (NDOG, University of Oxford, UK).

Table 4.1: Primer sequences used to perform direct mutation detection on individually-isolated sperm. (F: forward primer; R: reverse primer).

	H233L locus	H398P locus
Outer Primers (5'-3')	F: TTTTGCTAGTGCCCTTG R: AGGCTAAGCATTATAGGATTTGA	F: TGCCAAGACAATACATATCAAAA R: GTTACTTCTGCAACAACACTCAATATC
Inner Primers (5'-3')	F: ATTGACTGCTGGGATGGA	R: TCTTGGGGATTAATAATTAGAAGAG
Mini-sequencing primers (5'-3')	F: AACTGTTATCCAAGCTATAC	F: AGTCCATGAGTTTATTTTC

The QIAGEN Multiplex PCR kit (Qiagen, UK) was used to carry out the first round of amplification according to the conditions described in Table 2. Inner reactions were performed using HotMaster Taq DNA polymerase (5 prime, UK). The reaction mix contained PCR grade water, 1x HotMaster Taq Buffer (with 25 mM Mg^{2+}), dNTPs (200 μ M each), 0.8 μ M of each primer, 0.6 units HotMaster Taq DNA polymerase and 1 μ l of first-round amplified product for a final volume of 15 μ l. PCR amplification was carried out as described in Table 2. Inner reaction products were treated with EXOSAP-it (Affymetrix, UK) to remove unincorporated primers and/or dNTPs. Subsequently, mini-sequencing was carried out using the SNaPshot Multiplex kit (Applied Biosystems, UK). Mini-sequencing products were analysed using capillary electrophoresis on a 3130 genetic analyser (Applied Biosystems, UK), and data analysed using GeneMapper v4.0 software (Applied Biosystems, UK). Procedures were performed by Mr. M Konstantinidis (NDOG, University of Oxford, UK).

Table 4.2: Thermal cycling conditions for direct mutation detection in single sperm. Courtesy of M. Konstantinidis (NDOG, University of Oxford, UK).

		Outer Reaction – Multiplex*		Inner Reaction**	
PCR Steps		Temperature (°C)	Duration	Temperature (°C)	Duration
Repeated cycles	Holding	95	15 min	96	1 min
	Denaturation	94	30 sec	94	15 sec
	Annealing	58	90 sec	58	15 sec
	Extension	72	60 sec	65	45 sec
	Final Extension	60	10 min	65	2 min
Hold		4	Infinity	4	Infinity

* Multiplex 55 cycles

** 40 cycles

4.2.6 TaqMan SNP genotyping assay

Two genotyping assays employing sequence detection probes were developed using the ‘Custom TaqMan assay design tool’ (Applied Biosystems, UK) and used to screen a population of 100 fertile individuals for the presence of the H233L and H398P mutations. The resultant assays were carried out using GTXpress mastermix (Applied Biosystems, UK), with thermal cycling undertaken on a StepOne real-time PCR system (Applied Biosystems, UK) as detailed in Table 3. Results were analysed using StepOne v2.1 software and TaqMan Genotyper software (Applied Biosystems, UK). Procedures were performed by Mr. M Konstantinidis (NDOG, University of Oxford, UK).

Table 4.3: Thermal cycling conditions for the TaqMan SNP genotyping assay. Courtesy of M. Konstantinidis (NDOG, University of Oxford, UK).

		Outer Reaction – Multiplex		
PCR Steps		Temperature (°C)	Duration	Read
Pre-PCR Read		25	30 sec	Yes
DNA polymerase activation		95	20 sec	---
40 cycles* 35 cycles**	Denaturation	95*/92**	3 sec	---
	Annealing / Extension	60*/62**	20 sec	Yes
	Post-PCR read	25	30 sec	Yes

*H233L SNP assay

**H398P SNP assay

4.3 Results

4.3.1 Identification of a novel second mutation in PLC ζ from patient^{H398P}

In addition to the original PLC ζ ^{H398P} mutation described by Heytens *et al.* (2009), a base change in exon 6 of the PLC ζ gene was identified from patient^{H398P}, which resulted in the substitution of an Adenine (A) nucleotide at position 698 of the DNA ORF sequence to a Thymine (T) nucleotide (Chapter 3), corresponding to the substitution of histidine residue for a leucine residue in the catalytic X domain at position 233 (PLC ζ ^{H233L}) of the amino acid sequence (Figure 4.1A). A 3-dimensional (3D) model of PLC ζ ^{WT} and PLC ζ ^{H233L} protein (Figure 4.1B) was created based upon knowledge of the known structure of PLCdelta (PLC δ) derived previously by X-ray crystallography (Essen *et al.*, 1996) courtesy of Dr. R. Hamer and Dr. CM Deane (University of Oxford, UK). Computer modelling predicted that the PLC ζ ^{H233L} mutation is likely to reduce the number of contacts this residue has with other atoms, thus disrupting local protein folding. A multiple sequence alignment revealed that H233 is only marginally conserved across PLC ζ from various taxa (Figure 4.1C).

Micro-injection experiments further demonstrated that 100% of oocytes (16/16) exhibited Ca²⁺ oscillations in a pattern characteristic of fertilisation (>8 spikes per hour), when injected with PLC ζ ^{WT} cRNA, and resulted in the activation of 100% (8/8) of injected oocytes (from separate experiments). In contrast, while 67% of total oocytes (12/18) injected with PLC ζ ^{H233L} cRNA elicited oscillations compared to PLC ζ ^{WT} at concentrations of 352ng/ μ l and 344ng/ μ l respectively (Figure 4.2), these were severely altered in frequency and exhibited reduced amplitude, differing markedly from that normally seen at fertilisation. Injection of WT and H233L cRNA into mouse oocytes at a reduced

concentration (172ng/μl and 176ng/μl respectively; Figure 4.3) also resulted in a severely abnormal profile of Ca^{2+} in mutant cRNA injected oocytes (Figure 4.3).

While the amplitude of the first Ca^{2+} peak was significantly reduced ($p \leq 0.05$) following injection of H233L cRNA (~0.1 arbitrary units; a.u) compared to oocytes injected with WT cRNA (~0.25 a.u) at concentrations of 352ng/μl and 344ng/μl respectively, the interval between the first and second Ca^{2+} peaks following injection of H233L cRNA (~50mins) did not differ significantly from injection of WT cRNA (~40mins) at these concentrations (Figure 4.4A). However, injection of WT and H233L cRNA in mouse oocytes at a reduced concentration (172ng/μl and 176ng/μl respectively) resulted in a significantly decreased ($p \leq 0.05$) amplitude of the first Ca^{2+} peak following H233L cRNA injection (~0.15 a.u) as compared to WT cRNA injection (~0.3 a.u), as well as a significantly increased ($p \leq 0.05$) interval between the first and second Ca^{2+} peaks (~20mins and ~110mins respectively) following H233L and WT cRNA at these concentrations (Figure 4.4B). None of the oocytes injected with $\text{PLC}\zeta^{\text{H233L}}$ cRNA (0/8) activated.

4.3.2 Genetic modes of inheritance of the H233L and H398P $\text{PLC}\zeta$ mutations

Mini- and direct-sequencing of genomic DNA from patient^{H398P} revealed that $\text{PLC}\zeta^{\text{H233L}}$ and $\text{PLC}\zeta^{\text{H398P}}$ were heterozygous in nature as represented by the presence of two sequencing peaks for the same base (Figure 4.5; Figure 4.6). The mother of patient^{H398P} did not possess $\text{PLC}\zeta^{\text{H398P}}$, but was heterozygous for $\text{PLC}\zeta^{\text{H233L}}$. The father, however, was heterozygous for $\text{PLC}\zeta^{\text{H398P}}$. Interestingly, the daughter of patient^{H398P}, conceived via assisted reproductive technology and the use of Ca^{2+} ionophore-mediated

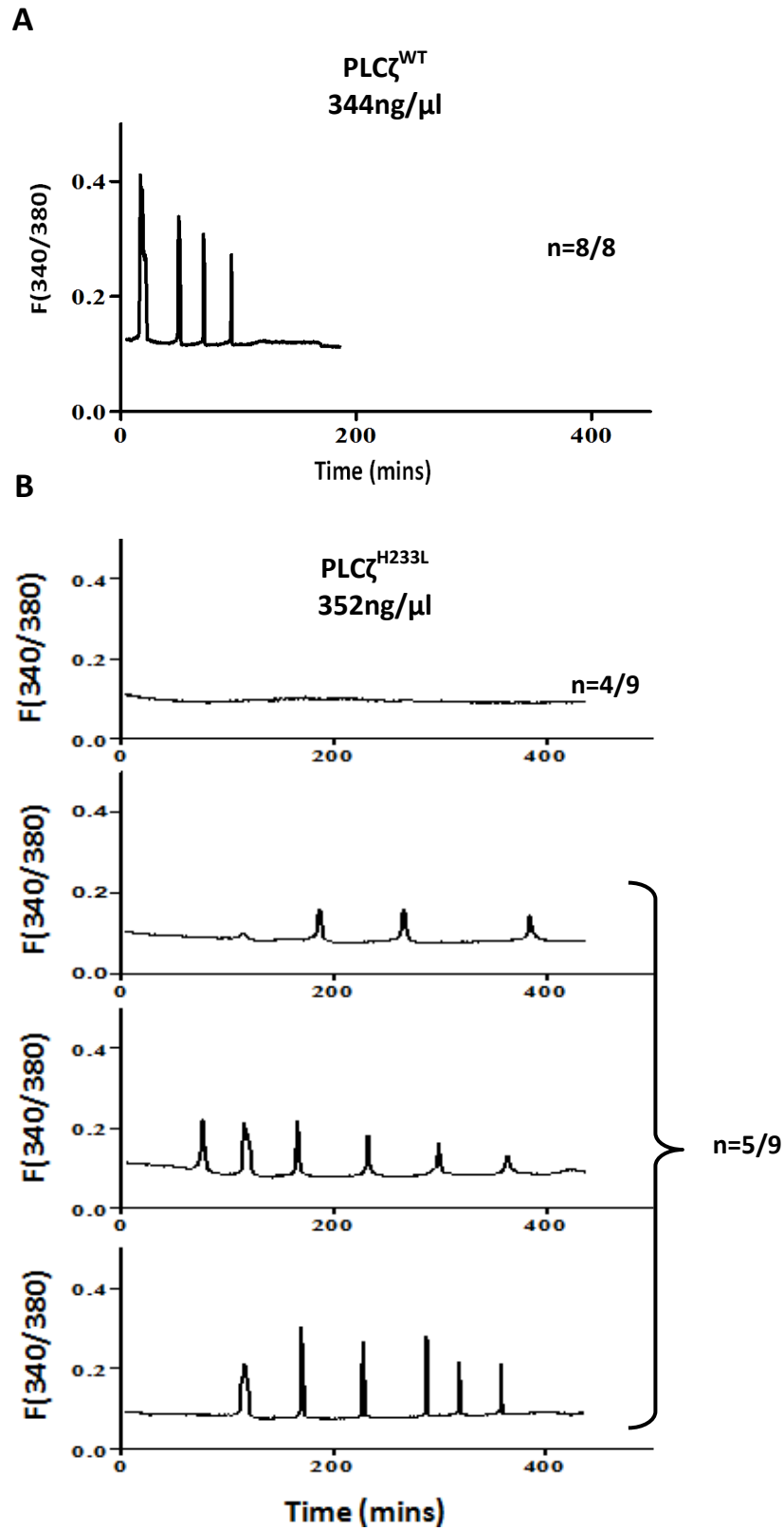


Figure 4.2: Micro-injection of PLC ζ^{WT} (A) and PLC ζ^{H233L} (B) cRNA into mouse oocytes at concentrations of 344ng/ μl and 352ng/ μl respectively, and resultant calcium release patterns. Courtesy of HC Lee and Professor RA Fissore (University of Massachusetts, USA). F(340/380) denotes ratio of the fluorescence obtained at wavelengths of 340nm and 380nm

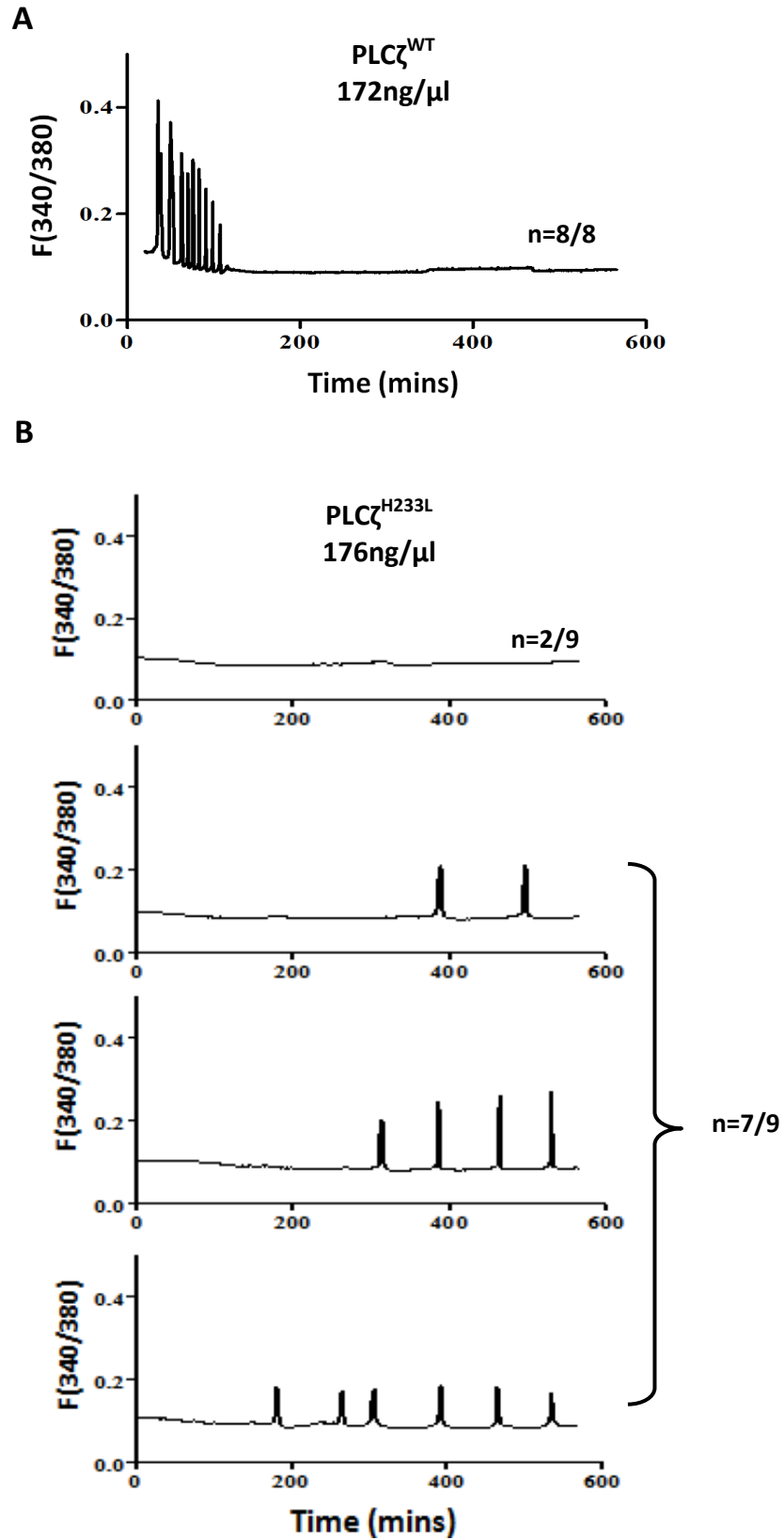


Figure 4.3: Micro-injection of PLC ζ^{WT} (A) and PLC ζ^{H233L} (B) cRNA into mouse oocytes at concentrations of 172ng/ μl and 176ng/ μl respectively, and resultant calcium release patterns. Courtesy of HC Lee and Professor RA Fissore (University of Massachusetts, USA). F(340/380) denotes ratio of the fluorescence obtained at wavelengths of 340nm and 380nm

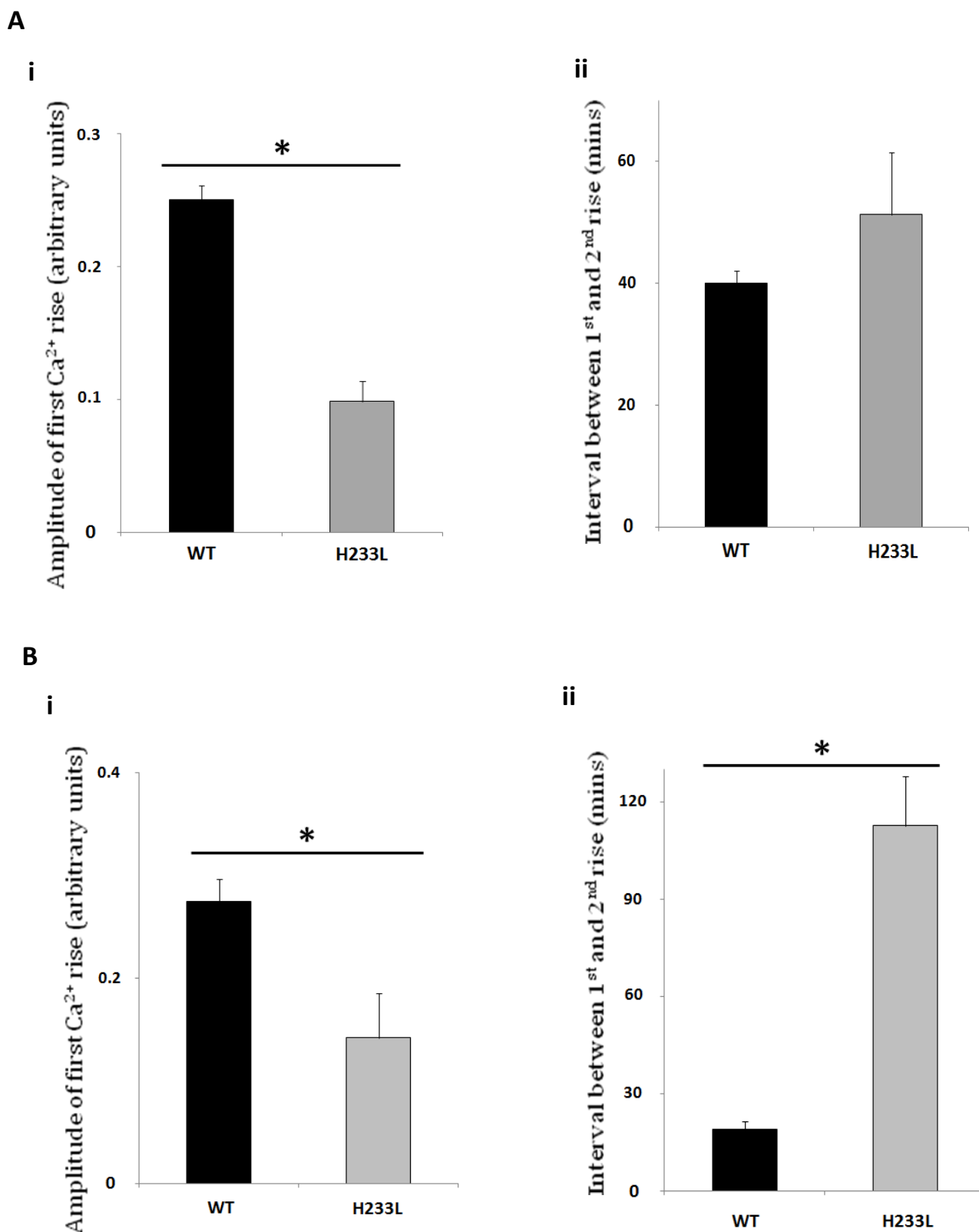


Figure 4.4: Analysis of Ca^{2+} peaks observed following microinjection of WT and H233L cRNA at 344ng/ μl and 352ng/ μl (**A**) and 172ng/ μl and 176ng/ μl (**B**). For both sets of histograms, the average amplitude of the first Ca^{2+} rise (**i**) and the average interval between the first and second Ca^{2+} peaks (**ii**) following microinjection are represented. The asterisk (*) on the histograms denotes a statistically significant difference ($p \leq 0.05$). Data are represented as mean $\pm$ SEM.

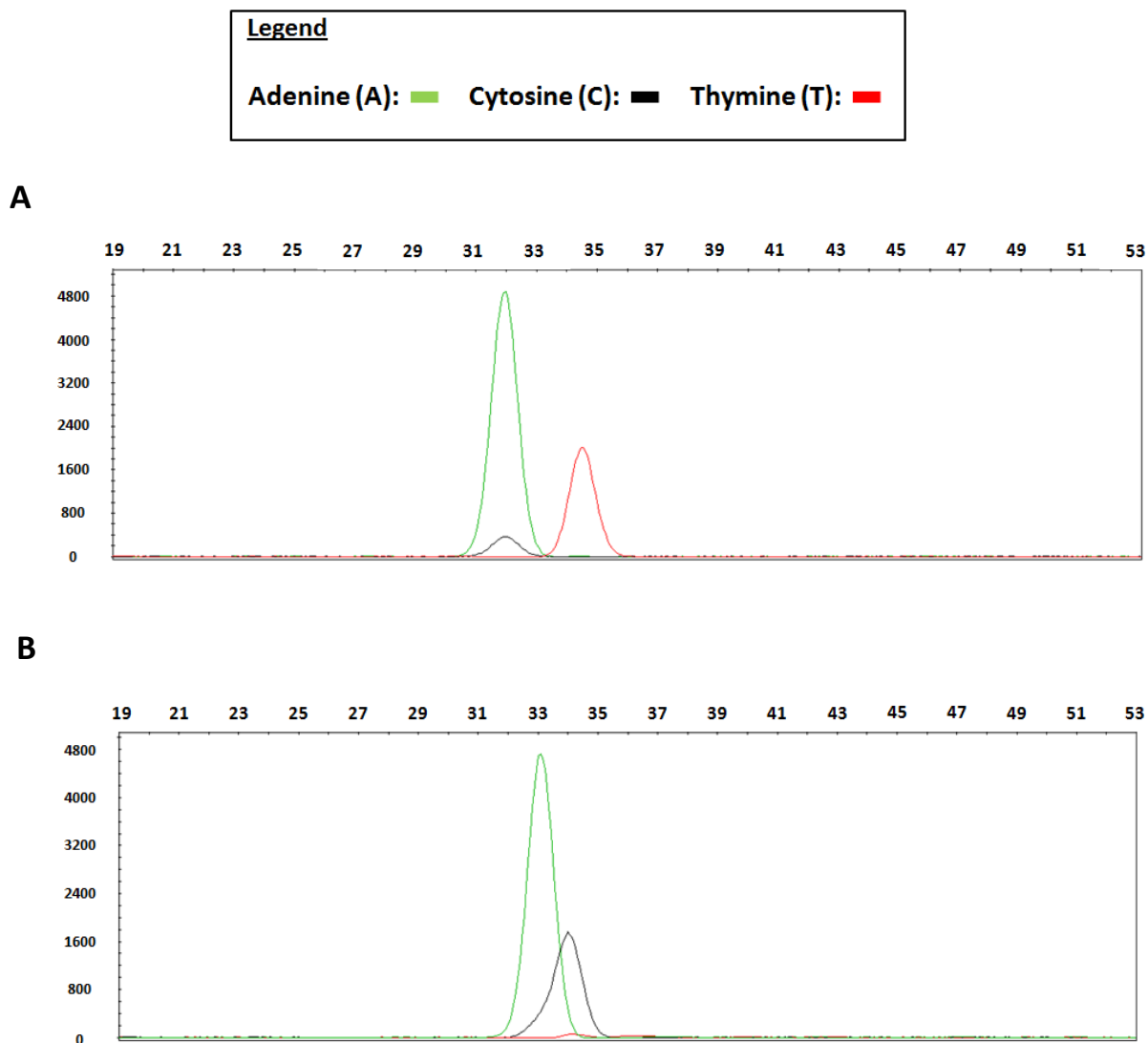


Figure 4.5: Mini-sequencing profiles of genomic DNA from patient^{H398P}. **(A)** Presence of adenine and thymine peaks indicates that PLC ζ^{H233L} is heterozygous, and **(B)** presence of adenine and cytosine peaks indicates that PLC ζ^{H398P} is heterozygous. Green peak: adenine; black peak: cytosine; red peak: thymine. Y-axis: relative fluorescence units; X-axis: amplicon base number.

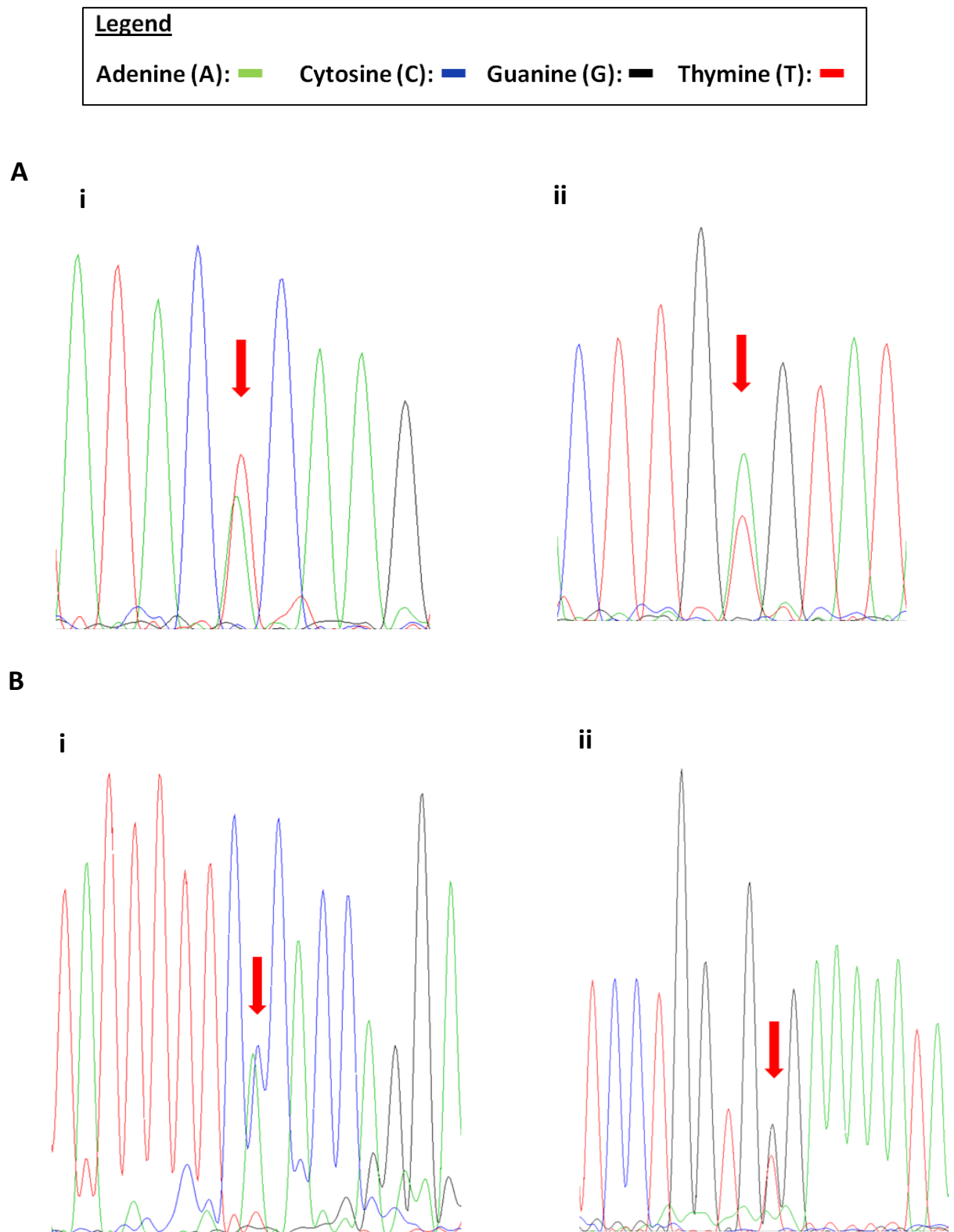


Figure 4.6: Genomic sequencing profile of patient^{H398P} identifying PLC ζ ^{H233L} and confirming that PLC ζ ^{H233L} (A) and PLC ζ ^{H398P} (B) are heterozygous as determined by the presence of adenine (A) and thymine (T) at position 698 (PLC ζ ^{H233L}), and the presence of A and cytosine (C) on position 1193 (PLC ζ ^{H398P}) of the PLC ζ ORF (indicated by red arrows). This was confirmed by both forward and reverse primers (i and ii for both mutations).

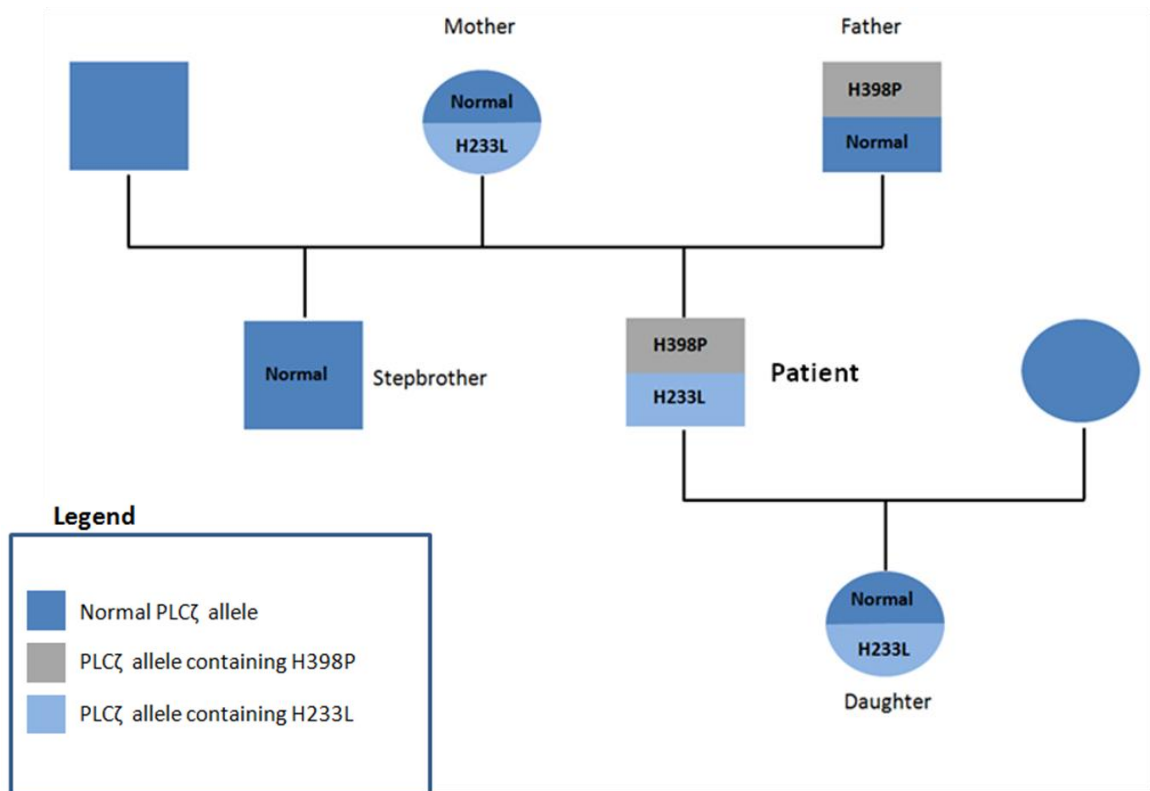


Figure 4.7: Pedigree showing the inheritance of PLC ζ^{H233L} and PLC ζ^{H398P} in patient^{H398P} and his immediate family.

4.3.3 Single sperm screening for H233L and H398P in patient^{H398P}

Mini-sequencing of single sperm from patient^{H398P} revealed that 58% (7/12) of sperm carried PLC ζ^{H233L} (Figure 4.8), while 42% (5/12) carried PLC ζ^{H398P} (Figure 4.9). Analysis revealed that the sperm screened possessed either PLC ζ^{H233L} (exhibiting either an adenine/normal peak or a thymine/mutant peak) or PLC ζ^{H398P} (exhibiting either an adenine/normal peak or a cytosine/mutant peak), but never both. This confirmed the independent inheritance of the H233L and H398P mutations in patient^{H398P}, demonstrating that he does not possess a normal copy of the PLC ζ gene (Table 4.4). While a clear bias towards a particular mutation was not indicated in the present analysis, results confirm that all sperm from patient^{H398P} would inevitably pass on a mutated version of PLC ζ .

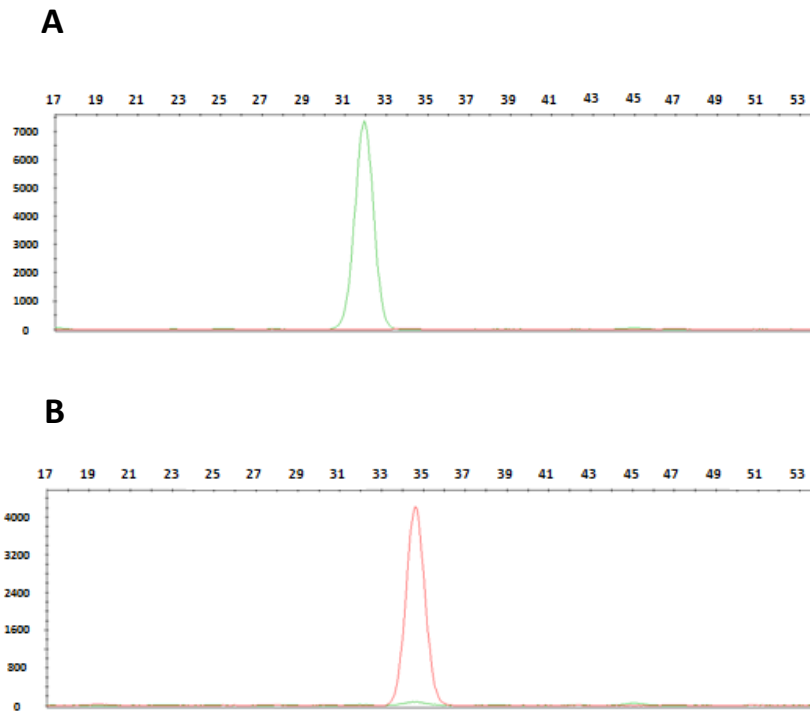


Figure 4.8: Representative mini-sequencing peaks of single sperm from patient^{H398P} for PLC ζ ^{H233L}. Single sperm exhibited either an adenine peak (**A**) or a thymine peak (**B**) upon screening for PLC ζ ^{H233L}. Y-axis: relative fluorescence units; X-axis: amplicon base number.

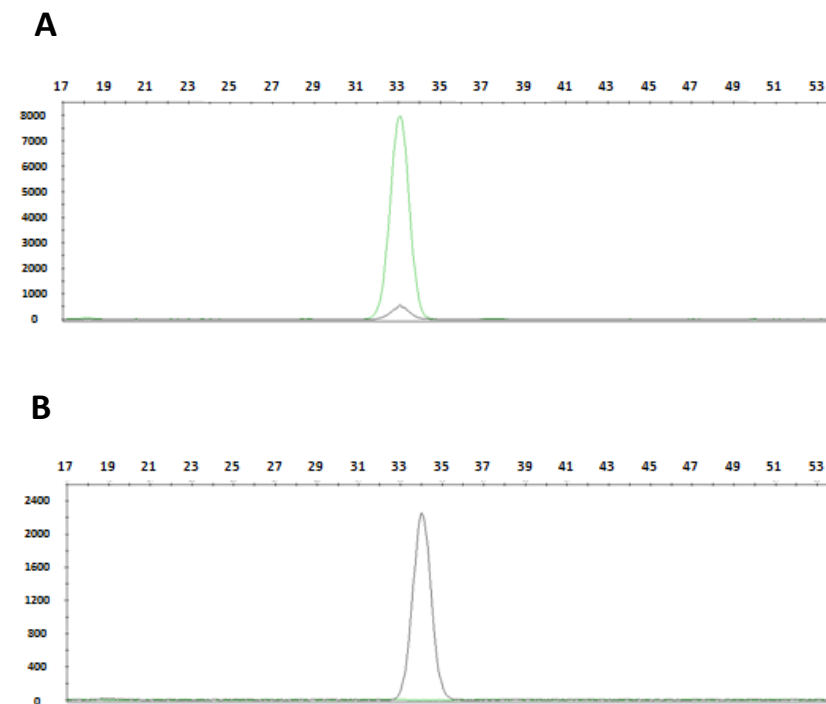


Figure 4.9: Representative mini-sequencing peaks of single sperm from patient^{H398P} PLC ζ ^{H398P}. Single sperm exhibited either an adenine peak (**A**) or a cytosine peak (**B**) upon screening for PLC ζ ^{H398P}. Y-axis: relative fluorescence units; X-axis: amplicon base number.

Table 4.4: Direct mutation detection from twelve individually isolated sperm from patient^{H398P} showing the prevalence of H233L and H398P PLC ζ mutations. Ticks indicate presence of mutation, while crosses indicate absence.

Sperm	Mutation present	
	H233L	H398P
1	-	+
2	+	-
3	-	+
4	-	+
5	+	-
6	-	+
7	-	+
8	+	-
9	+	-
10	+	-
11	+	-
12	+	-

4.3.4 Genetic screening of the prevalence of H233L and H398P in 100 fertile individuals and 8 patients diagnosed with oocyte activation deficiency

The SNP assays displayed results which were either heterozygous normal (both alleles normal; red circles), heterozygous affected (one normal allele and one abnormal; green circles), while negative controls displayed as blue squares. The SNP assays did not detect either the PLC ζ ^{H233L} or PLC ζ ^{H398P} mutation in any of the 100 fertile individuals (200 chromosomes), nor any of the 8 OAD patients screened (Figure 4.10; Table 4.5). SNP assays further confirmed that patient^{H398P}, his mother, and his daughter were all heterozygous for PLC ζ ^{H233L}, and that his halfbrother and father do not carry the H233L

mutation. However, analyses further demonstrated that patient^{H398P}'s father is heterozygous for the H398P mutation, suggesting that patient^{H398P} inherited the H233L and H398P mutations from his mother and father, respectively.

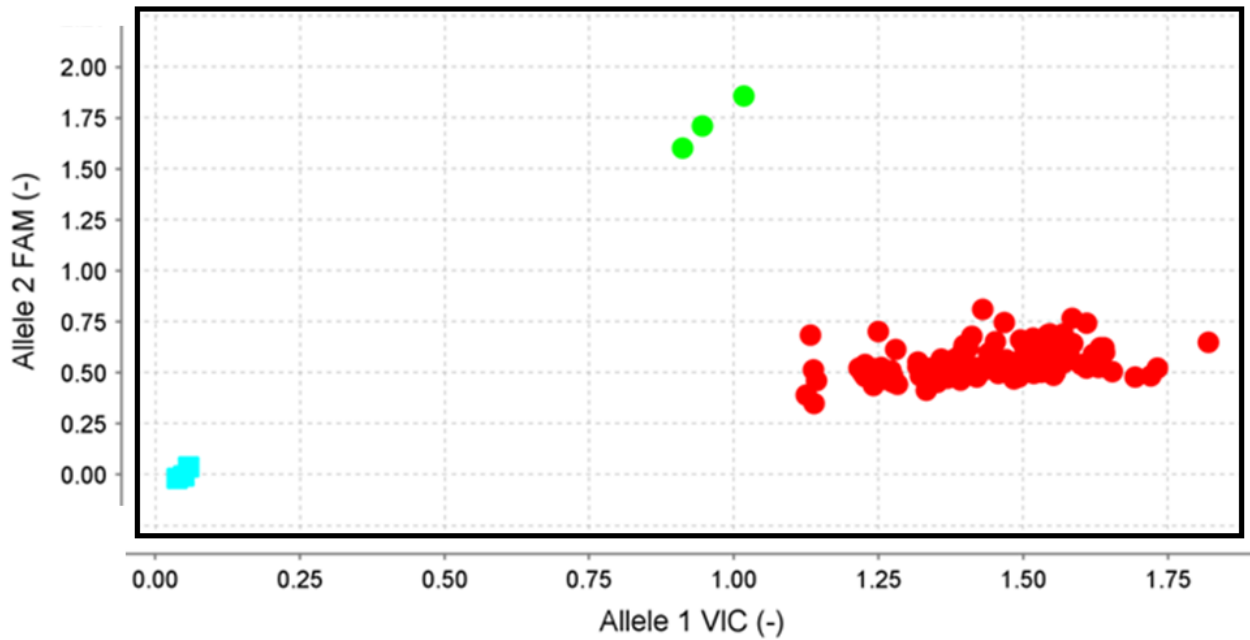


Figure 4.10: Representative scatter plot, indicating the normalised fluorescent intensities of the reporter dyes from each sample, showing screening results for 100 fertile individuals for PLC ζ ^{H233L} and PLC ζ ^{H398P} (overlapping red dots). Light blue squares represent negative controls, green dots represent heterozygous individuals (one normal PLC ζ allele, and one mutated PLC ζ allele), while red dots indicated homozygous unaffected individuals (both alleles contain normal PLC ζ). Allele 1 (x-axis) represents the fluorescence intensity of the normal allele labeled with VIC (red), while allele 2 (y-axis) represents the fluorescence intensity of the mutant allele labeled with FAM (blue). A homozygous sample for allele 1 would result in the display of fluorescence intensity for allele 1 (x-axis) and nothing for allele 2 (y-axis). The converse is true for samples that are homozygous for allele 2. A heterozygous sample, resulting in the binding of both probes to the template DNA, results in fluorescence from both reporter dyes (VIC/FAM), which is shown as green on the plot.

Table 4.5: Oocyte activation deficient patients screened for PLC ζ^{H233L} and PLC ζ^{H398P} . Plus (+) indicates presence of mutation, while minus (-) indicates absence.

Globozoospermic: Round-headed, acrosome-less sperm heads

Patient	Sperm morphology	H233L	H398P
1	Globozoospermic	-	-
2	Globozoospermic	-	-
3	Globozoospermic	-	-
4	Globozoospermic	-	-
5	Normal	-	-
6	Globozoospermic	-	-
7	Globozoospermic	-	-
8	Normal	-	-
H398P	Abnormal sperm head	+	+

4.4 Discussion

The first genetic link between OAD and PLC ζ , was reported by Heytens *et al.* (2009) who identified a histidine to proline residue substitution mutation (PLC ζ^{H398P}) within the Y domain (exon 11) of PLC ζ from an infertile male patient which resulted in compromised protein folding and abnormal Ca²⁺ release ability. However, confusion remains as to the precise genetic mechanisms underlying the existence and functional deficiency of the H398P mutation (Heytens *et al.*, 2009; Nomikos *et al.*, 2011). Chapter 3 of this thesis reported the identification of a novel mutation in PLC ζ (PLC ζ^{H233L}) from the same patient, which results in the substitution of a histidine for a leucine residue in the catalytic X domain (exon 6) at position 233 of the PLC ζ ORF. This Chapter aimed to characterise the effects of this new mutation on PLC ζ function. The genetic modes of inheritance of both H233L and H398P mutations were also investigated, shedding significant light upon the mechanisms by which PLC ζ deficiency, and potentially other male factor conditions, may be passed from parent to progeny.

4.4.1 A second novel loss-of-activity mutation in PLC ζ

While histidine is a polar and hydrophilic amino acid, leucine is a non-polar hydrophobic amino acid. Therefore, the H233L mutation is likely to disrupt local interactions within protein folding, in a manner similar to PLC ζ^{H398P} . Indeed, 3D modelling predicts that the histidine at position 233 of the PLC ζ amino acid sequence may serve a role in the maintenance of protein structure by holding the adjacent protein helix in place, and/or stabilising the position of a loop at the bottom of its adjacent helix. Micro-injection of cRNA corresponding to PLC ζ^{H233L} at a similar concentration (176ng/ μ l) resulted in an abnormal Ca²⁺ release profile, with the amplitude of the first Ca²⁺ peak and

the interval between the first and second Ca^{2+} peaks being significantly increased following H233L cRNA injection compared to WT cRNA injection, while also failing to activate oocytes.

However, while the injection of H233L cRNA at an increased concentration (352ng/ μl) resulted in no significant difference in the interval between the first and second Ca^{2+} peaks compared to WT cRNA injection at a similar concentration (344ng/ μl), the amplitude of the first Ca^{2+} peak was significantly reduced compared to WT cRNA injected oocytes. Collectively, the observed patterns of intracellular Ca^{2+} release indicated that some degree of functional activity is retained by $\text{PLC}\zeta^{\text{H233L}}$, and that the functional deficit is not as severe as that conferred by $\text{PLC}\zeta^{\text{H398P}}$, or its mouse equivalent H435P which totally abolished the ability of $\text{PLC}\zeta$ to trigger calcium oscillations in the mouse oocyte and prevented enzyme activity in a PIP2 hydrolysis assay (Nomikos *et al.*, 2011). Interestingly though, in light of this difference, while H398 is highly conserved among all known $\text{PLC}\zeta$ isoforms across a range of species (Heytens *et al.*, 2009), H233 is not. However, considering the abnormal profile of Ca^{2+} oscillations evident upon injection of $\text{PLC}\zeta^{\text{H233L}}$ cRNA into mouse oocytes which were insufficient for activation, it is clearly apparent that $\text{PLC}\zeta^{\text{H233L}}$ may exert reduced functionality, indicating that both mutations contributed towards the patient's infertility.

The discovery of $\text{PLC}\zeta^{\text{H233L}}$ from the same patient implies that the contribution of mutated $\text{PLC}\zeta$ protein to this particular patient's infertile status is more complex than previously thought. Both mutations were shown to exist on separate chromosomes (heterozygous), with $\text{PLC}\zeta^{\text{H398P}}$ inherited from the patient's father, and $\text{PLC}\zeta^{\text{H233L}}$ inherited from the mother. To the best of my knowledge, this represents the first description of an

autosomal point mutation to have been passed from a mother to her son, resulting in a deficit of sperm function and male infertility.

4.4.2 A maternally inherited autosomal mutation contributes to male infertility

While it is difficult to ascertain specific global causative factors for infertility, male factor and idiopathic (unknown) infertility are thought to be major contributors (each occurring in ~28% of cases). Accumulating evidence suggests that abnormalities in testicular gene expression may contribute towards idiopathic male infertility (Ambasudhan *et al.*, 2003; Coward *et al.*, 2007; Matzuk & Lamb, 2008; Avenarius *et al.*, 2009; Poongothai *et al.*, 2009; Kashir *et al.*, 2010). Major known causes of human male infertility include Y chromosome linked abnormalities and micro-deletions which are not associated with a syndrome but lead to impaired spermatogenesis, and mutations in X chromosome-linked genes such as *Akap4*, *Nxf2*, and *Tex11* involved in spermatogenesis (Wang & Pan, 2007; Zheng *et al.*, 2010). Due to the single-copy, hemizygous nature of these genes, damaging mutations would not be masked by a normal allele (Zheng *et al.*, 2010).

However, screening for X-linked gene mutations in infertile men has yet to reveal any causative factors (Zheng *et al.*, 2010). Furthermore, specific genes have not been identified for a number of autosomal male factor conditions. One such example is globozoospermia, an infertile condition involving round-headed and acrosome-less sperm, estimated to affect ~0.1% of infertile men (Dam *et al.*, 2007). While it is important to note that numerous studies indicate globozoospermia may be a multi-factorial genetic syndrome, involving mutation in many genes (Kullander & Rausing; 1975; Flörke-Gerloff *et al.*, 1984; Dale *et al.*, 1994; Kilani *et al.*, 2004; Heindryckx *et al.*, 2005), the specific

mode of inheritance of which remains unclear, although mutations within the SPATA16 gene, and recurrent deletions within the DPY19L2 region on chromosome 12 in humans, have been implicated (Dam *et al.*, 2007; Harbuz *et al.*, 2011; Kosciński *et al.*, 2011). One further example is the CFTR gene, thought to be mutated in 60-90% of patients presenting with congenital bilateral absence of the vas deferens (CBAVD), a type of obstructive azoospermia (O'Flynn O'Brien *et al.*, 2010). While this may be considered a maternally inherited autosomal disorder, the problem in this case is a structural defect of the male reproductive system rather than an abnormality in the mechanisms of gametogenesis or fertilisation.

The current data suggest that studies investigating the role of genes involved in male infertility should involve not only the father of an infertile male, but also the mother, since it now appears that infertile males may inherit mutated genes from either parent. Indeed, Sazci *et al.* (2005) described a case in which a son inherited a translocation (t(1;13)(q24;q10)) from his mother, associated with primary infertility in the son, but not the mother. This translocation was associated with a normal phenotype, but was theorised to affect meiosis during spermatogenesis, leading to azoospermia. While causing infertility, this was a chromosomal structural defect, and not an autosomal point-mutation as is the case here. Considering that many germ cell-specific genes are also localised at autosomes (Sazci *et al.*, 2005), the findings of this Chapter suggest that male infertility may be more multi-factorial and complex in nature than previously thought.

It seems apparent that mutations in PLC ζ may contribute not only towards male infertility but male sub-fertility. Indeed, mini-sequencing of individual sperm from the patient indicated that either PLC ζ^{H398P} or PLC ζ^{H233L} was present at any given time. This is

supported by the fact that the patient's daughter inherited only PLC ζ^{H233L} . Since the patient's daughter has inherited PLC ζ^{H233L} , it is possible that if she has male progeny in future, they may also inherit the same mutation and thus possibly experience sub-fertility. However, further work is required to determine the exact role of heterozygous mutations of PLC ζ in human sub-fertility, which may perhaps be accomplished by a heterozygous knock-in mouse model.

The incidence of both PLC ζ^{H233L} and PLC ζ^{H398P} was determined to be less than 1 in 100 individuals (200 chromosomes) within a general population, and were not identified in a small group of patients diagnosed with OAD, suggesting that these particular mutations may be relatively rare, or even unique. It is worth noting however, that six of these patients suffered from globozoospermia, a condition linked to a suspected genetic syndrome which results in the formation of sperm with abnormal morphology and without acrosomes (Dam *et al.*, 2007). It is therefore important that future mutation studies should predominantly target males exhibiting oocyte activation failure, without globozoospermia, and exhibiting normal sperm morphology. Such studies should attempt to correlate genetic analysis to the specific pattern of PLC ζ localisation in the sperm head. Whilst Grasa *et al.* (2009) mapped the distribution of PLC ζ to acrosomal, equatorial, and post-acrosomal regions of fertile human sperm, it remains possible that structural changes of the PLC ζ protein, occurring as a result of genetic mechanisms, may result in abnormal expression or localisation patterns with associated functional deficit. Indeed, high-resolution immunogold electron microscopy studies have highlighted important roles for the acrosomal membrane and perinuclear acroplaxome in harbouring a large number of structural and functional proteins (Kierszenbaum *et al.*, 2011), mutations of which may lead to defective sperm function and male infertility.

4.4.3 Mutations in PLC ζ may function in a recessive manner

It is interesting to note that while the patient's father was heterozygous for PLC ζ^{H398P} , the patient was conceived naturally without the aid of ART. Since PLC ζ^{H398P} and its mouse equivalent have been demonstrated to abolish the Ca²⁺ inducing ability of PLC ζ (Heytens *et al.*, 2009; Nomikos *et al.*, 2011), the natural conception of the patient demonstrates that loss-of-activity mutations in PLC ζ need to be present on both maternal and paternal alleles for complete infertility to occur. While speculative at this time, the most likely scenario by which sperm from the patient's father was able to fertilise oocytes from the mother successfully was if the fertilising sperm contained normal PLC ζ protein in addition to PLC ζ^{H398P} DNA.

While the first point of PLC ζ translation during human spermatogenesis has yet to be precisely identified, studies indicate that PLC ζ is first detectable at the round spermatid stage in porcine (Yondea *et al.*, 2006), hamster (Young *et al.*, 2009), and equine (Bedford-Guaus *et al.*, 2011) models. Since there is a possible interchange of mRNA transcripts via cytoplasmic bridges in developing spermatids and spermatocytes (Dadoune *et al.*, 2004; Heytens *et al.*, 2009), this could have led to the presence of functional wild type PLC ζ protein within spermatids and spermatocytes containing mutant PLC ζ (Figure 9; 10). Numerous studies indicate that the majority of protein transcription and translation occurs within the meiotic stages of spermatogenesis, and levels start to decline upon passage into the spermiogenesis stage (Eddy, 2002; Dadoune *et al.*, 2004; Bettegowda & Wilkinson, 2010). It is therefore a possibility that PLC ζ transcription occurs throughout the meiotic phase of spermatogenesis, potentially resulting in mixed populations of PLC ζ transcripts derived from both maternal and paternal alleles (Figure 10; 11).

It is possible that two mechanisms may be occurring. First, both PLC ζ^{H233L} and PLC ζ^{H398P} DNA and protein may be divided amongst the sperm population assuming that human PLC ζ is first transcribed at the spermatid stage. PLC ζ protein will be transcribed according to the chromatid present in the spermatid, and it is possible that transcripts may be swapped between spermatids via cytoplasmic bridges. Another possibility is that PLC ζ^{H233L} and PLC ζ^{H398P} DNA and protein may be divided amongst the sperm population assuming that human PLC ζ is transcribed throughout the meiotic phases of spermatogenesis, resulting in sperm with mixed populations of PLC ζ protein, where normal PLC ζ protein may override the deleterious effects of mutated PLC ζ , thereby rescuing the oocyte activating ability of the affected sperm. However, both these theories are speculation, and further, more in-depth experimental evidence is required to corroborate them.

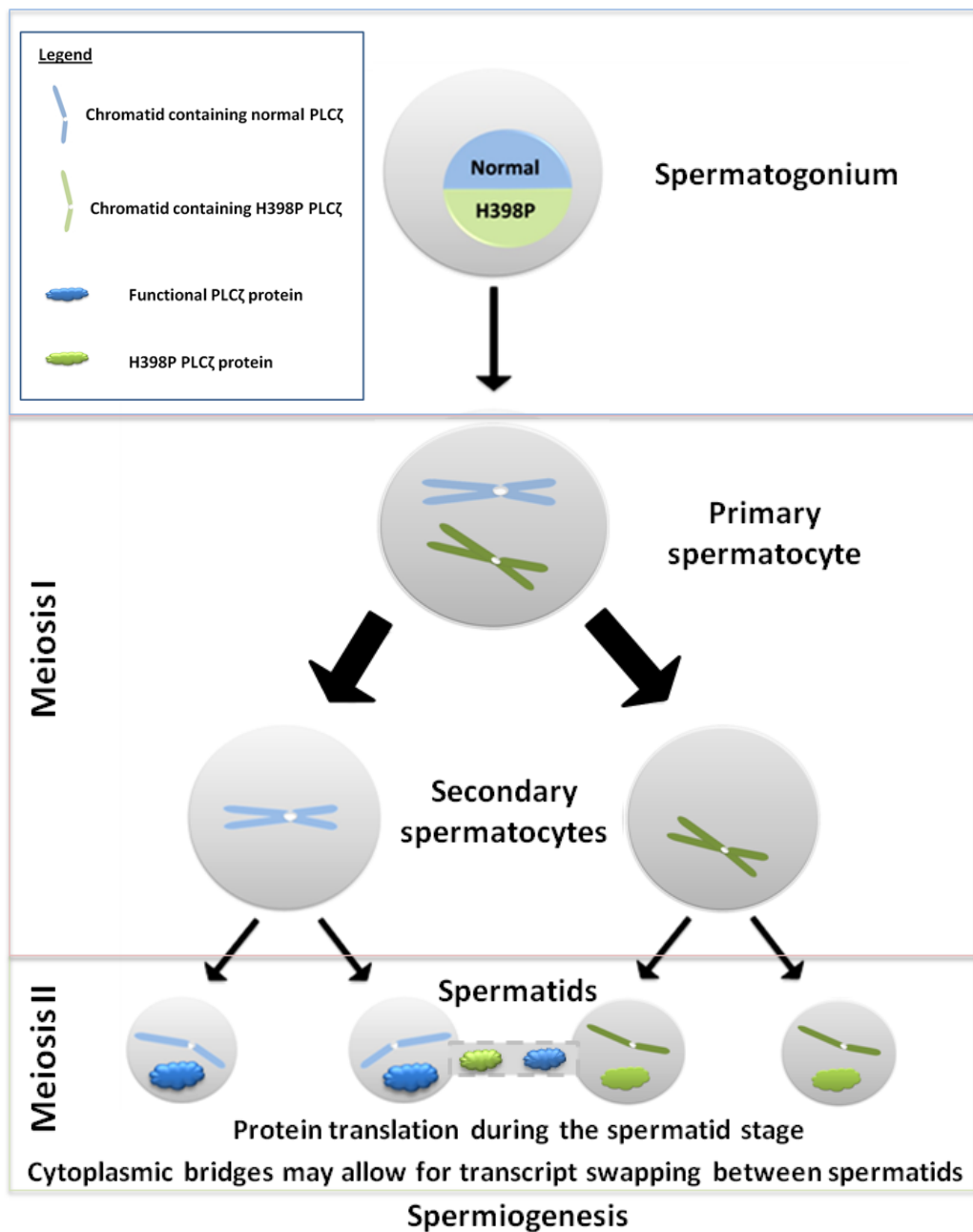


Figure 4.11: Schematic illustrating a possible model of spermatogenesis in the father of patient^{H398P}, indicating how both PLC ζ^{H233L} and PLC ζ^{H398P} DNA and protein may be divided amongst the sperm population assuming that human PLC ζ is first transcribed at the spermatid stage. PLC ζ protein will be transcribed according to the chromatid present in the spermatid, and it is possible that transcripts may be swapped between spermatids via cytoplasmic bridges. Schematic produced by JK.

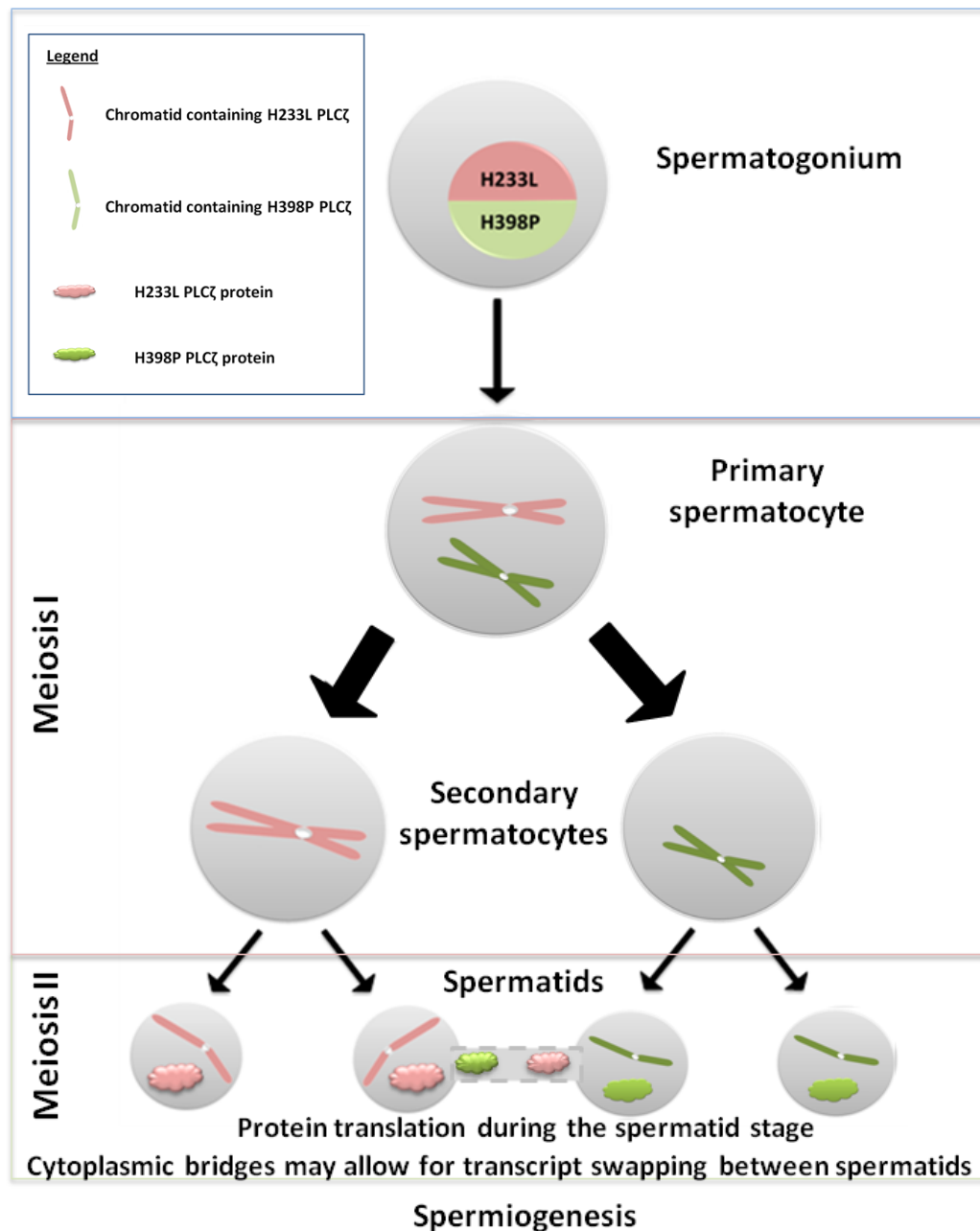


Figure 4.12: Schematic illustrating a possible model of spermatogenesis in patient^{H398P}, indicating how both PLC ζ ^{H233L} and PLC ζ ^{H398P} DNA and protein may be divided amongst the sperm population assuming that human PLC ζ is first transcribed at the spermatid stage. PLC ζ protein will be transcribed according to the chromatid present in the spermatid, and it is possible that transcripts may be swapped between spermatids via cytoplasmic bridges. Schematic produced by JK.

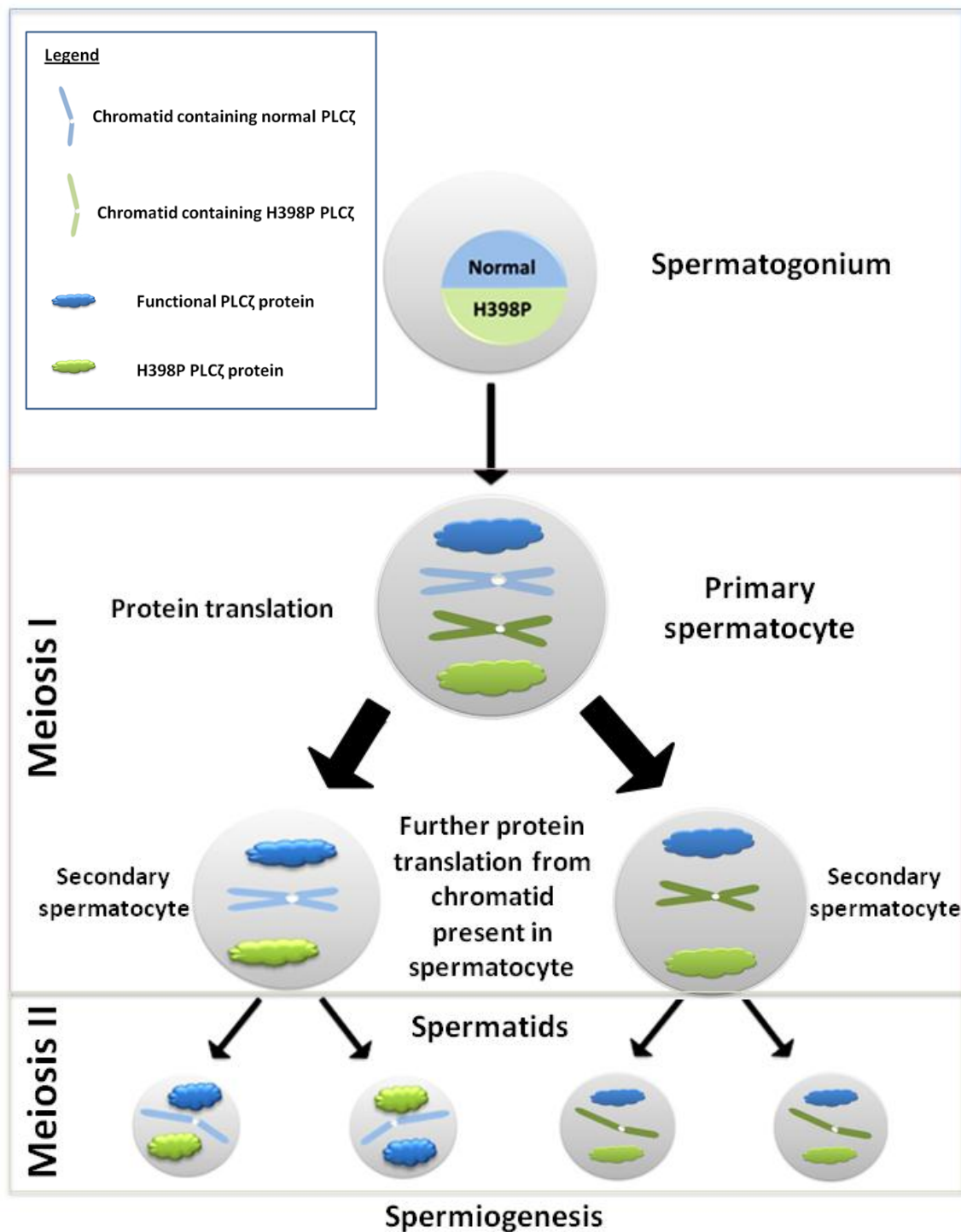


Figure 4.13: Schematic showing a possible model of spermatogenesis in the father of patient^{H398P}, indicating how both PLC ζ^{H233L} and PLC ζ^{H398P} DNA and protein may be divided amongst the sperm population assuming that human PLC ζ is transcribed throughout the meiotic phases of spermatogenesis, resulting in sperm with mixed populations of PLC ζ protein, where normal PLC ζ protein may override the deleterious effects of mutated PLC ζ , thereby rescuing the oocyte activating ability of the affected sperm. Schematic produced by JK.

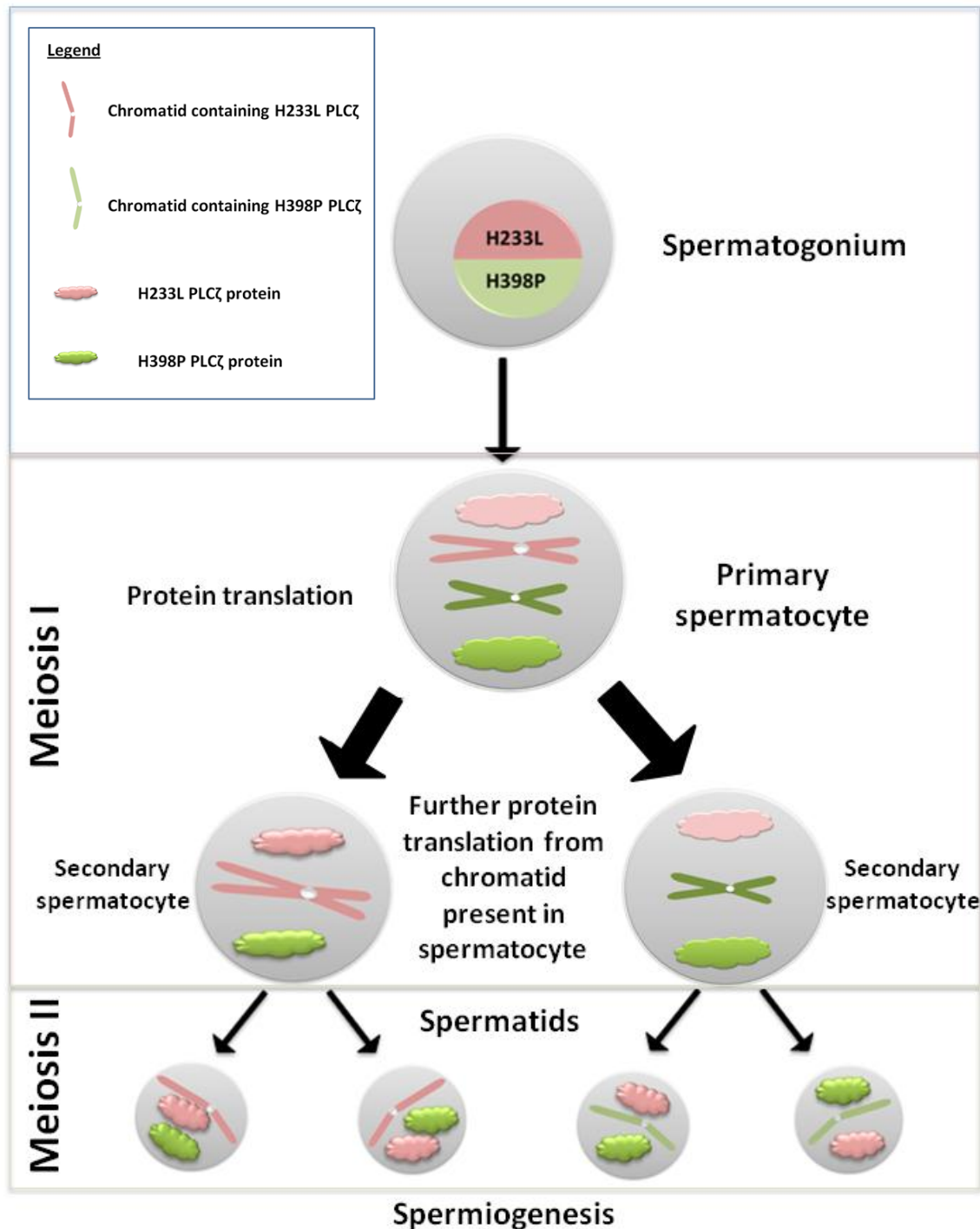
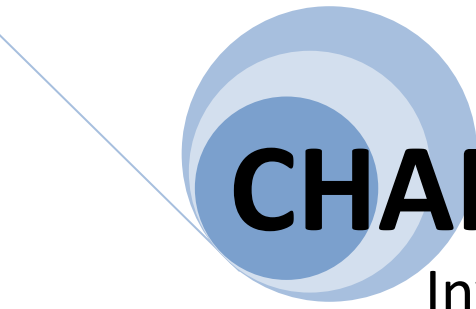


Figure 4.14: Schematics showing a possible model of spermatogenesis in patient^{H398P}, indicating how both PLCζ^{H233L} and PLCζ^{H398P} DNA and protein may be divided amongst the sperm population assuming that human PLCζ is transcribed throughout the meiotic phases of spermatogenesis, resulting in sperm with mixed populations of PLCζ protein, where normal PLCζ protein may override the deleterious effects of mutated PLCζ, thereby rescuing the oocyte activating ability of the affected sperm. Schematic produced by JK.

4.5 Conclusions and future work

In summary, a novel second mutation ($\text{PLC}\zeta^{\text{H233L}}$) of the oocyte activation factor $\text{PLC}\zeta$ was identified in an infertile patient, which severely reduced PLC activity. Both $\text{PLC}\zeta^{\text{H233L}}$ and $\text{PLC}\zeta^{\text{H398P}}$ mutations may have a low degree of occurrence within the general population, although study of a much larger population size is required before this can be said with certainty. It is possible that such mutations function in a recessive manner. The findings of this Chapter demonstrate for the first time, that the maternal inheritance of autosomal point mutations in male-specific proteins may result in certain forms of male factor infertility, highlighting the importance of studying maternal inheritance in male infertility.

It would be intriguing to investigate whether developing sperm exhibit bias towards a particular allele of $\text{PLC}\zeta$. It may also be of interest to examine whether there are any intrinsic mechanisms in place to prevent affected alleles from being translated, similar to mechanisms such as nonsense mediated mRNA decay (NMD), a eukaryotic mechanism of ‘quality-control’ which selectively degrades mRNAs harbouring premature termination codons (Chang *et al.*, 2007). It is also important that mutation studies predominantly target males exhibiting oocyte activation failure, without globozoospermia, and exhibiting normal sperm morphology. Such studies should attempt to correlate genetic analysis to the specific patterns of $\text{PLC}\zeta$ localisation in the sperm head, as was attempted in Chapter 3 of this thesis. Collectively, the findings presented in the current Chapter not only further support the physiological importance of $\text{PLC}\zeta$ in the oocyte activation process, but represent a considerable advancement in the understanding of the modes of inheritance of male infertility, and shed significant new light as to how such conditions may arise.



CHAPTER 5

Investigation of the potential clinician-induced (iatrogenic) damage upon levels of PLC ζ in human sperm by clinical ART protocols

5.1 Introduction

Currently, assisted reproductive technology (ART) protocols facilitate the treatment of most forms of infertility, and ART now accounts for 7% of all births in some developed countries (Nasr-Esfahani *et al.*, 2009). Worldwide, approximately 1 million ART treatments are performed each year and over eight million ART babies have been born worldwide (ICMART, 2009). Although ART plays a critical role in reducing infertility, there are still groups of infertile couples for which ART has not yet proven successful. Furthermore, whilst advances in ART have revolutionised infertility treatment, success rates vary, with pregnancy and delivery rates for *in vitro* fertilisation (IVF) and intracytoplasmic sperm injection (ICSI) rarely exceeding 30% and 23% respectively (ICMART 2009; de Mouzon *et al.*, 2010). Consequently, it is critical that ART protocols are constantly modified in line with scientific advances, so that clinics can consistently provide patients with the best chances of conception.

While it is well characterised that techniques such as cryopreservation can detrimentally affect sperm parameters such as viability, motility and morphology (Ozkavukcu *et al.*, 2008), Borges *et al.* (2007) showed that fertilisation rates following ICSI were higher using fresh sperm (73.8%) compared to cryopreserved sperm (68.7%), and that sperm from patients diagnosed with asthenozoospermia (reduced sperm motility) or oligoasthenozoospermia (reduced sperm count and motility), may be more susceptible to freeze/thaw damage, resulting in lower fertilisation rates. Cryopreservation is thought to generate sperm DNA fragmentation not only in fertile sperm, but exacerbate such conditions in sperm from patients seeking fertility treatment (de Paula *et al.*, 2006; Thomson *et al.*, 2009a; 2009b; Zribi *et al.*, 2010) through the increased presence of

reactive oxygen species and oxidative stress (Thomson *et al.*, 2009b). While sperm DNA fragmentation has been shown to have a negative effect upon the chances of conception and achieving pregnancy either naturally or through ART (Borini *et al.*, 2006; Benchaib *et al.*, 2007; Thomson *et al.*, 2009b), the possible consequences of a pregnancy achieved using sperm exhibiting DNA damage remains of great concern, which may result in defective embryo quality (Lewis & Aitken, 2005; Niu *et al.*, 2011).

Another important technique used in fertility treatment is density gradient washing (DGW). Following the development of classical swim-up methods, improved techniques such as DGW have been developed to improve the quality of sperm recovered for ART. Indeed, sperm quality has been shown to be superior following density gradient compared to swim-up processing (Allamaneni *et al.*, 2005; Niu *et al.*, 2011). However, several studies indicate that sperm separation methods may also isolate sperm with damaged or ‘nicked’ DNA and poorly condensed chromatin, however this will require further studies due to conflicting results (Gandini *et al.*, 2004; Bungum *et al.*, 2008; Chiamchanya *et al.*, 2010; Matsuura *et al.*, 2010; Niu *et al.*, 2011). Indeed, recent studies indicate that DGW and swim-up sperm isolation techniques may play a role in selecting sperm without DNA damage, and are able to select non-fragmented sperm even following cryopreservation (Gosálvez *et al.*, 2010; Enciso *et al.*, 2011).

Pérez-Llano *et al.* (2010) demonstrated that incubation of sperm at varying temperatures and lengths of time may also play a role in affecting porcine semen quality adversely, with incubation at 37°C appearing to result in an increased level of DNA fragmentation within sperm, indicating that routine clinical techniques such as overnight incubation of seminal fluid to test for infection may adversely affect seminal quality and

clinical outcome. Further to this, considering that the major cause behind DNA fragmentation is thought to be oxidative stress induced by an increased level of reactive oxygen species (ROS) which may occur through environmental changes (Agarwal & Allamaneni, 2004; Agarwal *et al.*, 2006), routine handling of gametes even within a stringently climate controlled environment as the ART laboratory, may impair gamete and embryo quality.

Whilst cryopreservation can reduce levels of some sperm proteins such as glutathione (Gadea *et al.*, 2010) in human sperm, little is known of the potential effects upon the oocyte activation factor, phospholipase C zeta (PLC ζ), which following release into the oocyte upon gamete fusion, induces oscillations in intracellular oocyte calcium (Ca²⁺), allowing meiotic resumption and exit; a process termed oocyte activation (Dale *et al.*, 2010; Kashir *et al.*, 2010). Immunofluorescence and immunoblot analysis have recently revealed that infertile, ICSI failed, sperm from patients were unable to elicit Ca²⁺ oscillations upon mouse oocyte microinjection, and exhibited absent/reduced levels of PLC ζ (Yoon *et al.*, 2008; Heytens *et al.*, 2009; Taylor *et al.*, 2010). While Heytens *et al.* (2009) observed reduced levels of PLC ζ in human sperm by immunoblot analysis following cryopreservation; the significance of this preliminary finding was not explored further. This Chapter aimed to use immunofluorescent analysis to investigate how cryopreservation and DGW affect PLC ζ levels in normal human sperm to investigate the potential effects of such techniques on levels of PLC ζ in human sperm to ascertain if this may be a contributory reason behind the relatively low success rates of ART following the use of these techniques.

5.2 Materials and methods

5.2.1 Fertile control semen samples

Semen samples were donated by healthy volunteers, who exhibited no previous history of infertility, with informed written consent and ethical approval as previously described (Chapter 2, Section 2.1). All samples were obtained and analysed independently from other samples used in this thesis. All ejaculates ($n = 7$) exhibited normal semen parameters (WHO, 2010; Chapter 2, Table 2.1).

5.2.2 Sperm density gradient washing

Sperm from fertile controls were subject to density gradient washing as previously described (Chapter 2, Section 2.14), and were divided into three fractions: unprocessed ('raw'); density gradient washed ('DGW'); and a fraction in which raw sperm were frozen, thawed and subjected to DGW ('PT-DGW').

5.2.3 Cryopreservation

Freeze/thaw fractions were treated with standard clinical protocols. 'SpermFreeze' (700 μ l; FertilPro, Belgium) was added drop-wise to 1ml of raw sperm whilst swirling. The cryoprotectant was allowed to equilibrate to RT for a minimum of 1 hour before use. Once the cryoprotectant was fully incorporated, the mixture was left at RT for 10 minutes and aspirated into labelled freezing straws (Cryo Bio System, France). Straws were subsequently sealed and suspended vertically in a holding tank containing liquid nitrogen in the vapour phase for 30 minutes. Straws were then plunged directly into the liquid. Samples were thawed by incubating straws at RT for 15 minutes, and subjected to DGW as before, but with only 40% PureSperm, to yield a post-thaw DGW ('PT-DGW') fraction.

5.2.4 Sperm PLC ζ immunofluorescence, microscopy, and fluorescence quantification

Sperm samples from all individuals were prepared for immunofluorescence and analysed using ImageJ software (NIH, USA) as described previously (Chapter 2, Sections 2.15, and 2.19). Fluorescence microscopy was carried out using a Leica DM IRE2 fluorescent microscope (x20 and x40 objectives) using the fluorescence filters mentioned previously (Chapter 2, Section 2.15), and images captured using SimplePCI software (Hamamatsu, UK).

5.2.5 Statistical analysis

Correlations between the proportion of raw and DGW sperm in which PLC ζ could be detected were determined for donors 2 - 7 ($n = 6$) but not donor 1 owing to insufficient ejaculate volume. Proportions (%) were arcsine transformed prior to statistical analysis. Correlations between the relative fluorescent intensity of total PLC ζ for DGW and PT-DGW fractions for all donors ($n = 7$) were determined using the Student's t -test (Graph-Pad InStat, San Diego, USA).

5.3 Results

5.3.1 Proportional analysis of PLC ζ immunofluorescence in fertile sperm following DGW

DGW resulted in a significant elevation ($p < 0.0001$, $n = 6$ donors) in the proportion of sperm in which immunoreactive PLC ζ patterns (Figure 5.1) could be detected (representing elevations of 26 – 81%) compared to raw fractions, indicating that raw samples contain a larger proportion of sperm devoid of immunoreactive PLC ζ (Figure 5.2).

5.3.2 Total PLC ζ immunofluorescence following cryopreservation

The proportions of PT-DGW sperm exhibiting PLC ζ immunoreactivity were similar to fresh DGW sperm, and the proportions of sperm exhibiting PLC ζ immunoreactivity were higher in the DGW and PT-DGW fractions compared to raw sperm. The immunofluorescence observed in the PT-DGW fractions were markedly lower compared to the DGW fractions (Figure 5.3), and the decrease in levels of total PLC ζ immunofluorescence observed varied between each individual donor. A highly significant reduction ($p < 0.0001$) in PLC ζ immunofluorescence was observed in sperm following cryopreservation compared to fresh DGW fractions. Levels were reduced by 20 - 56% in four out of seven fertile donors, two donors exhibited increased levels of immunofluorescence following cryopreservation ($p < 0.0001$) and one donor exhibited no change ($p \geq 0.05$) (Figure 5.4), suggesting a variable effect of cryopreservation over multiple semen samples.

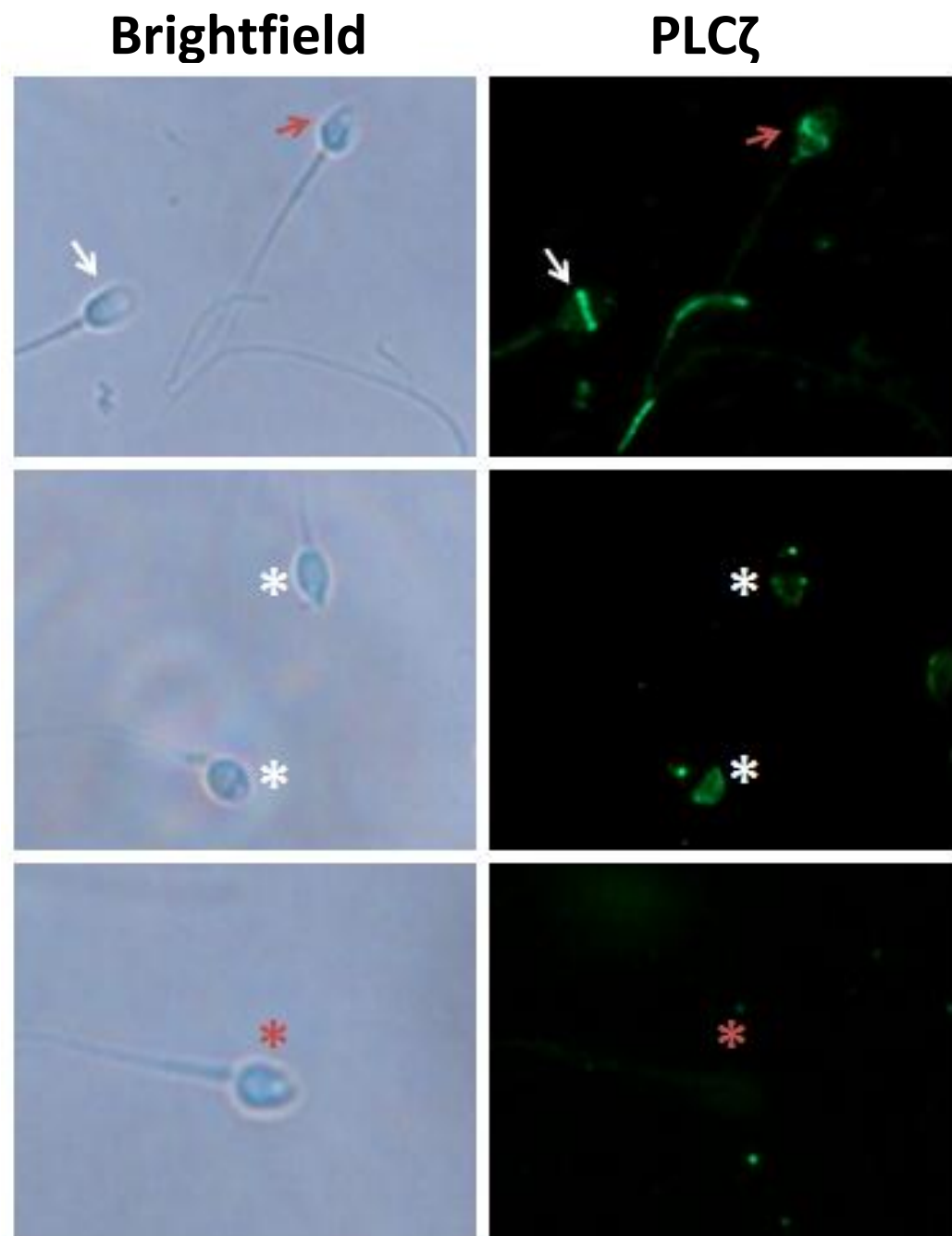


Figure 5.1: Representative bright-field (left panel) and corresponding PLC ζ immunofluorescence (right panel) of human sperm showing characteristic localisation patterns previously identified by Grasa *et al.* (2008). White arrows indicate equatorial localisation; red arrows indicate equatorial + post-acrosomal localisation; white asterisk (*) indicates equatorial + acrosomal localisation). Bottom panel shows a representative image of raw sperm not exhibiting PLC ζ immunofluorescence (red asterisk*). Images were captured at 40x magnification and enlarged digitally.

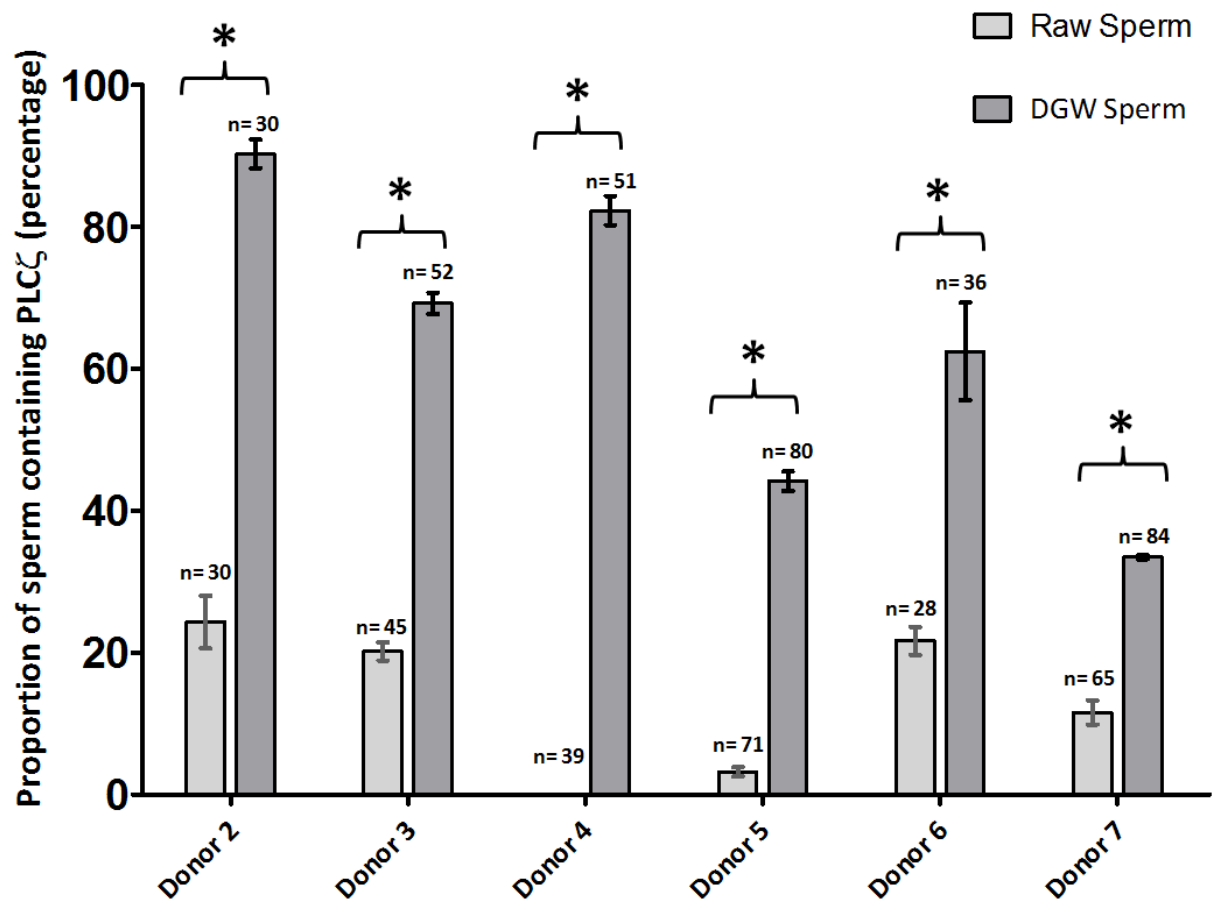


Figure 5.2: Relative proportions of sperm exhibiting PLC ζ fluorescence in raw and DGW fractions for donors 2 - 6. Asterisk (*) denotes statistically significant values ($p < 0.0001$) as determined by the students *t*-test. 'n' represents the number of sperm analysed for each donor for both fractions. Data were arcsine transformed prior to analysis and represent mean $\pm$ SEM.

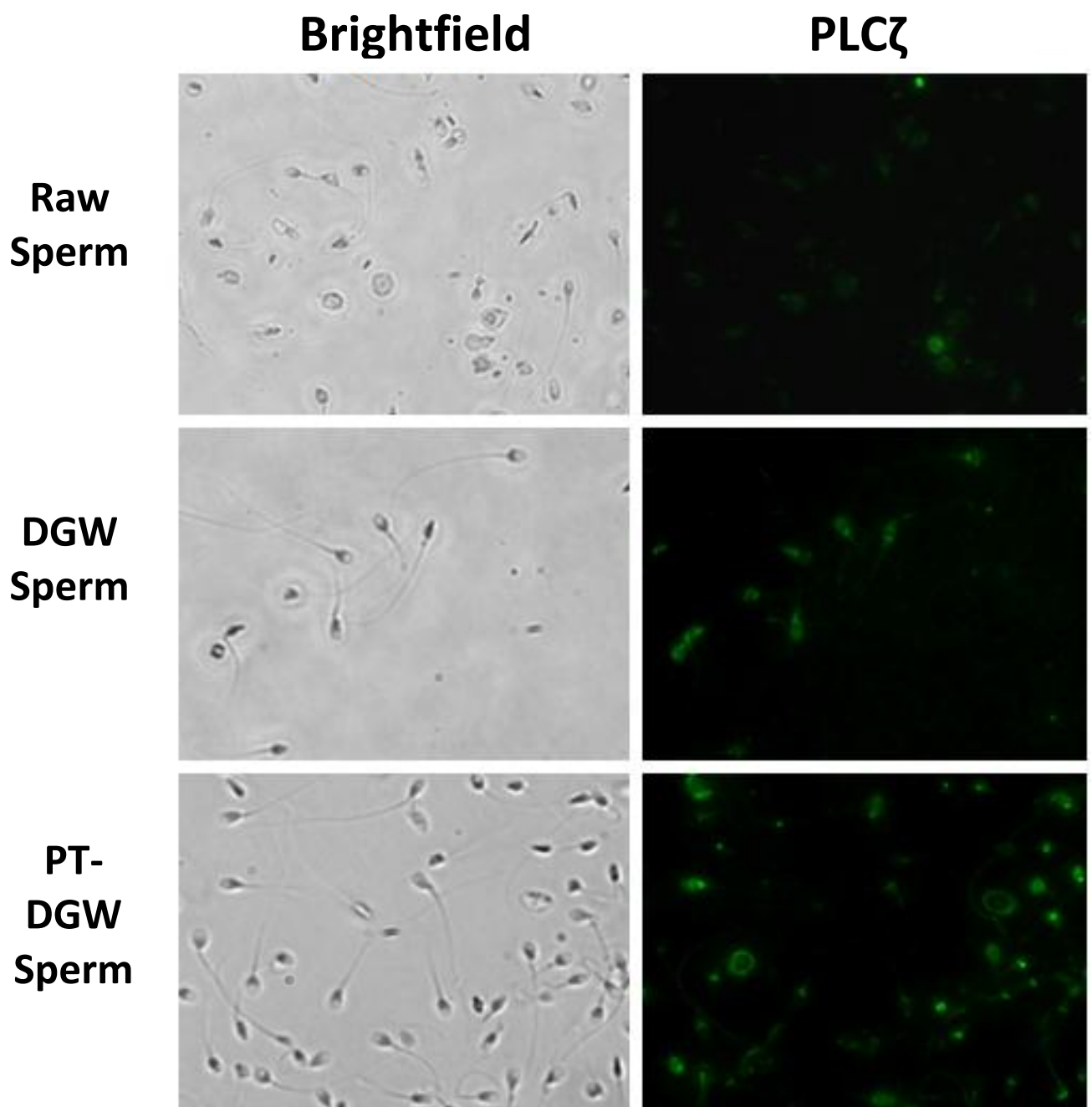


Figure 5.3: Representative bright-field (left panel) and corresponding PLC ζ immunofluorescence (right panel) of human sperm for all three sperm fractions (raw, DGW, and PT-DGW fractions), showing PLC ζ levels and localisation patterns. Images were captured at 40x magnification and enlarged digitally. Raw: unprocessed sperm; DGW: density gradient washed sperm; PT-DGW: frozen/thawed sperm.

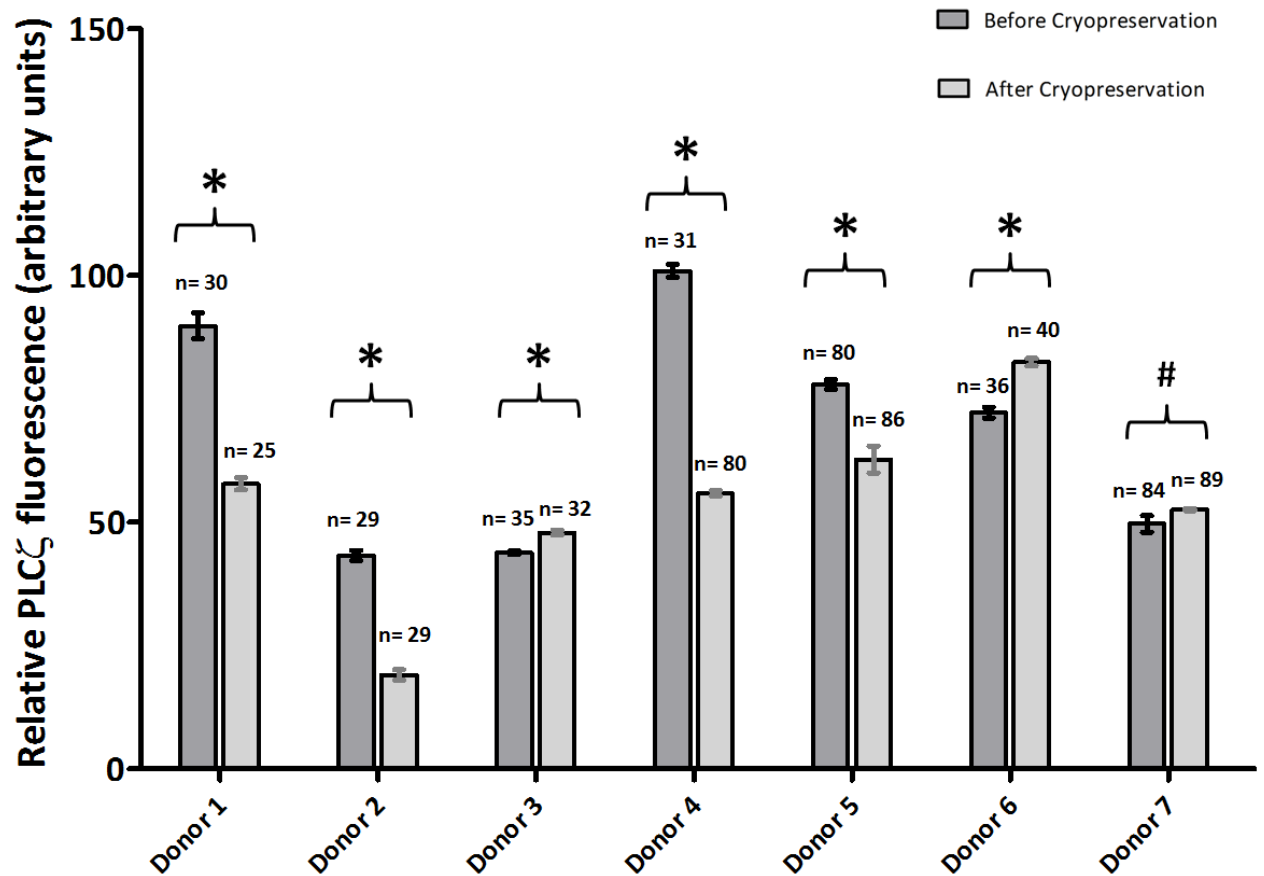


Figure 5.4: Relative PLC ζ fluorescence in sperm from donors 1 - 7 before and after cryopreservation. Fluorescent intensity was quantified in arbitrary units using ImageJ. Asterisk (*) denotes statistically significant differences ($p < 0.0001$) as determined by the students *t*-test. Hash (#) denotes values not statistically different ($p \geq 0.05$). 'n' represents the number of sperm analysed for each donor for both fractions. Data represents mean $\pm$ SEM.

5.4 Discussion

The rapid growth in ART in recent years has led to the development of a range of sperm preparation methods such as density gradient washing and cryopreservation, which have contributed towards the great success of ART, which now accounts for 7% of all births in some developed countries (Nasr-Esfahani *et al.*, 2009), and 1.4% of all births in the UK (HFEA register, 2007). However, ART has yet to prove successful for many infertile couples, with success rates requiring much need of improvement, ranging from success rates (defined as live births per cycle started) of 30% and 23% for IVF and ICSI respectively (ICMART 2009; de Mouzon *et al.*, 2010). It is, therefore, necessary that efforts are made to improve the efficacy of ART protocols in order to provide infertile couples with the best chances of conception. To this end, the effects of treatments such as DGW and cryopreservation upon vital sperm parameters such as motility, viability, structural integrity and DNA damage, are subject to increasing scrutiny (Ozkavukcu *et al.*, 2008; Zribi *et al.*, 2010). Here, the effects of such treatments on levels of the oocyte activation factor PLC ζ in human sperm were investigated.

5.4.1 DGW increases the proportion of sperm exhibiting PLC ζ immunofluorescence

The current analyses demonstrate that DGW resulted in a significant elevation in the proportion of sperm in which immunoreactive PLC ζ patterns could be identified, indicating that raw samples contained a higher proportion of sperm devoid of immunoreactive PLC ζ . This may be due to the removal of senescent and dead sperm from this fraction, along with particulate debris in the DGW fraction. Since DGW selects the best quality sperm for ART, this proportional increase in PLC ζ , which appears to vary between individuals, indirectly confirms the importance of PLC ζ at fertilisation, and

suggests that PLC ζ levels in sperm may represent a useful biomarker of fertility. However, while all donor sperm exhibited an increase in proportions of sperm exhibiting PLC ζ immunofluorescence, the amount of the increase varied between individuals, which may reflect the overall quality of the semen samples provided by the donors. Intriguingly, the results presented in this chapter provide support to those observed in Chapter 3, which indicated that examining the proportion of sperm which exhibited PLC ζ immunofluorescence may be a valid method of determining sperm fertility. Indeed, all donors included in this study had no previous history of infertility, and all exhibited a significantly large proportion (40 - 95%) of sperm exhibiting PLC ζ immunofluorescence following DGW, indicating that examining proportions of sperm exhibiting PLC ζ immunoreactivity may be a viable diagnostic for sperm oocyte activation capability. It should be noted that the analyses described was not able to examine immunofluorescence for other markers (such as β -actin or nuclear proteins) within donor sperm owing to ethical restrictions. It would be necessary for future studies to take this into account, to examine whether the methodology utilised remains consistent for other markers, and whether similar variability is observed per sperm.

5.4.2 Cryopreservation reduces levels of total PLC ζ immunofluorescence in human sperm

The mechanisms underlying the detrimental effects of cryopreservation upon sperm may be multifactorial, involving excessive reactive oxygen species (ROS) production during the freeze/thaw process, leading to subsequent changes in membrane function and structure (Gadea *et al.*, 2010), possibly resulting in disruption of PLC ζ localisation or leakage from the sperm head. Indeed, Nakai *et al.* (2011), who observed a similar decrease

in levels of PLC ζ in boar sperm following cryopreservation, indicated that the freeze/thaw process may have damaged the sperm head, resulting in protein leakage to the surrounding media. It is again intriguing to note that the effect of cryopreservation on sperm differed between different donors. While the majority of donors exhibited a significant decrease, one donor exhibited no significant change, while, for reasons not known, two donors exhibited an increase in levels of PLC ζ immunofluorescence.

It is worrying to observe that cryopreservation appears to have a detrimental effect upon levels of PLC ζ immunofluorescence in human sperm, indicating that cryopreservation may reduce the fertility of sperm which may have been more fertile before treatment. Indeed, multiple studies (Yoon *et al.*, 2008; Heytens *et al.*, 2009; Taylor *et al.*, 2010) have implicated absent/reduced levels of PLC ζ in human sperm to recurrent failure to activate oocytes, even following ICSI. However, a reduced level of PLC ζ immunofluorescence may not necessarily mean that the fertility of the sperm sample in question has been reduced. Indeed, the findings of Chapter 3 of this thesis indicate that levels of PLC ζ immunofluorescence in human sperm may not be a viable indicator of sperm fertility due to the large variability present between patient and control samples.

It is of great concern though, to note that the amplitude and frequency of Ca²⁺ oscillations at oocyte activation, dependant on levels of PLC ζ , are directly implicated in cell cycle progression and early embryo gene expression profiles (Ducibella *et al.*, 2002). It is, therefore, possible that the variability of levels of PLC ζ naturally present in sperm from different males may play a role in ensuring genetic/epigenetic diversity between individuals. Worryingly however, cryopreservation may be reducing levels of PLC ζ within human sperm, which while remaining fertile, may lead to an iatrogenic modification of

genetic and epigenetic diversity within populations of infertile couples whose treatment includes cryopreserved sperm. Of further concern is that there have not yet been any investigations with regards to the effects of oxidative stress on levels of PLC ζ within sperm.

5.4.3 Iatrogenic effects of novel ART techniques on gametes and markers of gamete function

This Chapter shows, for the first time, that routine clinical techniques such as cryopreservation and DGW may differentially affect levels of PLC ζ within human sperm. While the effects of such techniques on oocytes and embryos are relatively well classified, very little is known with regards to the effects of such techniques on other markers of sperm function. Potential markers include factors involved in sperm/oocyte fusion, such as the ADAM (A Disintegrin And Metalloprotease) family of integral membrane proteins, or characteristic proteins such as *Izumo*, a testis-specific protein, only detectable on acrosome-reacted sperm and when knocked out, renders male mice sterile, despite otherwise normal mating behaviour (Rubinstein *et al.*, 2006). Other markers include proteins involved in capacitation (a series of physiological, biochemical, and biophysical modifications that occur within the female reproductive tract and bestow fertility; Yoshida *et al.*, 2008), such as soluble adenylate cyclase (sAC). Moreover, comparison of oxygen consumption of frozen/thawed bull sperm indicated that sperm with higher levels of ATP and ZO₂ were more fertile. However, further investigation is required to establish the reasons behind such elevated production (Garrett *et al.*, 2008).

Additionally, similar markers of gamete function have been proposed for oocytes. Indeed, higher levels of activin A have been associated with increased oocyte quality (Lau *et al.*, 1999), while higher levels of GDF-9, an oocyte secreted factor, is associated with improved oocyte quality in culture (Yeo *et al.*, 2008; Gilchrist *et al.*, 2008). Analysis of aneuploid embryos demonstrated an altered amino acid turnover *in vitro*, in comparison to genetically normal embryos, indicating a potential association between amino acid turnover and chromosome aneuploidy *in vitro* (Picton *et al.*, 2010). Furthermore, modification of oocyte-secreted glycoproteins by oocyte-specific deletion of core 1-derived O-glycans increases litter size in mice, thought to be mediated by increased follicle survival (Williams & Stanley, 2008). While it is unknown which oocyte-derived glycoprotein(s) is responsible for this increase, such oocyte generated glycoprotein(s), along with analysis of secreted oocyte factors such as amino acids, may represent a marker for enhanced oocyte quality (Sturmey *et al.*, 2008).

However, it has not yet been established whether factors such as PLC ζ within the sperm play a role with regards to respiratory, or amino acid turnover rates of the developing zygote/embryo. Indeed, the microfilament-dependant redistribution of mitochondria during oocyte maturation is thought to correlate to bursts of ATP production (Yu *et al.*, 2010), while Ca²⁺ oscillations have been directly linked to rhythmic actomyosin-driven contractions induced by mouse gamete fusion (Ajduk *et al.*, 2011), indicating a possible involvement of the respiratory status of the zygote at the initial stages of fertilisation. It would be important for future studies to investigate potential links between routinely used ART techniques on markers of sperm fertility, and potential correlations with oxidative stress.

Several further sperm selection methods have been developed with the objective of improving sperm preparation protocols used during ART, aiming to isolate mature, structurally intact and non-apoptotic spermatozoa with high DNA integrity (Said & Land, 2011). One group of new selection techniques separates sperm based on size and electronegative charge. Considered a relatively rapid method of sperm selection (Ainsworth *et al.*, 2005; Said & Land, 2011), and not involving centrifugation, such methods may avoid the generation ROS thought to be associated with this procedure (Aitken & Clarkson, 1988; Said & Land, 2011). Another sperm preparation technique, based on the externalisation of phosphatidylserine (PS) to the outer surface of the sperm membrane, a feature of early apoptosis, has been proposed as a basis for selection of non-apoptotic spermatozoa (Said & Land, 2011). While still not as efficient as routine techniques such as DGW or swim up in selecting a large proportion of viable sperm, combination of DGW and such techniques resulted in the selection of sperm with a 50% reduction in DNA fragmentation rates (Donnelly *et al.*, 2000), and significantly higher rates of survival following cryopreservation-thawing compared to samples prepared by DGW alone (Said *et al.*, 2005).

Morphological selection techniques have also been proposed based on sperm ultramorphology. While it is debatable whether morphological evaluation of randomly stained cells from an ejaculate at $\times 1000$ magnification is of value during ICSI where the sperm is selected unstained at $\times 400$ magnification (Bartoov *et al.*, 2002), a sperm selection method has been developed based on the inclusion of normal sperm only assessed using real-time motile sperm organelle morphology examination (MSOME) at a magnification of $\times 6300$ (Bartoov *et al.*, 2002; Said & Land, 2011). Assessing the sperm acrosome, post-acrosomal lamina, neck, tail and mitochondria and nuclear shape and chromatin content,

MSOME has been utilised in a modification of ICSI termed intracytoplasmic morphologically selected sperm injection (IMSI; Bartoov *et al.*, 2003). While such an approach particularly benefits situations where identification of specific sperm organelles is required, such as the acrosomal components in cases of globozoospermia (Check *et al.*, 2007), debate rages with regards to the clinical viability of MSOME followed by IMSI, considering that it is an elaborate, highly skilled procedure that involves prolonged sperm manipulation, adding significantly to routine ICSI processing times.

While there are contradictory reports regarding whether ART outcomes may be improved by using such advanced sperm selection methods, it would be important for future studies to investigate the potential effects of these selection techniques on markers of sperm fertility such as PLC ζ , if such techniques are to be utilised confidently within a clinical setting. Additionally, further to advances in sperm selection, improvements in oocyte/embryo culture conditions are being sought (Fukui *et al.*, 1996; Cimetta *et al.*, 2009; Heo *et al.*, 2010; Swain & Smith, 2011). It would be essential that the potential iatrogenic damage of such techniques upon markers of zygote and embryo viability are investigated, along with their potential effect on proteins/factors essential for embryogenesis, of which currently little is known.

Towards this end, sensors have been developed to measure real-time changes in levels of oxidative stress within microfluidic devices (Amatore *et al.*, 2007), embryo oxygen consumption (O'Donovan *et al.*, 2006) or embryo secretions (Urbanski *et al.*, 2008). However, while the effects of such culture techniques have been exhaustively studied on oocyte and embryos, there is little work in relation to sperm function (Swain & Smith, 2011). It would be vital that the potential iatrogenic effects of such techniques be

examined on markers of sperm fertility, not only for routine clinical use, but to gain an increased understanding of the intricate mechanisms involved during gamete fusion. Indeed, the physical environment during assisted reproduction may affect gamete and resulting embryo quality. Examination of iatrogenic effects on gamete markers of viability may illuminate their *in vivo* physical requirements, leading to the improvement of current and novel ART procedures, subsequently improving patient chances of conception (Swain & Smith, 2011).

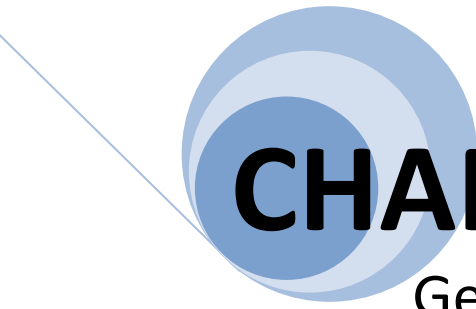
5.5 Conclusions and future work

In summary, this Chapter shows that DGW yields a processed sample with a significantly greater number of sperm exhibiting detectable PLC ζ protein than in the raw fractions. Furthermore, cryopreservation was also shown to affect levels of PLC ζ in sperm; an effect that may extend to other proteins involved in sperm function. Whilst this effect may vary between individuals following DGW, the majority of donors (57%) exhibited significantly reduced levels of PLC ζ following cryopreservation. Given that reduced levels of PLC ζ have been linked to male infertility, the data indicate that a more substantial study, involving a larger population of donors, is highly warranted. Furthermore the effects of cryopreservation on levels of PLC ζ in human sperm should also be confirmed utilising more techniques such as immunoblotting, and the functional effect of reduced levels of PLC ζ investigated using mouse oocyte microinjection of cryopreserved sperm and following the resulting Ca²⁺ oscillation profiles.

In particular, the findings of this Chapter indicate that a study of the effects of variable levels of PLC ζ in human sperm on genetic and epigenetic inheritance patterns is highly warranted to understand previously unknown iatrogenic affects of ART upon the patient population. Indeed, iatrogenic oxidative stress and DNA damage seem to be worryingly high within ART, and it would of great interest to correlate the prevalence of these conditions with levels of PLC ζ . In conclusion, however, this Chapter demonstrates that PLC ζ provides a powerful tool to investigate sperm quality in ART, which may contribute towards improving current clinical protocols and chances of conception. It also indicates that a similar approach could be used to measure the iatrogenic effect of ART on

other markers of sperm fertility to understand better the nature of the effects of ART upon gametes.

Considering that the effect of iatrogenic damage on sperm proteins is largely uninvestigated, it is important that the findings of this Chapter are extended to other markers of sperm fertility involved in structural integrity and sperm function. It is also necessary to apply findings of this study other routine clinical techniques within ART such as overnight incubation, *in vitro* maturation (IVM), or effects of culture media upon markers of gamete function and viability. Future studies should also investigate potential links between increasing oxidative stress and their effects on markers of sperm fertility.



CHAPTER 6

Generation of active recombinant human PLC ζ protein using bacterial and mammalian expression systems

6.1 Introduction

Oocyte activation is a fundamental step in fertilisation, involving cortical granule exocytosis, electrical modifications of the plasma membrane, and ultimately, the release of the oocyte from meiotic arrest (Tosti & Boni, 2004; Whitaker, 2006) in response to inositol 1,4,5-trisphosphate (IP₃)-mediated changes in intracellular calcium (Ca²⁺) (Stricker, 1999; Whitaker, 2006; Berridge, 2009). In mammals, this manifests as a series of characteristic Ca²⁺ oscillations beginning soon after gamete fusion and persisting beyond the completion of meiosis (Jones, 2005). Numerous studies suggest that the agent responsible for the induction of such intracellular Ca²⁺ oscillations, and subsequent oocyte activation, is a sperm-specific protein introduced into the oocyte upon gamete fusion; a novel phospholipase C (PLC), PLCzeta (PLCζ) (Swann *et al.*, 2006; Parrington *et al.*, 2007; Saunders *et al.*, 2007; Kashir *et al.*, 2010).

Injection of recombinant mouse PLCζ RNA and protein into mouse oocytes induced Ca²⁺ oscillations similar to those observed at fertilisation, triggering embryonic development to the blastocyst stage (Cox *et al.*, 2002; Saunders *et al.*, 2002; Kouchi *et al.*, 2005), while immuno-depletion of PLCζ from sperm extracts abolished their ability to elicit Ca²⁺ release (Saunders *et al.*, 2002). Sperm fractionation experiments indicated that the presence of PLCζ in sperm extracts correlates to Ca²⁺ oscillatory ability within the oocyte (Fujimoto *et al.*, 2004; Kurokawa *et al.*, 2005). In addition, RNA interference (RNAi) experiments produced transgenic mice with significantly reduced expression of PLCζ in the testis (Knott *et al.*, 2005); when injected into mouse oocytes, sperm derived from these animals induced severely abnormal Ca²⁺ oscillation profiles, which ended prematurely. Collectively, these studies provide significant support for PLCζ as the oocyte

activation factor. Furthermore, PLC ζ appears to play a similar role during fertilisation in a variety of non-mammalian species including chicken (Coward *et al.*, 2005), fish (Ito *et al.*, 2008; Coward *et al.*, 2011), and quail (Mizushima *et al.*, 2009), suggesting the existence of a universal mechanism of activation involving PLC ζ that is common to all vertebrates.

Considering the vital function of PLC ζ at oocyte activation, it is very likely that abnormal forms or aberrant function of PLC ζ may be the underlying cause of certain forms of human male factor infertility and oocyte activation failure. Recent studies have demonstrated that sperm from infertile men which consistently fail routine clinical procedures such as *in-vitro* fertilisation (IVF) and ICSI are unable to produce Ca²⁺ oscillations upon injection into mouse oocytes, or those which do are uncharacteristic of oscillations observed from fertile men, being reduced in both frequency and amplitude (Yoon *et al.*, 2008; Heytens *et al.*, 2009). In addition, Yoon *et al.* (2008) reported the absence of PLC ζ in both normal and round-headed sperm of a partial globozoospermic patient (mixed populations of morphologically normal and round-headed sperm).

Although it is difficult to compile accurate data for the incidence of global infertility, it is believed that infertility affects 70 million couples worldwide, the majority of whom reside in developing countries (Ombelet *et al.*, 2008). Current ART methods facilitate the treatment of most forms of infertility. For example, ICSI can rectify a number of male factor problems, such as oligospermia (low sperm count), transcending the need for normal sperm count/motility (Mangoli *et al.*, 2008; McVeigh *et al.*, 2008). While ICSI results in average fertilisation success rates of 70% (Heindryckx *et al.*, 2005), complete or virtually complete fertilisation failure still occurs in 1-5% of ICSI cycles (Flaherty *et al.*, 1998; Mahutte & Arici, 2003; Yanagida, 2008). The incorrect injection of sperm,

expulsion of the injected sperm from the oocyte, and the failure of sperm head decondensation are not considered to contribute substantially to fertilisation failure after ICSI (Yanagida, 2008). A deficiency in the mechanism of oocyte activation is regarded as the principal cause of fertilisation failure, or abnormally low fertilisation after ICSI, and can recur in several cycle attempts (Sousa & Tesarik, 1994; Heindryckx *et al.*, 2008), and can only be resolved using assisted oocyte activation (AOA).

Numerous reports have demonstrated that ICSI combined with AOA greatly increased the success rate of fertilisation and subsequent pregnancy (Rybouchkin *et al.*, 1997; Kim *et al.*, 2001; Tesarik *et al.*, 2002; Heindryckx *et al.*, 2005; 2008; Dirican *et al.*, 2008; Tejera *et al.*, 2008; Kyono *et al.*, 2009; Taylor *et al.*, 2009). However, several studies in animals have demonstrated that the number and amplitude of Ca^{2+} transients not only affects activation efficiency, but has a profound influence on subsequent embryonic development (Ducibella *et al.*, 2002; Ozil *et al.*, 2006; Kim *et al.*, 2011), blastocyst quality (Bos-Mikich *et al.*, 1997), the implantation potential of rabbit parthenogenotes (Ozil & Huneau, 2001) and mouse zygotes (Ozil *et al.*, 2006), and results in altered embryonic gene expression (Ozil *et al.*, 2006), due to which there is significant concern over the clinical use of such agents.

However, there are artificial stimuli which induce Ca^{2+} oscillations similar to those observed at normal fertilisation. The most efficient of these is strontium (Sr^{2+}), the use of which resulted in successful pregnancy of normozoospermic patients diagnosed with oocyte activation deficiency (Chen *et al.*, 2010). However, Sr^{2+} treatment is varied in terms of oocyte activation efficacy depending on the species in which it is used (Yanagida, 2008) with success rates being relatively low in humans (Chen *et al.*, 2005). Furthermore, little is

known as to the specific downstream effects of Sr^{2+} treatment on signalling molecules such as PKC and DAG, which is particularly worrying considering that Ca^{2+} signalling is also a feature of DAG action, which may have downstream effects on PKC. It would, therefore, be prudent to examine methods of AOA which elicit Ca^{2+} release in a manner as close as possible to the physiological pattern, to circumvent these concerns. An ideal example of this would be to use a purified recombinant version of human PLC ζ protein, the principle of which has been previously demonstrated by Yoon *et al.* (2008), who used mouse recombinant cRNA to ‘rescue’ the activating ability of ICSI failed patient sperm.

Indeed, Rogers *et al.* (2004) demonstrated that the microinjection of cRNA corresponding to recombinant human PLC ζ induced prolonged Ca^{2+} oscillations in aged human oocytes which had failed to fertilise during IVF or ICSI, and was able to induce parthenogenetic activation of these oocytes. Resultant embryos were able to proceed through the cleavage stages, and form blastocysts. Collectively, while these studies indeed signify the therapeutic potential of PLC ζ , problems such as uncontrollable expression and reverse transcriptase activity within oocytes (which potentially may convert introduced cRNAs into PLC ζ cDNA that might then become incorporated into the genome; Spadafora, 2004) pose significant obstacles for the therapeutic use of PLC ζ cRNA. It would, therefore, be necessary to obtain an active, purified version, of recombinant human PLC ζ protein. However, while active recombinant mouse PLC ζ protein has been purified and shown to elicit Ca^{2+} oscillations in a manner similar to sperm microinjection (Kouchi *et al.*, 2004; 2005; Nomikos *et al.*, 2005), no progress has been made regarding the generation of active human PLC ζ protein.

Thus, considering the documented links between PLC ζ and certain types of male factor infertility, the aim of this Chapter was to generate a purified form of recombinant human PLC ζ protein which retained PLC activity, and to investigate its viability as a more physiological therapeutic replacement for current AOA protocols that would override current concern surrounding such agents. Moreover, an increased understanding of the structure-function relationships underlying human PLC ζ protein, and its mechanism of action, may permit the development of an inhibitor(s) that could be utilised as a novel non-hormonal male contraceptive. Such research is vital if the therapeutic applicability of PLC ζ is to be appropriately investigated, and research translated from the laboratory into the clinic. Furthermore, such investigations may also provide a powerful tool with which to further current understandings of the molecular properties of the PLC ζ enzyme, as well as its biochemical mode of action.

6.2 Materials and Methods

6.2.1 DNA templates, vectors and constructs

All constructs used herein are shown in Table 6.1. PLC ζ^{WT} and PLC ζ^{H398P} were cloned into pTracer-EF/Bsd (Invitrogen, UK) and pcDNA 3.1 (Invitrogen, UK) to permit recombinant protein expression and purification. Cloning of constructs was performed as previously described (Chapter 2, Section 2.7). DNA sequences encoding PLC ζ^{WT} and PLC ζ^{H398P} were amplified from the original corresponding pGEX constructs (previously constructed in Dr. Parrington's laboratory and donated to the Coward laboratory) using PCR and the High Fidelity Master Kit (Roche Biosciences, UK) as described in Chapter 2, Section 2.3. Studies also utilised an Empty vector (i.e. no PLC ζ insert) version of each construct, which was used as a further control. For more information on restriction enzymes and primer sequences utilised, refer to Table 6.2. For more detailed vector and construct information, refer to Supplementary Appendices 4-6. All molecular constructs were verified by DNA sequencing (Geneservice, Oxford). A series of overlapping sequencing primers were employed to ensure the prevention of sequencing errors (Supplementary Appendices 7-8).

Table 6.1: The various constructs utilised throughout this chapter, showing PLC ζ isoform, vector used, and system targeted. Asterisk (*) denotes constructs provided by Dr. John Parrington. pTracer and pcDNA3.1 constructs were cloned in-house, and verified by sequencing.

Construct name	Vector used	Description	System Transfected/Transformed
*pGEX-PLC ζ ^{WT}	pGEX-6P-2	Wild Type Human Recombinant PLC ζ fused with N-Terminal GST	<i>E. coli</i> BL21
*pGEX-PLC ζ ^{H398P}	pGEX-6P-2	H398P mutant Human Recombinant PLC ζ fused with N-Terminal GST	<i>E. coli</i> BL21
pcDNA3.1-PLC ζ ^{WT}	pcDNA3.1/V5-His	Wild Type Human Recombinant PLC ζ fused with C-Terminal 6xHis tag	HEK293T
pcDNA3.1-PLC ζ ^{H398P}	pcDNA3.1/V5-His	H398P mutant Human Recombinant PLC ζ fused with C-Terminal 6xHis tag	HEK293T
pTracer-PLC ζ ^{WT}	pTracer	Wild Type Human Recombinant PLC ζ produced alongside a reporter GFP	HEK293T
pTracer-PLC ζ ^{H398P}	pTracer	H398P mutant Human Recombinant PLC ζ produced alongside a reporter GFP	HEK293T

Table 6.2: Summary of primer sequences and restriction enzymes used during molecular cloning.

Construct name	Restriction sites	Primer sequences
pcDNA 3.1-PLC ζ ^{WT} pcDNA 3.1-PLC ζ ^{H398P} pTracer-PLC ζ ^{WT} pTracer-PLC ζ ^{H398P}	5'-EcoRI 3'-NotI	5'-GATCGAATTCACCATGGAAATGAGATGGTTTTTG-3' (Forward) 5'-GATCGCGGCCCATCTGACGTACCAACATAAAC-3' (Reverse)

6.2.2 Bacterial transformation and protein purification

pGEX-PLC ζ^{WT} and pGEX-PLC ζ^{H398P} were transformed into BL21 chemically competent *E. coli* cells following the manufacturer's instructions (Invitrogen, UK). Transformed colonies were cultured overnight in Luria Bertani media containing 100 $\mu\text{g/ml}$ ampicillin and 17 $\mu\text{g/ml}$ chloramphenicol (LB-Amp+CM) at 225rpm (37°C), diluted (1 in 100) with LB-Amp+CM, and cultured to an optical density of 0.6 (absorbance 600nm). 0.5mM isopropylthiogalactoside (IPTG; Sigma, UK) was added, and cultures incubated for a further 4 hours (25°C). Cells were harvested by centrifugation (6,000 rpm, 20 minutes at 4°C), and pellets re-suspended in phosphate buffered saline (PBS) and stored at -80°C. Frozen suspensions were thawed slowly on ice and sonicated, following addition of 1mM phenylmethanesulfonyl-fluoride (PMSF), 4 times (15 Hz) for 90 seconds on ice, with 60 second rest periods between each sonication. Sonicated samples were centrifuged at 19,000rpm for 20 minutes (4°C).

Supernatant was applied to Glutathione Sepharose-4-Fast beads (GE Healthcare, UK) in columns previously washed twice with PBS, and allowed to flow with gravity. Columns were washed with PBS, and GST cleaved with PreScission protease and collected in PBS. Purified proteins were dialyzed overnight, using snake-skin dialysis tubing (8kDa cut-off; VWR, UK), against 120mM KCL/ 20mM HEPES at 4°C. Protein band detection was performed utilising polyacrylamide gel electrophoresis using pre-cast gels (10% NuPAGE Bis-Tris Pre-cast Gels, Invitrogen, UK) following the manufacturer's instructions. Gels were run at 150V for ~1.5 hours, and stained with coomassie G-250 stain (SimplyBlue SafeStain, Invitrogen, UK). Molecular weights were deduced by comparison with the Novex Sharp protein standard (Invitrogen, UK).

6.2.3 Protein identification via Liquid Chromatography/Mass Spectrometry (LC/MS)

One of the most powerful methods for protein identification is a combination of liquid chromatography (LC) and mass spectrometry (MS), also termed LC/MS. After purification and separation on a gel, proteins are digested into peptides by enzymes at theoretically predictable positions. Each protein generates a unique combination of peptides with different masses. Thus, by determining the mass list of a protein, or “protein mass fingerprint”, a specific protein can be identified utilising a protein fingerprint database (Zhang *et al.* 2009).

In brief, proteins were separated using polyacrylamide gel electrophoresis, and stained using coomassie G-250 stain (SimplyBlue SafeStain, Invitrogen, UK) as previously described (Chapter 2, Section 2.16). Stain was removed from the gel using fresh double-distilled water for each wash, and bands of interest excised from the gel using a sterilised scalpel, taking care not to introduce keratin contamination. Excised bands were then submitted for identification by LC/MS; a research service provided courtesy of Dr. Mariola Edelman and Dr. Benedikt Kessler (University of Oxford, UK). For an example of the output format, refer to Supplementary Appendix 9.

6.2.4 Sea urchin egg homogenate (SUEH)

The SUEH is a well established model system used to characterise intracellular Ca^{2+} mobilisation and release mechanisms, whose sensitivity to Ca^{2+} release makes it useful to study the properties of factors such as cADPR and NAADP (Thomas *et al.*, 2001; Jones & Swann, 2007). Concentrated SUEH was diluted with glucamine intracellular medium (2mM ATP, 20mM phosphocreatine and 20U/ml creatine phosphokinase) performed sequentially at 17°C to achieve a final 2.5% concentration. Fluo-3 (3µM; Sigma Aldrich,

UK) was added to the final preparation. 2µl of protein was added at a concentration of 120µg/ml, or following prior addition of 1mM PIP2 (Sigma Aldrich, UK), into 600µl of diluted SUEH. Changes in Ca^{2+} levels were determined using a luminescence spectrophotometer (Perkin Elmer LS 50B, USA).

6.2.5 Mammalian cell culture

Transformed human embryonic kidney (HEK293T) cells were cultured under standard conditions and transfected using established calcium phosphate precipitation protocols, and the jetPEI transfection kit (Poly-plus transfection, USA) as previously described (Chapter 2, Section 2.10). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with high glucose, and supplemented with 10% fetal bovine serum (FBS), 4mM glutamine, 100U/ml penicillin, and 0.1mg/ml streptomycin. Cells were incubated at 37°C with 5% CO₂ in a humidified chamber.

6.2.6 Calcium phosphate precipitation transfection

HEK293T cells were grown in T-175 flasks (Nuclon, Denmark), and transfected at 60-70% confluency. One volume of 2X Ca (200mM CaCl_2) /DNA (60µg) was added drop-wise to one volume of 2X HBS solution (280mM NaCl, 1.5mM Na_2PO_4 , 50mM HEPES, pH = 7.12), and the precipitate added to the cell culture medium. 150µl of precipitate was added for every 1ml of medium.

6.2.7 Fluorescence microscopy

In order to investigate transfection efficiency and maximal expression of PLCζ isoforms following transfection into HEK293T cells, cells were cultured and transfected with pTracer-PLCζ^{WT} and pTracer-PLCζ^{H398P} as previously described using the jetPEI

transfection kit (Chapter 2, Section 2.10), and subject to fluorescence microscopy at time-points of 8, 24, 26, 48 and 56 hours post-transfection as previously described in Chapter 2, Section 2.19.

6.2.8 Protein expression and purification using a mammalian system

HEK293T cells were transfected with either calcium phosphate precipitation, or using the jetPEI transfection kit (Poly-plus transfection, France) as previously described (Chapter 2, Section 2.10). 24 hours post-transfection, medium was replaced with DMEM without FBS, and cells harvested 56 hours post-transfection following medium removal and washing (All PLC ζ constructs exhibited ~70% transfection as indicated by proportional fluorescence analysis). Cells were lysed in lysis buffer (50mM NaH₂PO₄, 300mM NaCl, 10mM imidazole, 0.05% Tween 20, pH 8.0) containing protease inhibitors (Roche Biosciences, UK). The supernatant (transfected HEK293T cell lysate) was removed following centrifugation at 19,000rpm for 20 minutes at 4°C. This was added to Poly-Prep chromatographic columns (Bio-Rad, UK) packed with Ni-NATA Superflow Magnetic Agarose beads (Qiagen, UK), previously equilibrated by washing with three column volumes (CV) of lysis buffer, and allowed to flow with gravity. After two additions of the supernatant, the column was washed twice with 2 CV of wash buffer (32mM Na₂HPO₄, 300mM NaCl, 20mM Imidazole, 0.05% Tween 20, pH = 8.0) and eluted with 1 CV of elution buffer (50mM Na₂HPO₄ 300mM NaCl, 250mM Imidazole, 0.05% Tween 70, pH = 8.0). Purified protein was then dialyzed into 120mM KCL/ 20mM HEPES as previously described in this Chapter (Section 6.2.2).

6.2.9 Mouse oocyte injection of recombinant cRNA and human protein

To test functional ability to induce Ca^{2+} release of synthesised cRNA and recombinant protein, these were microinjected into mouse oocytes, following their respective methods of preparation, as previously described in this Chapter (Section 6.2.8) and Chapter 2 (Section 2.10). Ca^{2+} was then monitored as also described in Chapter 2. The reagents injected into mouse oocytes for this Chapter were $\text{PLC}\zeta^{\text{WT}}$ cRNA, $\text{PLC}\zeta^{\text{WT}}$ and $\text{PLC}\zeta^{\text{H398P}}$ purified from pGEX transformed *E. coli*, and lysates obtained from HEK293T cells transfected with pTracer- $\text{PLC}\zeta^{\text{WT}}$ and pTracer- $\text{PLC}\zeta^{\text{H398P}}$. Concurrent with injection of lysates obtained from HEK293T cells transfected with pTracer- $\text{PLC}\zeta^{\text{WT}}$, oocytes were also co-injected with Cyclohexamide (CHX; 20 $\mu\text{g}/\mu\text{l}$) to prevent translation of any $\text{PLC}\zeta^{\text{WT}}$ cRNA, present within the lysates, to protein. Procedures were performed by Mr. HC Lee and Professor Rafael Fissore (University of Massachusetts, USA).

6.2.10 Immunoblotting

In order to identify protein bands separated on a polyacrylamide gel, immunoblotting was performed as previously described (Chapter 2, Section 2.16). Membranes were incubated with a monoclonal anti-His antibody conjugated with horseradish peroxidase (HRP) (Invitrogen, UK) applied at a dilution of 1:5000 to identify His-tagged protein, as well as with a polyclonal anti- $\text{PLC}\zeta$ antibody (25 $\mu\text{g}/\text{ml}$), followed by a HRP conjugated anti-rabbit secondary antibody (1:5000; Invitrogen, UK) to identify $\text{PLC}\zeta$ protein. An anti-human HRP conjugated primary antibody against β -actin was also used as a loading control (1:15000; Sigma-Aldrich, UK; refer to Supplementary Appendix 10). $\text{PLC}\zeta$ band intensity was determined by relative densitometry using ImageJ (National Institute of Health, USA), and normalised against the bands obtained for β -actin, and was done so for two subsequent replicates. Statistical correlations between band intensity for cell lysates

obtained from cells transfected with pTracer-PLC ζ^{WT} and pTracer-PLC ζ^{H398P} were determined with the Students' *t*-test.

6.2.11 Reverse transcription PCR (RT-PCR)

To examine the status of PLC ζ isoforms being transcribed by HEK293T cells, total RNA from transfected cells were subject to RT-PCR. Cells were transfected with the mCherry-PLC ζ^{WT} construct (produced PLC ζ tagged with a red fluorescent mCherry on the N-terminus; construct created by Dr. Margarida Ruas, University of Oxford, UK, and donated to the Coward laboratory) as previously described (Chapter 2, Section 2.10). Cells were harvested 56 hours post-transfection, and lysed by repeated aspiration with a blunt 20 gauge needle and syringe. Total RNA was then isolated from lysed cells using the RNeasy Mini Kit, following the manufacturer's protocol (QIAGEN, UK) as previously described in Chapter 2, Section 2.12. Total RNA was then divided into single use aliquots and stored at -80°C until required.

cDNA was transcribed using the Omniscript reverse transcription kit (QIAGEN, UK), utilising an oligo-dT primer (QIAGEN, UK), following the manufacturer's protocol. Briefly, total RNA (1.5 μ g) was added to 10x buffer RT, dNTP mix (5mM), and Oligo dT primer (10mM; QIAGEN, UK), RNase inhibitor (10 units/ μ l; QIAGEN, UK), and RNase-free water, and incubated at 37°C for 1 hour. Following reverse transcription, reactions (2.5 μ l of RT reaction mix per PCR reaction) were subject to PCR cycling conditions as previously described in Chapter 2, Section 2.3, utilising a combination of different primer sets (Table 6.3; Figure 6.1). Potential RNA splicing sites were also investigated using SplicePort online software (Dogan *et al.*, 2007), to which corresponding primers were then designed. Resultant amplicons were analysed using 1% agarose gel electrophoresis as

previously described (Chapter 2, Section 2.5), and results confirmed by three independent replicates.

Table 6.3: Primer names and sequences used to amplify reverse transcription products.

Primer	Construct/PLCζ domain targeted	Primer sequence (5' → 3')
P1	mCherry	TATGGATCCATGGTGAGCAAGGGCGAG
P2	Beginning of C2 domain	TATGAATTCTTATGTAATTGGCATACCCTC
P3	Predicted C2 splice site 1	TATGAATTCTTACAACCTGGATACCACTGAT
P4	Predicted C2 splice site 2	TATGAATTCTTATGAATCACCTTTGTTAGA
P5	Before end of PLCζ ORF	TATGAATTCTTAAAGGCTCTACCCATTCT
P6	End of PLCζ ORF	TATGAATTCTTATCTGACGTACCAAACATA

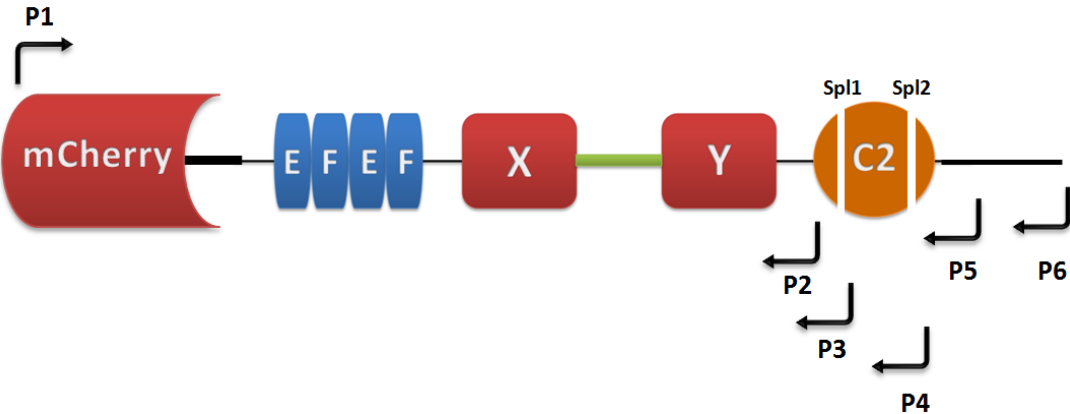


Figure 6.1: Schematic representation of the PLCζ ORF illustrating the primer sets utilised and their respective binding sites on the PLCζ domain ORF. Primer P1 was utilised as the forward primer and P2-P6 utilised as reverse primers during for the PCR following RT. All primer sets would yield amplicons of ~ 2.0 – 2.1Kb. Spl1 and Spl2 denote two predicted splice sites within the C2 domain of the PLCζ ORF.

6.3 Results

6.3.1 Bacterial expression system

The molecular identity of a protein (molecular weight ~74kDa) purified from bacteria expressing pGEX-PLC ζ^{WT} (Figure 6.2) was confirmed as human PLC ζ by liquid chromatography mass spectrophotometry (LC/MS). The identity of the protein as PLC ζ was also indicated by a reduction in molecular weight (~100kDa to ~75kDa) following cleavage of the 25kDa GST-tag (Figure 6.3).

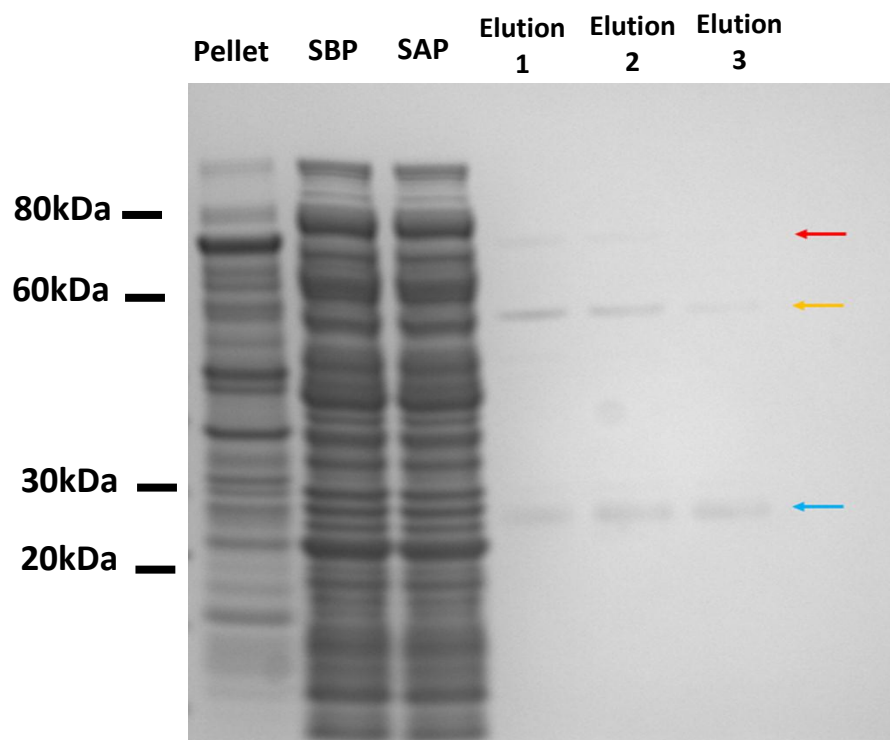


Figure 6.2: Polyacrylamide gel electrophoresis and coomassie blue staining of PLC ζ^{WT} cultures before and after purification. Bands indicated by arrows were excised and subjected to LCMS which identified them as Red arrow: PLC ζ ; Orange arrow: Heat shock protein 70; Blue arrow: GST. SBP: supernatant before purification; SAP: supernatant after purification

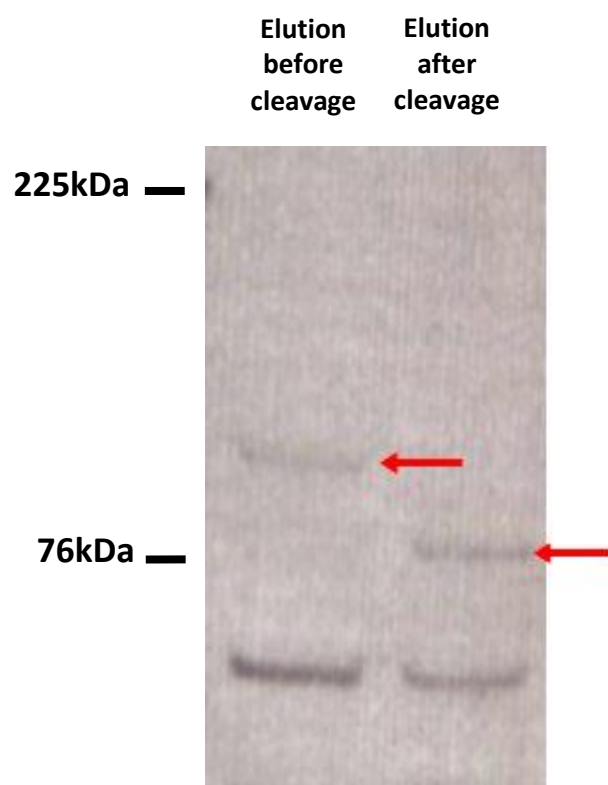
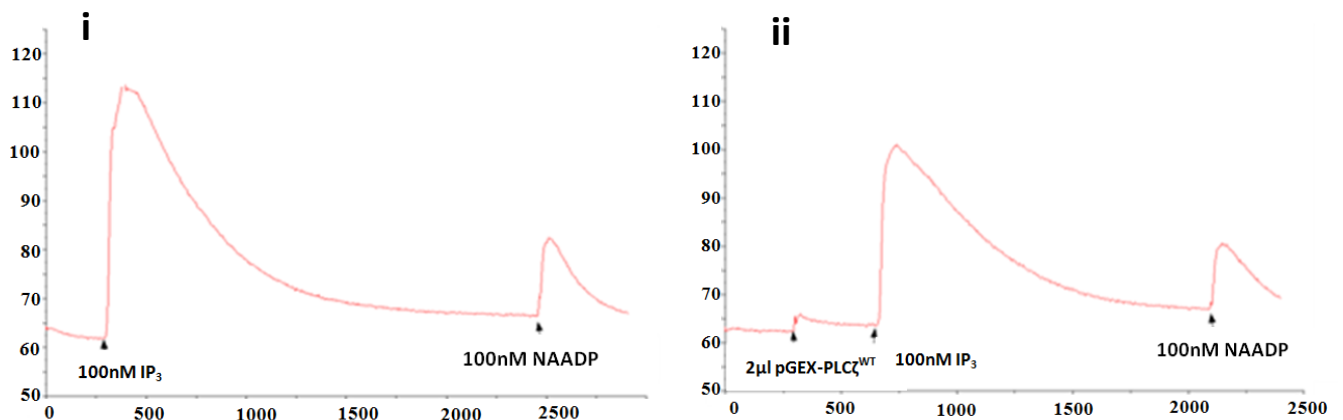


Figure 6.3: Polyacrylamide gel electrophoresis and coomassie blue staining of PLC ζ^{WT} cultures before and after cleavage of the GST tag following purification. Bands highlighted by the red arrows indicate a reduction in size ($\sim 25\text{kDa}$) following cleavage of the GST from the elution.

However, the addition of purified PLC ζ from bacteria transformed with pGEX-PLC ζ^{WT} or pGEX-PLC ζ^{H398P} (120 $\mu\text{g/ml}$) to the SUEH, an established bioassay of Ca^{2+} release (Parrington *et al.*, 2007), resulted in negligible Ca^{2+} release. Subsequent addition of 100nM IP_3 indicated that IP_3 receptors (IP_3Rs) had not been desensitised. Prior addition of 1mM PIP_2 to ensure substrate was present, had little effect (Figure 6.4A). Moreover, only 27% of mouse oocytes injected with pGEX-PLC ζ^{WT} eluates (courtesy of Professor RA Fissore, University of Massachusetts, USA) showed significant changes in intracellular Ca^{2+} , which were immediate but transient in nature (Figure 6.4B; Table 6.4), indicating these peaks were due to factors other than active PLC ζ protein in the eluate such as Ca^{2+}

contamination, or artifacts due to eluate addition to the SUEH. Of 26 injected oocytes, only 1 exhibited activation as deduced by the presence of pronuclei, and only 4 proceeded to the 2-cell stage, indicating that activation may have occurred in these cases due to the injection process itself and not the injected protein.

A



B

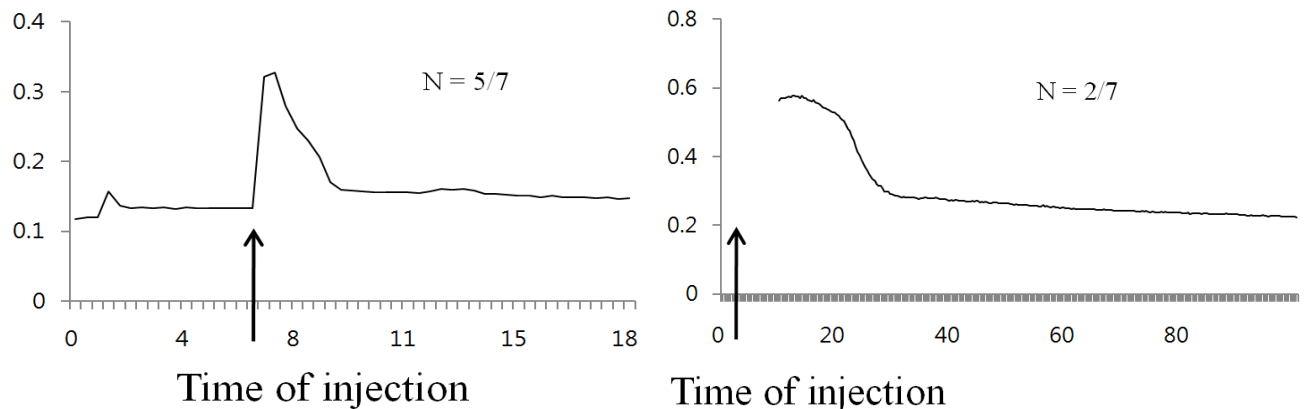


Figure 6.4: **A)** Representative Ca^{2+} peaks from the SUEH activity bioassay with recombinant human PLC ζ proteins obtained from the bacterial expression system. Arrows indicate sample addition. Two distinctive peaks observed when 100nM IP_3 and NAADP were added (**i**). No significant Ca^{2+} peak observed following the addition of 2 μl of 120 $\mu\text{g}/\text{ml}$ PLC ζ^{WT} (**ii**). **B)** Changes in intracellular Ca^{2+} concentration resulting from injection of PLC ζ^{WT} recombinant protein, obtained from the bacterial system, in mouse oocytes. Arrows indicate the time of protein injection. Ca^{2+} concentration increased immediately following protein injection and decreased to baseline measurement with time. (Mouse oocyte microinjection data courtesy of Professor Rafael Fissore, University of Massachusetts, USA). Y axis: ratio of relative fluorescence units obtained at wavelengths of 340nm and 380nm ($F_{340/380}$). X axis: Time (seconds).

Table 6.4: Fate of all 26 injected oocytes with purified protein obtained from the prokaryotic system. (Injected: number of oocytes injected; Survival: number of oocytes surviving injection; 2PB: oocytes exhibiting 2 polar bodies; PN: oocytes exhibiting pro-nuclei; 2-cell: oocytes reaching the 2-cell stage). (Data courtesy of Professor Rafael Fissore, University of Massachusetts, USA).

Injected	Survival	2PB	2PN	2-cell
26	23	2	1	4

6.3.2 Mammalian expression system

A reduced level of fluorescence was observed for HEK293T cells transfected with pTracer-PLC ζ^{H398P} compared to pTracer-PLC ζ^{WT} transfected cells (Figure 6.5). For both constructs, the fluorescent signal became visible from 24 hours post-transfection, reaching maximal levels at 48 and 56 hours. Proportional analysis of cells exhibiting a fluorescence signal indicated a transfection efficiency of $\geq 70\%$ for both constructs. Immunoblotting with an anti-His monoclonal antibody was unable to identify bands at the expected size of PLC ζ (~74kDa) at any stage of the purification process for both pTracer and pcDNA 3.1 constructs. No bands were detectable for both constructs in the un-purified cell lysates, whereas a single band at ~57kDa could be observed in the eluates for both constructs (Figure 6.6A). Immunoblotting using an anti-PLC ζ polyclonal antibody was able to detect bands at the expected size of PLC ζ in un-purified cell lysates obtained from cells transfected with pTracer-PLC ζ^{WT} and pTracer-PLC ζ^{H398P} (Figure 6.6B). Relative densitometry indicated that levels of PLC ζ^{WT} (1.3 arbitrary units; a.u) present in the lysates were ~ 6 times higher than that of PLC ζ^{H398P} (0.2 a.u) (Figure 6.7).

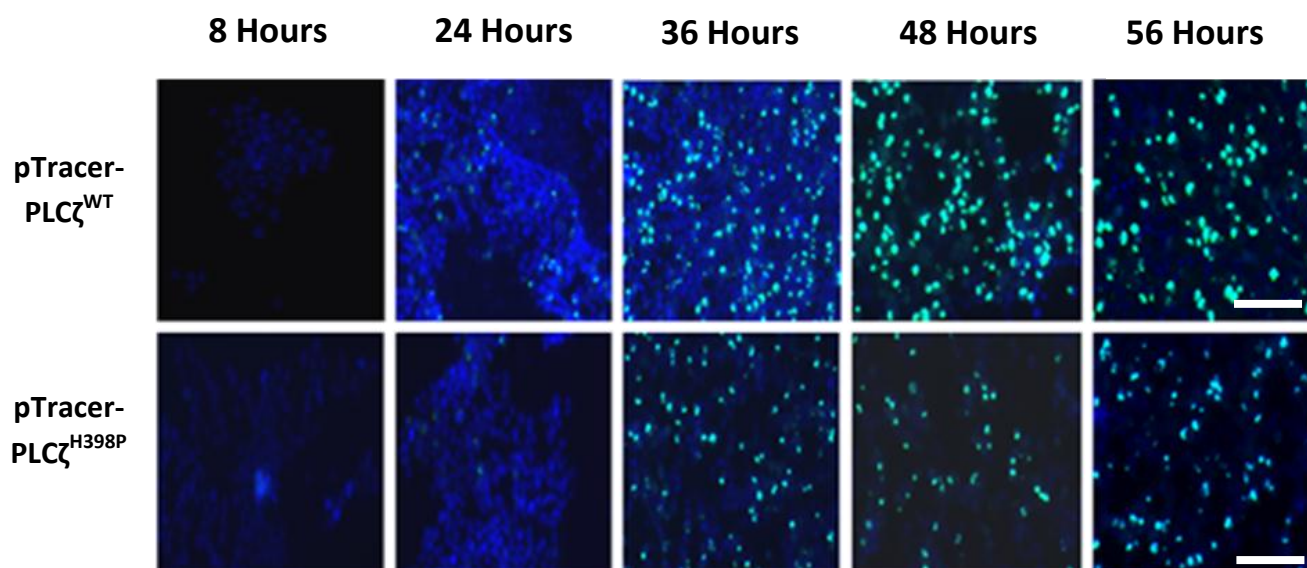


Figure 6.5: Representative fluorescence microscopy image taken across 8, 24, 36, 48, and 56 hour time points for HEK293T cells transfected with pTracer-PLC ζ constructs. Images were captured at a magnification of 10X, and were repeated 3x3 times. Images represent overlays of DAPI and FITC fluorescence. White scale bars indicate 50 μ m.

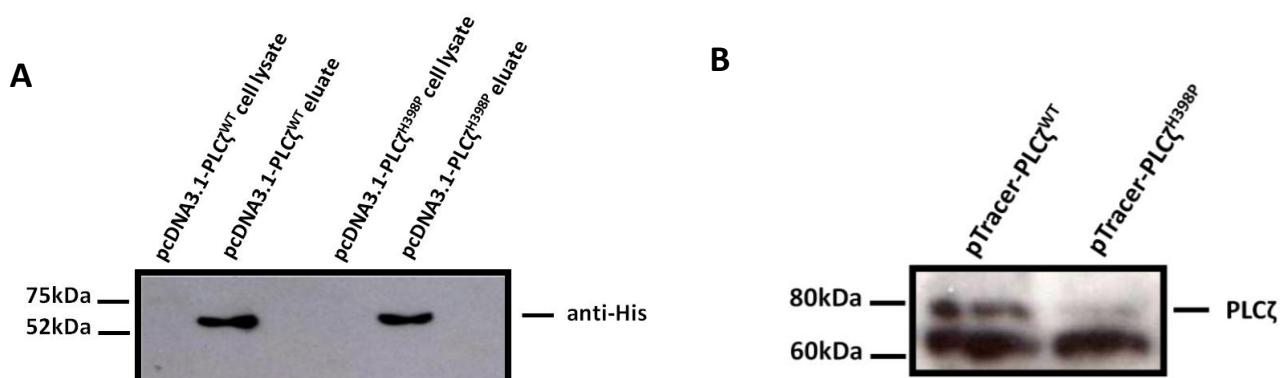


Figure 6.6: **A)** Representative immunoblot using anti-His monoclonal antibody with purification samples from pcDNA and pTracer constructs. No bands are identifiable in pre-purification samples, with bands only being identified in the elutions. **B)** Representative PLC ζ immunoblotting for HEK293T cells transfected with pTracer-PLC ζ^{WT} , and – PLC ζ^{H398P} . Bands corresponding to ~74kDa were successfully detected, which was the expected size for the expressed PLC ζ protein.

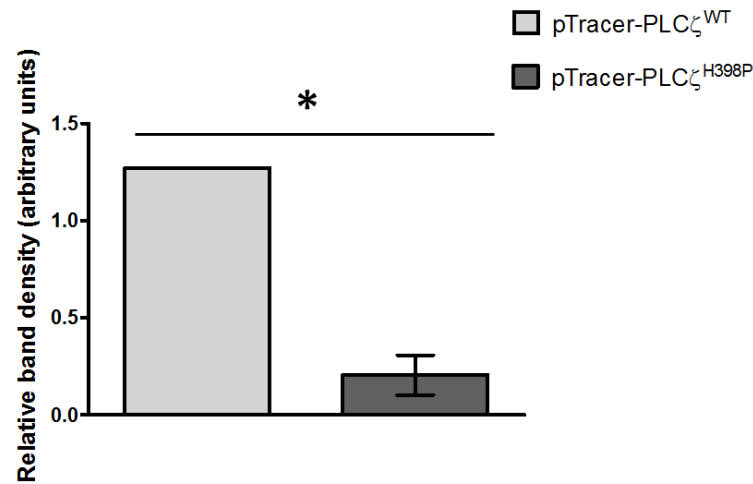


Figure 6.7: Relative densitometry values illustrating the intensity of PLC ζ bands obtained for HEK293T cells transfected with pTracer-PLC ζ^{WT} , and -PLC ζ^{H398P} . Asterisk (*) denotes a statistically significant difference ($p \leq 0.05$) as determined by the *t*-test. Data are represented as mean $\pm$ S.E.M.

6.3.3 Reverse Transcription (RT) – PCR

To investigate the isoform of PLC ζ being produced by transfected HEK293T cells, RT-PCR was utilised which suggested that PLC ζ cRNA produced within transfected HEK293T cells exhibited a C-Terminal truncation after the C2 domain and just before the end of the PLC ζ ORF (Figure 6.8A). Primer combinations of P1 with P2-P6 did not amplify any bands following RT of total RNA from HEK293T cells transfected with mCherry-empty vector (mCherry vector without a PLC ζ isoform). However, the same primer combinations were able to produce amplicons up until the P1+P5 primer combination, but with P1+P6 failing to amplify anything (Figure 6.8B).

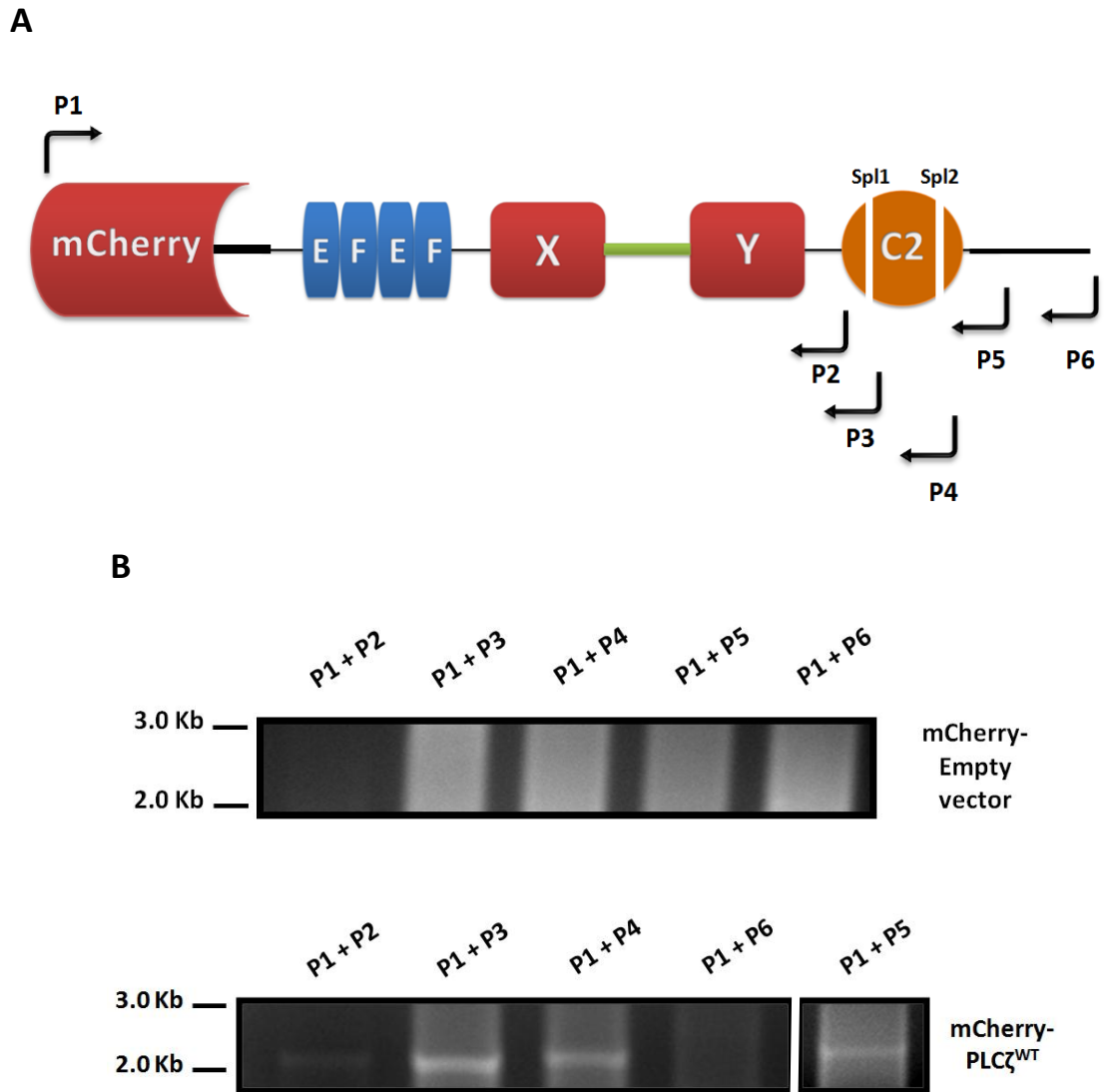


Figure 6.8: **A)** Schematic illustrating the primer sets utilised and their respective binding sites on the PLC ζ domain ORF. Primer P1 was utilised as the forward primer and P2-P6 utilised as reverse primers during for the PCR following RT. All primer sets would yield amplicons of $\sim 2.0 - 2.1$ Kb. **B)** Representative 1% agarose gel pictures of RT-PCR using primer sets P1-P6. No bands were detected following RT-PCR with RNA isolated from mCherry-Empty vector transfected HEK293T cells, while primer sets P2-P5 with P1 exhibited bands at the expected sizes, while RT-PCR with the P1+P6 primer set did not yield any amplicon. Spl1 and Spl2 denote two predicted splice sites within the C2 domain of the PLC ζ ORF.

6.3.4 Mouse oocyte microinjection

Microinjection of PLC ζ^{WT} cRNA into mouse oocytes induced Ca $^{2+}$ oscillations in 100% (16/16) of injected oocytes, while those injected with PLC ζ^{H398P} cRNA did not exhibit Ca $^{2+}$ responses (14/14 and 5/5 respectively). The injection of lysates prepared from HEK293T cells transfected with pTracer-PLC ζ^{WT} (2.6 $\mu\text{g}/\mu\text{l}$) resulted in Ca $^{2+}$ oscillations typical of human PLC ζ , while the injection of whole cell lysates prepared from HEK293T cells transfected with pTracer empty vector (6.7 $\mu\text{g}/\mu\text{l}$) and pTracer-PLC ζ^{H398P} (5.8 $\mu\text{g}/\mu\text{l}$) failed to induce a Ca $^{2+}$ response (Figure 6.9A). Injection of lysates prepared from HEK293T cells transfected with pTracer-PLC ζ^{WT} at increased concentration (22.1 $\mu\text{g}/\mu\text{l}$) also resulted in Ca $^{2+}$ release, while injection of increased concentrations of lysates prepared from HEK293T cells transfected with pTracer empty vector (15.8 $\mu\text{g}/\mu\text{l}$) and pTracer-PLC ζ^{H398P} (15.1 $\mu\text{g}/\mu\text{l}$) failed to induce a Ca $^{2+}$ response.

Additionally, co-injection of lysates prepared from HEK293T cells transfected with pTracer-PLC ζ^{WT} and cyclohexamide (CHX; 20 $\mu\text{g}/\mu\text{l}$) also resulted in Ca $^{2+}$ oscillations, indicating that the Ca $^{2+}$ oscillations observed were induced by PLC ζ protein and not related to the translation of PLC ζ RNA present within the lysate (Figure 6.9B). While PLC ζ^{WT} lysates were able to elicit Ca $^{2+}$ oscillations effectively upon injection into mouse oocytes at a concentration of just 2.6 $\mu\text{g}/\mu\text{l}$, mutant lysates could not. Increasing the concentration of PLC ζ^{H398P} lysate injected by a factor of either x2, x3, or x8 (15.1 $\mu\text{g}/\mu\text{l}$) also failed to induce oscillations, indicating that the inability to elicit Ca $^{2+}$ oscillations was due to the mutant protein having defective PLC ζ activity as opposed to a lack of sufficient PLC ζ^{H398P} protein being injected.

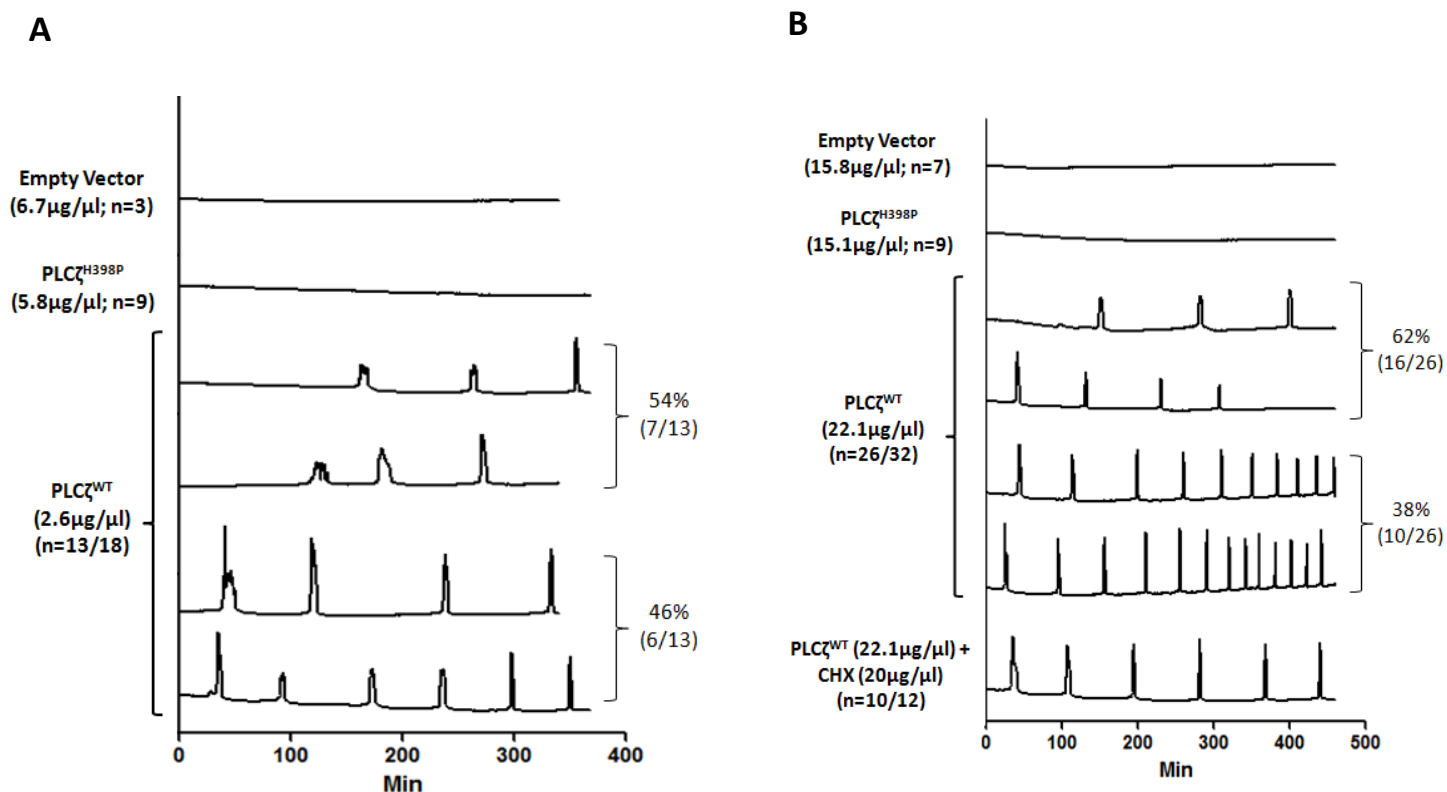
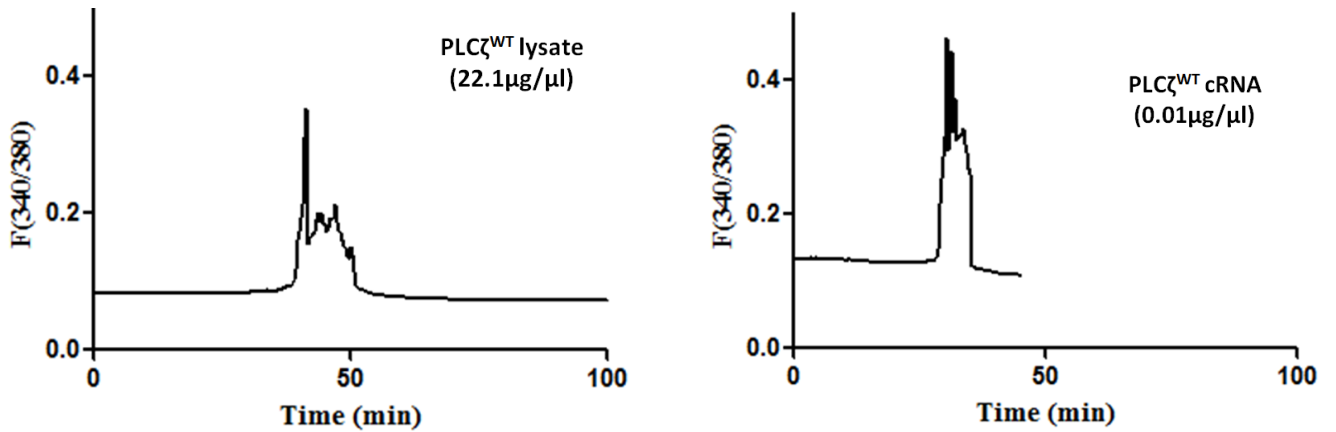


Figure 6.9: Representative Ca^{2+} oscillation profiles of mouse oocytes injected with lysates at **(A)** low concentrations and **(B)** higher concentrations, prepared from HEK293T cells transfected with pTracer empty vector, pTracer-PLCζ^{H398P}, pTracer-PLCζ^{WT}. Baseline values for all Ca^{2+} traces shown were similar, and traces are stacked to facilitate visualisation of the responses. Y-scale ratios therefore do not reflect absolute 340/380 fluorescence ratios, and were added to note progression of the scale. Courtesy of HC Lee and Professor RA Fissore (University of Massachusetts, USA).

Overall, Ca^{2+} peaks resulting from lysates containing PLCζ^{WT} micro-injection were of low frequency, low amplitude, and were delayed in their initiation (occurring ~60mins post-injection), a profile similar to oocytes injected with low concentrations of human PLCζ mRNA, as were the shape and amplitude of the first peaks (which are indicative of fertilisation; Figure 6.10B, C). Following microinjection experiments over three separate occasions, 79% (49/62) of total oocytes injected with lysates prepared from cells transfected with pTracer-PLCζ^{WT} exhibited Ca^{2+} oscillations, while 45% (13/29) injected oocytes exhibited pronuclei formation, indicating successful activation. In contrast, none

of the oocytes injected with lysates prepared from pTracer-empty vector or pTracer- $\text{PLC}\zeta^{\text{H398P}}$ exhibited Ca^{2+} oscillations or oocyte activation

A



B

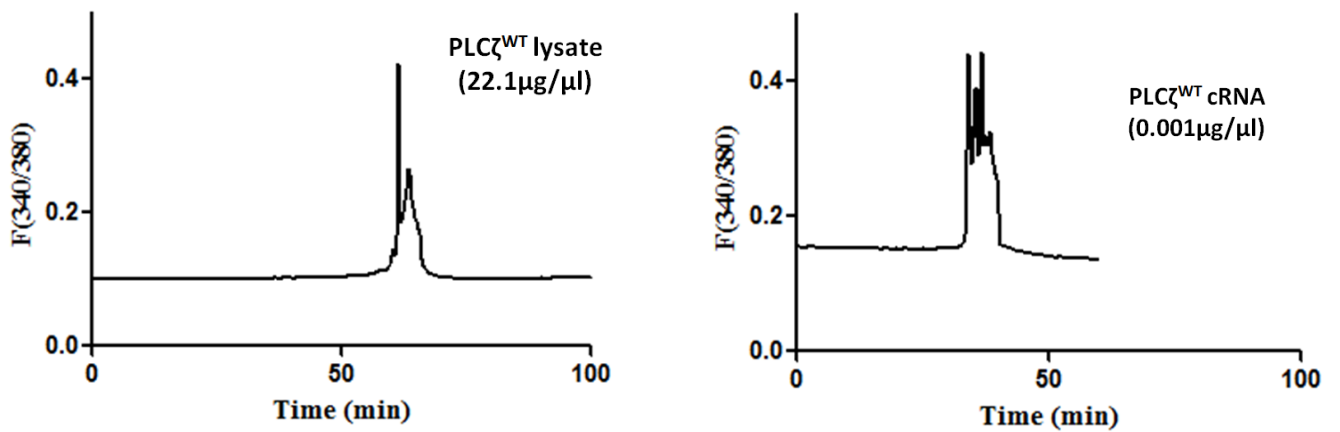


Figure 6.10: Representative comparison of first Ca^{2+} peak between mouse oocytes injected with the high concentration of lysate (22.1 $\mu\text{g}/\mu\text{l}$) from HEK293T cells transfected with pTracer- $\text{PLC}\zeta^{\text{WT}}$, and oocytes injected with human $\text{PLC}\zeta^{\text{WT}}$ cRNA at concentrations of (A) 0.01 $\mu\text{g}/\mu\text{l}$, and (B) 0.001 $\mu\text{g}/\mu\text{l}$. Courtesy of HC Lee and Professor RA Fissore (University of Massachusetts, USA).

Table 6.5: A) Number of mouse oocytes injected with lysates prepared from HEK293T cells transfected with pTracer-empty vector, -PLC ζ^{WT} , and -PLC ζ^{H398P} exhibiting Ca²⁺ oscillations and pronuclei formation as indicated on three separate occasions. **B)** Total number of oocytes injected exhibiting Ca²⁺ oscillations and pronuclei formation. N/A indicates incidences where data was not available. Data courtesy of HC Lee and Professor RA Fissore (University of Massachusetts, USA).

A

Lysate injected	Number of Oocytes Injected	Number of Oocytes exhibiting Ca ²⁺ Oscillations (%)	Number of Oocytes Injected	Number of Oocytes exhibiting Ca ²⁺ Oscillations (%)
1st Injection Session				
Empty Vector (6.7 µg/µl)	3	0(0)	3	0(0)
PLC ζ^{WT} (2.6 µg/µl)	18	13(72)	7	4(57)
PLC ζ^{H398P} (5.8 µg/µl)	9	0(0)	9	0(0)
2nd Injection session				
Empty Vector (15.8 µg/µl)	7	0(0)	9	0(0)
PLC ζ^{WT} (22.1 µg/µl)	32	26(81)	22	9(41)
PLC ζ^{H398P} (15.1 µg/µl)	9	0(0)	16	0(0)
3rd Injection session				
PLC ζ^{WT} (22.1 µg/µl) + CHX (20. µg/µl)	12	10(83)	N/A	N/A

B

Lysate injected	Oocytes Injected	Oocytes exhibiting Ca ²⁺ Oscillations (%)	Oocytes Injected	Oocytes exhibiting Pro-nuclei (%)
Empty Vector	10	0(0)	12	0(0)
PLC ζ^{WT}	62	49(79)	29	13(45)
PLC ζ^{H398P}	18	0(0)	25	0(0)

6.4 Discussion

Whilst ART has played a critical role in reducing global infertility, there are still groups of infertile couples for which ART has not yet proven successful, particularly infertile males whose sperm are unable to activate oocytes, even following procedures such as ICSI. A deficiency in the oocyte activation mechanism is regarded as the principal cause of fertilisation failure, or abnormally low fertilisation after ICSI, and can currently only be resolved using assisted oocyte activation (AOA). However, while numerous cases have demonstrated that ICSI combined with AOA greatly increased the success rate of fertilisation and subsequent pregnancy of sperm from such infertile men, great concerns still remain with regards to the non-physiological nature of AOA stimulus, and how this might affect embryogenesis in terms of altered patterns of embryonic gene expression (Ducibella *et al.*, 2002). It would, therefore, be extremely desirable to obtain a more physiological alternative to current AOA protocols. The aims of the present study were to develop an active recombinant human PLC ζ protein via expression in a prokaryotic (*E. coli*) system, and a eukaryotic (HEK293T) system, which could be of potential clinical use for patients whose sperm are unable to cause oocyte activation, and a more physiological alternative to current AOA protocols.

6.4.1 Recombinant human PLC ζ protein is successfully expressed by a prokaryotic system in an inactive state, and expressed in an active form by a eukaryotic system

Recombinant human PLC ζ was successfully purified following the successful expression of PLC ζ^{WT} using a bacterial system. However, this protein was functionally inactive as indicated by SUEH analysis and mouse oocyte injection. Lack of chaperone proteins, errors in/lack of post-translational modifications (PTMs), and inability to be targeted to sub-cellular locations in prokaryotic systems can all result in protein misfolding and aggregation (Dyson *et al.*, 2004), which may have contributed towards the observed inactivity of the PLC ζ protein.

However, injection of lysates obtained from HEK293T cells transfected with pTracer-PLC ζ^{WT} in mouse oocytes successfully elicited Ca²⁺ release and activation, thus indicating the presence of active PLC ζ^{WT} protein, in contrast to injection of pTracer-PLC ζ^{H398P} and pTracer empty vector lysates which failed to exhibit Ca²⁺ release. However, it is interesting to note that bands of appropriate molecular weight were not detected in any pre-purification lysate following immunoblotting (including lysates which exhibited Ca²⁺ release following mouse oocyte injection). Kuroda *et al.*, (2006) suggest that the functional structure of PLC ζ involves the association of the EF hands with the C2 domain (see Figure 1.6B; Chapter 1), which may have hidden the C-Terminal 6xHis tag, explaining the failure to detect pre-purification bands. It is possible that purification causes the full length protein to undergo cleavage, giving rise to the truncated bands (~55kDa) observed post-purification.

Indeed, the stability of His-tagged protein interactions with its purifying ligand is known to be influenced by tag accessibility (Block *et al.*, 2009), and the insertion of metal binding tags can lead to unstable or degraded protein products (Rosales & Lee, 2000; Ueda *et al.*, 2003). However, the current results indicate that it is more likely that PLC ζ undergoes a C-Terminal cleavage at the RNA level following expression in HEK293T cells. While it was possible to amplify PLC ζ amplicons, using RT-PCR, up until the end of the C2 domain, indicating that all functional domains are successfully transcribed, it was not possible to amplify the entire mCherry-PLC ζ fragment, suggesting that a fragment of DNA was missing. However, when a reverse primer corresponding to a segment just before the end of the PLC ζ ORF (P5) was used, a band at the expected size was amplified successfully. This may explain why PLC ζ protein was undetectable at any stage of the purification process, since the C-terminal tags (6x His tag; V5 epitope) may not have been present, or at least attached to the PLC ζ RNA transcript. Concurrently, this may also explain why bands corresponding to the expected size for PLC ζ (~74kDa) following immunoblotting utilising an anti-His monoclonal antibody were not apparent. It would be interesting to investigate whether such truncation events are repeated physiologically within the testes and sperm as well. Indeed, if such truncation of PLC ζ cRNA is present within testes and sperm, this would not have been detected by previous studies since the truncation would be too small to detect at the protein level (~50bp; ~5kDa).

However, it is not yet clear whether such truncation is a requirement for PLC ζ activity, or simply represents a by-product of the cellular translational machinery employed by the HEK293T cells used herein. Interestingly though, as was indicated by injection of cell lysates into mouse oocytes, this truncation did not seem to have an effect upon PLC ζ activity. However the significance of this truncation needs to be investigated

further, considering that the truncation identified did not occur within any functional domain. One must also consider that such truncation may be a cell-line specific by-product, which may not be present *in vivo* within testes. Furthermore such a truncation may result in changes to the 3D folding of the enzyme, which may influence altered activity. It is vital that such questions are answered before PLC ζ protein can be utilised within the clinic as a viable replacement for current AOA protocols.

6.4.2 Human PLC ζ protein generated in a human cell line induces Ca²⁺ oscillations in mouse oocytes, and may require post-translational modifications in the oocyte to obtain an active state

The successful production of active recombinant human PLC ζ protein has not yet been described within the literature, and it is essential that this is obtained for the viable therapeutic use of PLC ζ within the clinic. Injection of lysates created from HEK293T cells transfected with pTracer-PLC ζ^{WT} into mouse oocytes successfully elicited Ca²⁺ release and oocyte activation, indicating the presence of active PLC ζ^{WT} protein, representing a fundamental step forward in the area of research. PLC ζ^{WT} protein activity was confirmed by the co-injection of lysates with cyclohexamide (CHX), which would have suppressed the translation of any PLC ζ RNA transcripts that may have been present in the lysates, thus proving that any resultant Ca²⁺ release was the sole result of protein activity. In contrast, the injection of lysates prepared from cells transfected with pTracer-PLC ζ^{H398P} and pTracer-empty vector failed to exhibit Ca²⁺ release.

While levels of PLC ζ^{H398P} seemed significantly lower in equal concentrations of lysates compared to PLC ζ^{WT} , PLC ζ^{WT} lysates were able to elicit oscillations effectively while the mutant lysates could not following injection of double the amount (5.8 $\mu\text{g}/\mu\text{l}$) compared to the PLC ζ^{WT} lysates (2.6 $\mu\text{g}/\mu\text{l}$). Furthermore, tripling the concentration of mutant lysate (15.1 $\mu\text{g}/\mu\text{l}$) used, compared to the of the PLC ζ^{WT} lysate (2.6 $\mu\text{g}/\mu\text{l}$), was still unable to induce oscillations following injection, indicating that inability to elicit Ca $^{2+}$ oscillations was due to loss of PLC ζ activity as opposed to lack of PLC ζ^{H398P} protein. It is possible that PLC ζ^{H398P} protein cannot be transcribed as efficiently as PLC ζ^{WT} protein by the mammalian system used herein with PLC ζ^{H398P} protein possibly being transcribed at a slower rate than PLC ζ^{WT} , which may explain the discrepancy in terms of protein levels between the two isoforms. However, as discussed in more detail in Chapters 7 and 8, this discrepancy may also have been due to the presence of the H398P mutation itself, which may have led to increased protein instability, leading to protein degradation.

Curiously, there was a noticeable delay between injection and the onset of Ca $^{2+}$ release following the injection of PLC ζ^{WT} lysates, with the first Ca $^{2+}$ spikes not observed until one hour post-injection. Otherwise, the oscillation profile observed following lysate injection was similar to that observed following the injection of low concentrations of PLC ζ^{WT} cRNA, possibly indicating that the levels of active PLC ζ^{WT} within the lysate were of low concentration. However, injection of PLC ζ^{WT} lysates at increased concentration resulted in similar Ca $^{2+}$ profiles to that of PLC ζ^{WT} lysates injected at lower concentrations, indicating that the concentration of PLC ζ^{WT} protein within the lysates was unlikely to be the reason for the observed delay in initial response. The apparent delay between cell lysate injection and the onset of Ca $^{2+}$ oscillations may imply that PLC ζ protein within the lysate required post-translational modification, or interactions with proteins inside the

oocyte, before attaining the functional ability to release Ca^{2+} . Indeed, post-translational modification has been suggested as being a key requirement for endogenous PLC ζ activity (Cooney *et al.*, 2010). There is mounting speculation over the possible role of protein cleavage in the functional ability of PLC ζ . Moreover, spontaneous proteolysis of PLC ζ was apparent in both mouse and porcine sperm (Kurokawa *et al.* 2005; 2007), with cleaved products able to re-associate to form functional heterodimers with increased levels of activity (Kurokawa *et al.*, 2007). Cleavage was reported to occur at various sites within the PLC ζ X-Y linker (Kurokawa *et al.*, 2007), a region susceptible to proteolysis in other PLCs (Ellis *et al.*, 1993). Moreover, a 62kDa PLC ζ isoform lacking EF1-3 hand domains was previously described in mouse sperm (Kouchi *et al.*, 2004; 2005; Kurokawa *et al.*, 2005; 2007; Nomikos *et al.*, 2005), and reported to activate mouse oocytes and support normal development to term (Nakanishi *et al.*, 2008).

Furthermore, purified recombinant mouse PLC ζ did not undergo cleavage, retained activity *in vitro*, but was far less efficient at inducing Ca^{2+} oscillations in mouse oocytes (Kouchi *et al.*, 2004; 2005; Nomikos *et al.*, 2005). Intriguingly, Phillips *et al.* (2011) recently showed that mouse PLC ζ protein over-expressed in Chinese hamster ovarian (CHO) cells did not exhibit activity within these cells, but did so following injection of whole cell lysates into mouse oocytes, further indicating that mouse PLC ζ protein may require oocyte specific modifications/interactions in order to exhibit activity. Finally, it is particularly interesting to note that immunoblots of sperm extracts from fertile men revealed specific profiles of PLC ζ expression including three identifiable bands at ~70kDa, ~55kDa, and ~40kDa, a pattern not apparent in infertile patients diagnosed with oocyte activation deficiency (Heytens *et al.*, 2009). However, a delay in the onset of Ca^{2+} oscillations was not observed by Phillips *et al.* (2011) as in the case of the current study. It

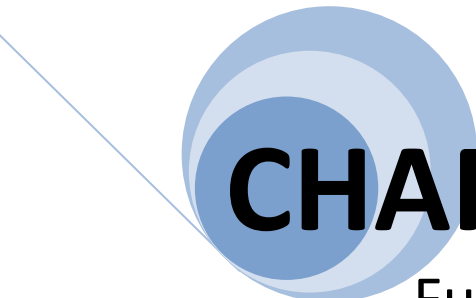
is possible that human PLC ζ requires more modifications compared to mouse PLC ζ to reach an active state. It is also possible that human PLC ζ requires certain covalent modifications in addition to PTMs. It is vital, therefore, that future studies elucidate whether post-translational or covalent modifications such as cleavage are a necessary requirement for endogenous PLC ζ activity. Increased understandings of such mechanisms are crucial for developing PLC ζ as a novel therapeutic agent for the treatment of infertility.

6.5 Conclusions and future work

In summary, this Chapter describes for the first time, the successful production of active recombinant human PLC ζ protein within lysates obtained from eukaryotic cells, which induced characteristic patterns of Ca²⁺ release when injected into mouse oocytes, representing a major step forward within this area of research. However, a male-infertility linked mutation in PLC ζ protein (PLC ζ^{H398P}) abolished these oscillations, indicating that the H398P mutation also causes loss of PLC activity of PLC ζ . It was possible to purify PLC ζ protein from a prokaryotic system, but this did not retain activity. It is possible that this was due to the absence of required PTMs, as is suggested by the injection of transfected eukaryotic cell lysates into mouse oocytes. Furthermore, it was not possible to purify PLC ζ protein from cell lysates obtained from transfected HEK293T cells, most likely due to a non-domain C-terminal truncation, which led to the absence of C-terminal tags. Furthermore, the current data indicate a key role for interactions with sperm or oocyte based factors for PLC ζ to obtain Ca²⁺ inducing activity.

It is important that future studies continue the efforts of this chapter in order to purify PLC ζ from these cell lysates in an active state before use in the clinic as an AOA. It may be prudent to switch purification tags from the C- to the N-terminus to avoid possible truncation problems. It may also be beneficial to utilise vectors containing secretion signals, inducing transcribed protein to be secreted into the culture medium, enabling easier purification and potentially a higher yield. Another interesting avenue of research would be to investigate whether RNA transcripts within sperm and testes are also prone to similar truncations, or whether this is a mechanism exclusive to the HEK293T cells utilised in this study. It is crucial to investigate whether such truncation ‘activates’ the PLC

activity of PLC ζ , or whether such a mechanism represents a negligible side effect of an *in-vitro* expression system. Importantly, however, it would be critical for the lysates produced by this study, as well as future purified versions to be tested with human oocytes to establish the efficacy of human PLC ζ protein as a viable replacement for current AOA protocols and agents. Such questions need to be answered urgently before the therapeutic viability of PLC ζ can be fully utilised within the clinic.



CHAPTER 7

Functional characterisation of wild type
and mutant isoforms of PLC ζ in HEK293T
cells

7.1 Introduction

Compelling evidence now implicates failure of the sperm to activate the oocyte in an appropriate manner in certain types of human male infertility (Yoon *et al.*, 2008; Heytens *et al.*, 2009). Molecular analysis of the genomic DNA encoding PLC ζ in human males diagnosed with oocyte activation deficiency previously identified a point mutation in one patient that resulted in the substitution of a conserved histidine residue at position 398 of the protein sequence for a proline residue (PLC ζ^{H398P}), leading to abnormal Ca²⁺ release profiles and reduced oocyte activation efficiency and providing the first genetic link between oocyte activation failure and PLC ζ deficiency (Heytens *et al.*, 2009). Similarly, utilising a modified version of the protocol employed by Heytens *et al.* (2009), Chapters 3 and 4 herein identified a novel mutation in PLC ζ from the same patient, resulting in the substitution of a histidine residue to a leucine residue at position 233 (PLC ζ^{H233L}) of the PLC ζ ORF, which negatively affected PLC ζ activity in a manner similar to PLC ζ^{H398P} .

While numerous studies support PLC ζ as the endogenous mammalian sperm factor (Saunders *et al.*, 2002; Fujimoto *et al.*, 2004; Knott *et al.*, 2005; Kouchi *et al.*, 2005; Kurokawa *et al.*, 2005), many questions remain regarding the spatial and temporal mechanism of action of human PLC ζ , particularly within the activating oocyte. Following fertilisation in mouse oocytes, Ca²⁺ oscillations cease at the time of pronuclei formation (Marangos *et al.*, 2003) with subsequent oscillations observed in mouse zygotes during mitosis (Carroll, 2001; Marangos *et al.*, 2003). Such cell-cycle dependant termination and resumption, may be explained by the localisation of mouse PLC ζ to the pronuclei during interphase owing to a nuclear localisation signal (NLS) within the protein. Resumption of oscillations occurs following pronuclear envelope breakdown during the fertilised oocyte's

entry into mitosis (Deguchi *et al.*, 2000; Larman *et al.*, 2004; Yoda *et al.*, 2004; Sone *et al.*, 2005; Kuroda *et al.*, 2006; Swann *et al.*, 2006; Ito *et al.*, 2008). While it remains possible that this represents a mechanism for sequestering mouse PLC ζ within the nucleus in the zygote and possibly implies a functional role within the nucleus itself, human PLC ζ does not translocate to the pronucleus when injected into mouse oocytes (Ito *et al.*, 2008b), and thus the relevance of these findings for human fertilisation remains unclear. While the functional and physiological properties of the mouse PLC ζ NLS have been exhaustively investigated (Larman *et al.*, 2004; Yoda *et al.*, 2004; Sone *et al.*, 2005; Kuroda *et al.*, 2006), similar studies have yet to be performed on any such human PLC ζ region. However, human PLC ζ does not yet have a confirmed NLS within its ORF, possessing only a theorised region which may act in a manner similar to the mouse NLS (Nomikos *et al.*, 2005; 2007; Saunders *et al.*, 2007).

The injection of fluorescently tagged human and mouse PLC ζ cRNA into mouse oocytes demonstrated a universal distribution of PLC ζ throughout the oocyte cytoplasm, suggesting that its substrate PIP₂ may be targeted at intracellular membranes (Rice *et al.*, 2000; Yoda *et al.*, 2004; Sone *et al.*, 2005; Kuroda *et al.*, 2006; Ito *et al.*, 2008; Yu *et al.*, 2008a; 2008b). Furthermore, the precise status of PLC ζ within sperm is not yet clear, as is the means by which PLC ζ is maintained in an active state within such gametes until fertilisation, or whether PLC ζ needs to be ‘activated’ in some way prior to fertilisation to allow it to perform its PLC activity.

Without a PH domain, it remains unclear how PLC ζ targets its membrane-bound substrate PIP₂. Intriguingly, Lee *et al.* (2004) suggested that the PH domain may not be integral to the membrane localising ability of PLC δ 4, suggesting that the PH domain may not be an absolute requirement for PLC localisation to its substrate. Mammalian PLC ζ isoforms have four EF-hand motifs at their N-termini, which appear to play an important structural role in PIP₂ targeting and enzyme activity (Fujimoto *et al.*, 2004; Kouchi *et al.*, 2004; Yoda *et al.*, 2004; Kurokawa *et al.*, 2005; Nomikos *et al.*, 2005; Swann *et al.*, 2006; Nakanishi *et al.*, 2008). Interestingly, however, Bi *et al.* (2009) claimed the presence of a novel isoform of PLC ζ , which they inexplicably named NYD-SP27, within the acrosome of mouse and human sperm. This isoform was thought to be lacking the four EF hands from the N-terminus (PLC $\zeta^{\Delta\text{EF}}$), and was proposed to play a role in capacitation and the acrosome reaction. However, while this role was previously proposed for the acrosomal population of PLC ζ by Grasa *et al.*, (2008) and Young *et al.*, (2009) for human and mouse sperm respectively, further evidence is required before the existence a specific role of such an isoform can be confirmed.

Thus, the aims of this chapter were to examine localisation patterns of fluorescently tagged human PLC ζ isoforms in HEK293T cells, utilising wild type PLC ζ , PLC ζ^{H398P} , and the novel mutation (PLC ζ^{H233L}) previously identified and characterised in Chapters 3 and 4 of this thesis. The present study also utilised an artificially induced frameshift mutation, causing a partial Y and complete C2 domain deletion (PLC $\zeta^{\Delta\text{YC2}}$) to characterise their differential response to treatments in comparison to PLC ζ^{WT} . In addition, the response of HEK293T cells transfected with these PLC ζ isoforms to ATP-induced Ca²⁺ release was also investigated, in order to ascertain their respective activity upon over-expression in HEK293T cells. Localisation patterns of a fluorescently tagged version of PLC $\zeta^{\Delta\text{EF}}$, and a

tagged version of just the EF hands of PLC ζ (PLC ζ^{EF}), were also investigated in order to determine the possible role of the EF hands for PLC ζ localisation. Collectively, these experiments aimed to further understanding of the molecular mechanisms of activity and regulation of PLC ζ , which could be important in the development of PLC ζ as a therapeutic agent for treatment of oocyte activation failure.

7.2 Materials and Methods

7.2.1 Molecular cloning of fluorescently tagged constructs

For more detailed information on the molecular constructs used herein, refer to Table 7.1. Fluorescent mCherry tags were N-terminally fused via a linker sequence, containing a *XhoI* restriction site, to PLC ζ^{WT} and two mutant isoforms (PLC ζ^{H398P} and PLC $\zeta^{\Delta\text{YC2}}$), which were cloned previously by Dr. Margarida Ruas (University of Oxford, UK). mCherry tagged versions of PLC ζ^{H233L} and PLC $\zeta^{\text{H233L+H398P}}$ were created via site-directed mutagenesis using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, USA) following the manufacturers protocol and using mCherry-PLC ζ^{WT} and mCherry-PLC ζ^{H398P} constructs as templates respectively as described in Chapter 2, Section 2.8. For schematics of the PLC ζ isoforms utilised to create mCherry-tagged constructs, refer to Supplementary Appendix 6. For details of the primers used for site directed mutagenesis, refer to Table 7.2. To create the mCherry tagged version of PLC $\zeta^{\Delta\text{EF}}$ and PLC ζ^{EF} , the corresponding sequences were amplified using PCR (as previously described in Chapter 2, Section 2.3) with primer sets and restriction enzymes described in Table 7.3, using mCherry-PLC ζ^{WT} as a template.

7.2.2 Mammalian cell culture

HEK293T cells were cultured under the conditions previously described in Chapter 2 (Section 2.10), to perform the majority of experiments within this Chapter.

Table 7.1: The various constructs utilised throughout this chapter, showing PLC ζ isoform, vector used, and system targeted. Asterisk (*) denotes constructs provided by Dr. Margarida Ruas. All other constructs were created by JK, and verified by DNA sequencing.

Construct name	Vector used	Description	Cell line Transfected
*mCherry-PLCζ^{WT}	pcDNA 5/T0	Wild Type Human Recombinant PLC ζ fused with N-Terminal mCherry fluorescent protein	HEK293T
*mCherry-PLCζ^{H398P}	pcDNA 5/T0	H398P mutant Human Recombinant PLC ζ fused with N-Terminal mCherry fluorescent protein	HEK293T
*mCherry-PLCζ^{ΔYC2}	pcDNA 5/T0	Δ YC2 mutant Human Recombinant PLC ζ fused with N-Terminal mCherry fluorescent protein	HEK293T
*mCherry empty vector	pcDNA 5/T0	pcDNA 5/T0 with only mCherry sequence inserted and no PLC ζ	HEK293T
mCherry-PLCζ^{H233L}	pcDNA 5/T0	H233L mutant Human Recombinant PLC ζ fused with N-Terminal mCherry fluorescent protein	HEK293T
mCherry-PLCζ^{H233L+H398P}	pcDNA 5/T0	Human Recombinant PLC ζ containing both H233L and H398P mutations fused with N-Terminal mCherry fluorescent protein	HEK293T
mCherry-PLCζ^{ΔEF}	pcDNA 5/T0	Human Recombinant PLC ζ lacking all four N-terminal EF hands fused with N-Terminal mCherry fluorescent protein	HEK293T
mCherry-PLCζ^{EF}	pcDNA 5/T0	Only the N-terminal EF hands of Human Recombinant PLC ζ fused with N-Terminal mCherry fluorescent protein	HEK293T

Table 7.2: Summary of constructs created via site-directed mutagenesis, and the templates and primers used to create them.

Construct name	Template used	Primer sequences
mCherry-PLCζ^{H233L}	mCherry-PLC ζ ^{WT}	5'-TTATCCAAGCTATACTCAAGTATGCATT CAT-3' (Forward) 5'-ATGAATGCATACTTGAGTATAGCTTGGATAA-3' (Reverse)
mCherry-PLCζ^{H233L+H398P}	mCherry-PLC ζ ^{H398P}	5'-TTATCCAAGCTATACTCAAGTATGCATT CAT-3' (Forward) 5'-ATGAATGCATACTTGAGTATAGCTTGGATAA-3' (Reverse)

Table 7.3: Summary of primer sequences and restriction enzymes used during the molecular cloning of mCherry-PLC $\zeta^{\Delta EF}$ and mCherry-PLC ζ^{EF} isoforms.

Construct name	Restriction sites	Primer sequences
mCherry-PLC $\zeta^{\Delta EF}$	5'- <i>XhoI</i> 3'- <i>Apal</i>	5'-TATCTCGAGTCGAAGAAATTATTGAGA-3' (Forward) 5'-TATGGGCCCTTATCTGACGTACCAAAC-3' (Reverse)
mCherry-PLC ζ^{EF}	5'- <i>BamHI</i> 3'- <i>Apal</i>	5'-TATGGATCCATGGTGAGCAAGGGCGAG-3' (Forward) 5'-TATGGGCCCTTATCTGTGCGTGATAAT-3' (Reverse)

7.2.3 Imaging Ca²⁺ release in live HEK293T cell preparations

HEK293T cells were grown on poly-D-lysine (Sigma, UK)-treated glass coverslips in 6-well plates (Nuclon, Denmark) and transfected with mCherry constructs as previously described (Chapter 2, Section 2.10). Forty eight hours after transfection, cells were loaded with 2 μ M fura-2/AM in 10% pluronic acid (Invitrogen, UK) for 30 minutes at RT, followed by a 30 minute de-esterification period. Coverslips were mounted on an imaging chamber and placed on an Olympus IX71 microscope (20x water immersion objective). Fura-2/AM fluorescence was monitored using 340 and 380nm dual wavelength excitation, and emission data were collected at >510 nm. Reference images were captured by excitation at 560nm and measuring emission at wavelengths >610nm to visualise transfected cells. Data acquisition was performed at 2.5 second intervals and the 340/380 ratio calculated by OptoFluor 7.6.3.0 software (Cairn Imaging, UK). Coverslips were super-fused manually with a Ca²⁺ containing buffer (121mM NaCl, 5.4mM KCl, 0.8mM MgCl₂, 2mM CaCl₂, 6mM NaHCO₃, 5.5mM D-Glucose, 25mM HEPES, pH = 7.3), and observed for 2-3 minutes to assess baseline stability. Adenosine triphosphate (ATP) was

applied for periods of 3 minutes at 1, 5 and 100 μ M concentrations followed by 5 minute perfusions with Ca²⁺ buffer to allow re-filling of Ca²⁺ stores.

Intracellular Ca²⁺ store integrity was also explored by depleting Ca²⁺ stores using thapsigargin (TG), an inhibitor of Ca²⁺ uptake by sarcoplasmic/endoplasmic reticulum Ca²⁺ ATPase (SERCA). Cell and coverslip preparation, along with subsequent imaging, were carried out as described previously in this section. Coverslips were super-fused manually with a Ca²⁺ free buffer containing EGTA (121mM NaCl, 5.4mM KCL, 0.8mM MgCl₂, 1mM EGTA, 6mM NaHCO₃, 5.5mM D-Glucose, 25mM HEPES, pH = 7.3), and incubated for 5 minutes to observe baseline stability. Coverslips were then super-fused with 5 μ M LaCl₃ (an inhibitor of Ca²⁺ transport across the plasma membrane) in Ca²⁺ free buffer, and incubated for a further 1 minute to restore baseline stability. Subsequently, 5 μ M TG in Ca²⁺ free buffer containing LaCl₃ was applied until a drop in the observed Ca²⁺ was observed, at which point coverslips were super-fused with LaCl₃ in Ca²⁺ free buffer without TG. One further replicate was carried out with each construct at room temperature and Ca²⁺ signalling monitored only in cells confirmed to have been transfected (i.e. exhibiting mCherry fluorescence). At least 100 cells were analysed per construct.

Post-acquisition analysis was performed using Prism 5.0 (GraphPad, USA). All experiments were performed at room temperature, and Ca²⁺ was monitored only in cells confirmed to have been transfected (i.e. those cells exhibiting mCherry fluorescence). At least 100 cells were analysed per construct per coverslip. Three coverslips were analysed per construct, and the experiment was repeated on three separate days. Statistical significance was determined by one way ANOVA followed by Bonferroni post-hoc testing. It was only possible to perform one repeat of TG stimulation, and thus it was not

possible to determine statistical significance on this occasion. Consequently, data are presented as mean $\pm$ SEM for only 'area under curve' analysis, and not the percentage of responding cells.

7.2.4 Immunoblotting

50 μ g of cell lysate (prepared as described in Chapter 2, Section 2.11) was loaded and separated on 4-12% Tris-Glycine gels (Invitrogen, UK) and immunoblotting performed as previously described in Chapter 2, Section 2.16, using a monoclonal anti-human primary antibody against the type I IP₃ receptor (IP₃R-1; the main receptor involved in Ca²⁺ release during oocyte activation; Wakai *et al.*, 2011) raised in mouse (1:5000; Millipore, UK), and with a horseradish peroxidase (HRP) conjugated anti-mouse IgG secondary antibody (1:25000; Millipore, UK) and an anti-human HRP conjugated primary antibody against β -actin as a loading control (1:15000; Sigma-Aldrich, UK). Band intensity was determined through relative densitometry using ImageJ as previously described in Chapter 6, Section 6.2.10.

7.2.5 Analysis of transfected HEK293T cells by fluorescent and confocal microscopy

HEK293T cells were grown on 25x75x1mm glass coverslips in 4-well plates (VWR, UK) and transfected at ~60% confluency with mCherry constructs (1 μ g) using the jetPEI kit (Poly-plus transfection, France), following the manufacturer's instructions as previously described in Chapter 2 Section 2.10. Control cells were treated in an identical fashion. Cells were analysed at time-points of 8, 24, 36, 48, and 56 hours post-transfection to determine the point of maximal transfection and transcription (as described in Chapter 2, Section 2.19). Fixed cells were additionally incubated for 10 minutes at room temperature (RT) with 10 μ g/ml Alexa Fluor 488, (or Alexa Fluor 555 where indicated)

wheat germ agglutinin conjugate (WGA, Invitrogen U.K.) to stain the plasma membrane. To stain the endoplasmic reticulum (ER) instead of the plasma membrane, live cells on the coverslips were incubated with 5 μ M ER-Tracker Green (Invitrogen, UK) for 30 minutes at 37°C following removal of media and an initial HBSS washing step. Coverslips were then washed with HBSS a second time and fixed as described earlier (Chapter 2, Section 2.19). Coverslips were mounted with Vectashield mounting medium containing 4'-6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, USA) and slides stored at 4°C. Fluorescence levels in HEK293T cells transfected with mCherry constructs were imaged using a Nikon Eclipse 80i fluorescence microscope (20x objective) using the DAPI (nuclei), FITC (WGA-Alexa Fluor 488), and TRITC (WGA-Alexa Fluor 555) filters. All PLC ζ constructs exhibited ~70% transfection throughout the experimentation.

Total fluorescence per cell was quantified using Image J (NIH, USA) for at least 300 cells per construct. Only cells visibly exhibiting red mCherry fluorescence were analysed, which indicated successful expression of recombinant protein. Transfected cells were additionally imaged by confocal microscopy using a 63x oil immersion objective (Zeiss, Germany) to examine intracellular localisation in a higher resolution. Wavelengths utilised were 350nm (DAPI), 488nm (WGA-Alexa Fluor 488), and 560nm (mCherry-PLC ζ isoform fluorescence/WGA-Alexa Fluor 555). Data were acquired and analysed by the Zeiss LSM Image browser (Zeiss, Germany). Statistical significance between fluorescence intensity for different PLC ζ isoforms was determined by one way ANOVA and Bonferroni *post-hoc* testing using Prism 5.0 (Graphpad, USA).

7.3 Results

7.3.1 Live cell Ca^{2+} imaging of transfected HEK293T cells

HEK293T cells transfected with mCherry-PLC ζ^{WT} exhibited a significantly reduced response to intermediate and high concentrations of ATP (5 μM and 100 μM respectively), as compared to cells transfected with other mCherry constructs (Figure 7.1). The number of responsive cells transfected with mCherry-empty vector, mCherry-PLC ζ^{H398P} , and mCherry-PLC $\zeta^{\Delta\text{YC2}}$ was significantly higher than mCherry-PLC ζ^{WT} transfected cells upon stimulation with 5 μM ATP (86%, 85%, 86%, and 19% respectively) and 100 μM ATP (96%, 95%, 95%, and 23% respectively). Cells transfected with mCherry-empty vector, mCherry-PLC ζ^{H398P} and mCherry-PLC $\zeta^{\Delta\text{YC2}}$ also exhibited a greater amplitude of ATP-induced responses than mCherry-PLC ζ^{WT} transfected cells at concentrations of 5 μM ATP (normalised 340/380 ratios of 2.033, 1.856, 2.23, and 0.68 respectively) and 100 μM ATP (normalised 340/380 ratios of 2.56, 2.28, 2.23 and 0.7 respectively). Moreover, cells transfected with mCherry-empty vector, mCherry-PLC ζ^{H398P} and mCherry-PLC $\zeta^{\Delta\text{YC2}}$ also exhibited increased amounts of Ca^{2+} release (determined by calculating the area under the curve, AUC) compared to those transfected with mCherry-PLC ζ^{WT} when stimulated with 5 μM ATP (60.55, 62.95, 64.08, and 5.72 respectively) and 100 μM ATP (118.09, 103.76, 120.63 and 8.44 respectively) (Figure 7.2). The number of responsive cells transfected with mCherry-empty vector, mCherry-PLC ζ^{H398P} , and mCherry-PLC $\zeta^{\Delta\text{YC2}}$ was higher than mCherry-PLC ζ^{WT} transfected cells upon TG stimulation (Figure 7.3) (95%, 100%, 100%, and 67.95% respectively). Moreover, cells transfected with mCherry-empty vector, mCherry-PLC ζ^{H398P} and mCherry-PLC $\zeta^{\Delta\text{YC2}}$ also demonstrated increased amounts of Ca^{2+} release (determined by calculating the area under the curve, AUC) compared to those

transfected with mCherry-PLC ζ^{WT} when stimulated with TG (115.50, 84.23, 123.59, and 33.02 respectively) (Figure 7.4).

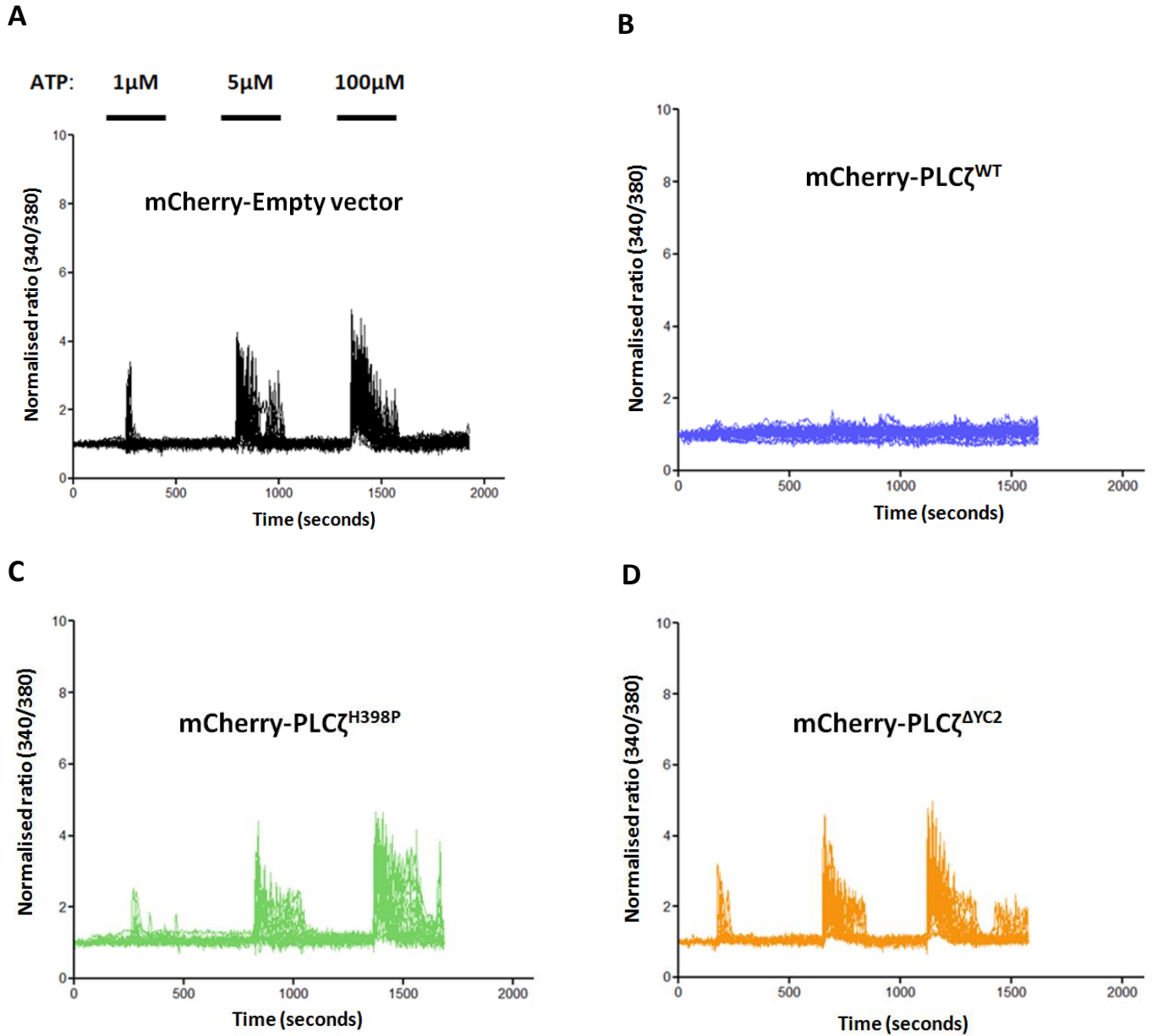


Figure 7.1: Representative Ca^{2+} responses of individual HEK293T cells transfected with mCherry (A) -empty vector, (B) -PLC ζ^{WT} , (C) -PLC ζ^{H398P} and (D) -PLC $\zeta^{\Delta\text{YC2}}$ constructs stimulated with 1μM, 5μM, and 100μM of ATP.

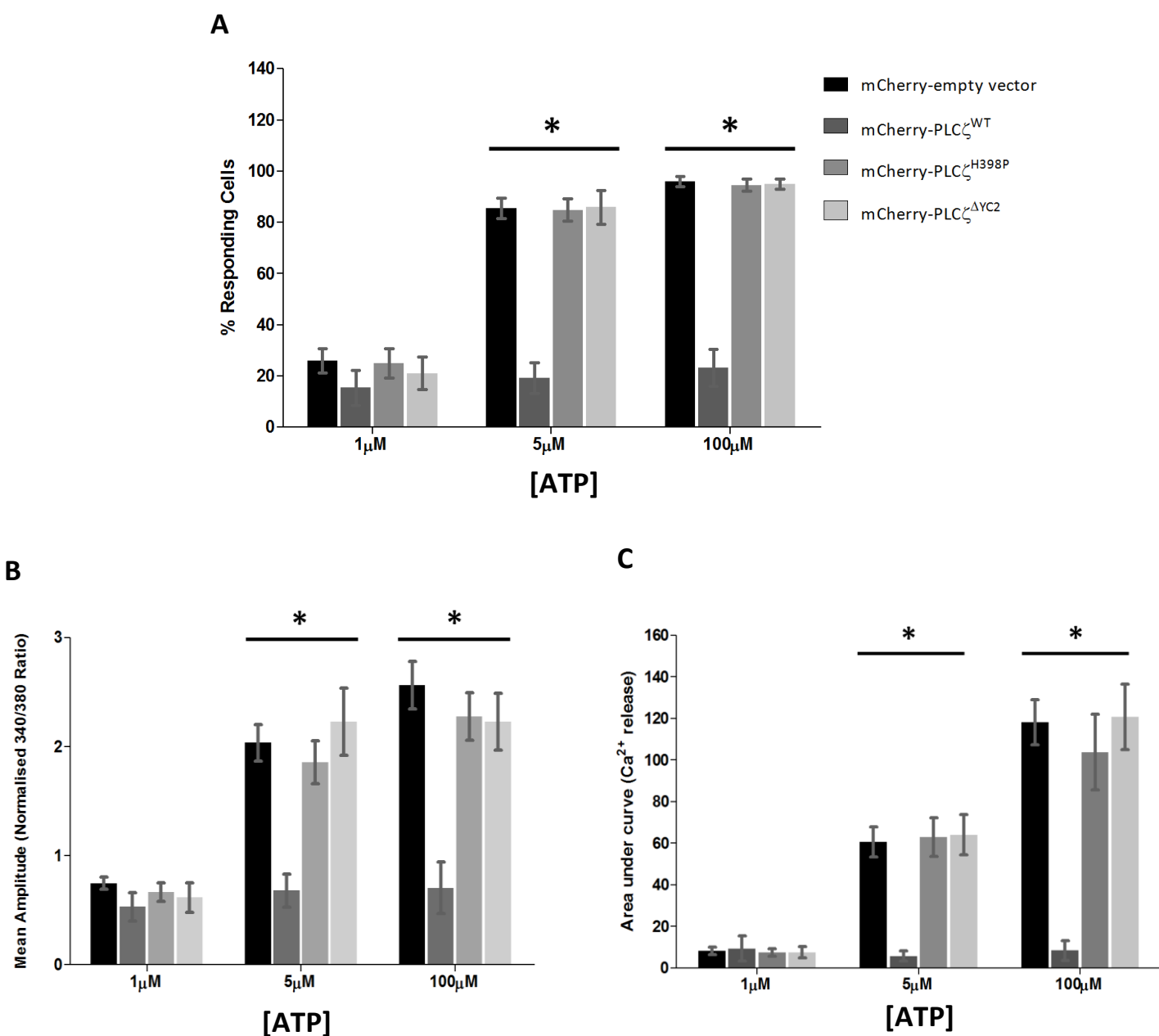


Figure 7.2: **A)** Mean data illustrating the proportion of cells responding to different ATP concentrations for cells transfected with mCherry-PLC ζ constructs. Data represent mean $\pm$ SEM. Asterisk (*) indicates $p < 0.05$. Statistical analysis was performed on arcsin transformed values of the proportions of responding cells. **B)** Mean data from three independent experiments showing the amplitude of Ca^{2+} responses of HEK293T cells transfected with mCherry-PLC ζ constructs in response to different ATP concentrations. Mean amplitude is shown for each ATP concentration. Data represent mean $\pm$ SEM. Asterisk (*) indicates $p < 0.05$. **C)** Mean from three independent experiments showing the amount of Ca^{2+} release by HEK293T cells transfected with mCherry-PLC ζ constructs in response to the different ATP concentrations, as calculated by the area under the curve (AUC) for each ATP addition. Mean AUC is shown for each ATP concentration. Data represent mean $\pm$ SEM. Asterisk (*) indicates $p < 0.001$.

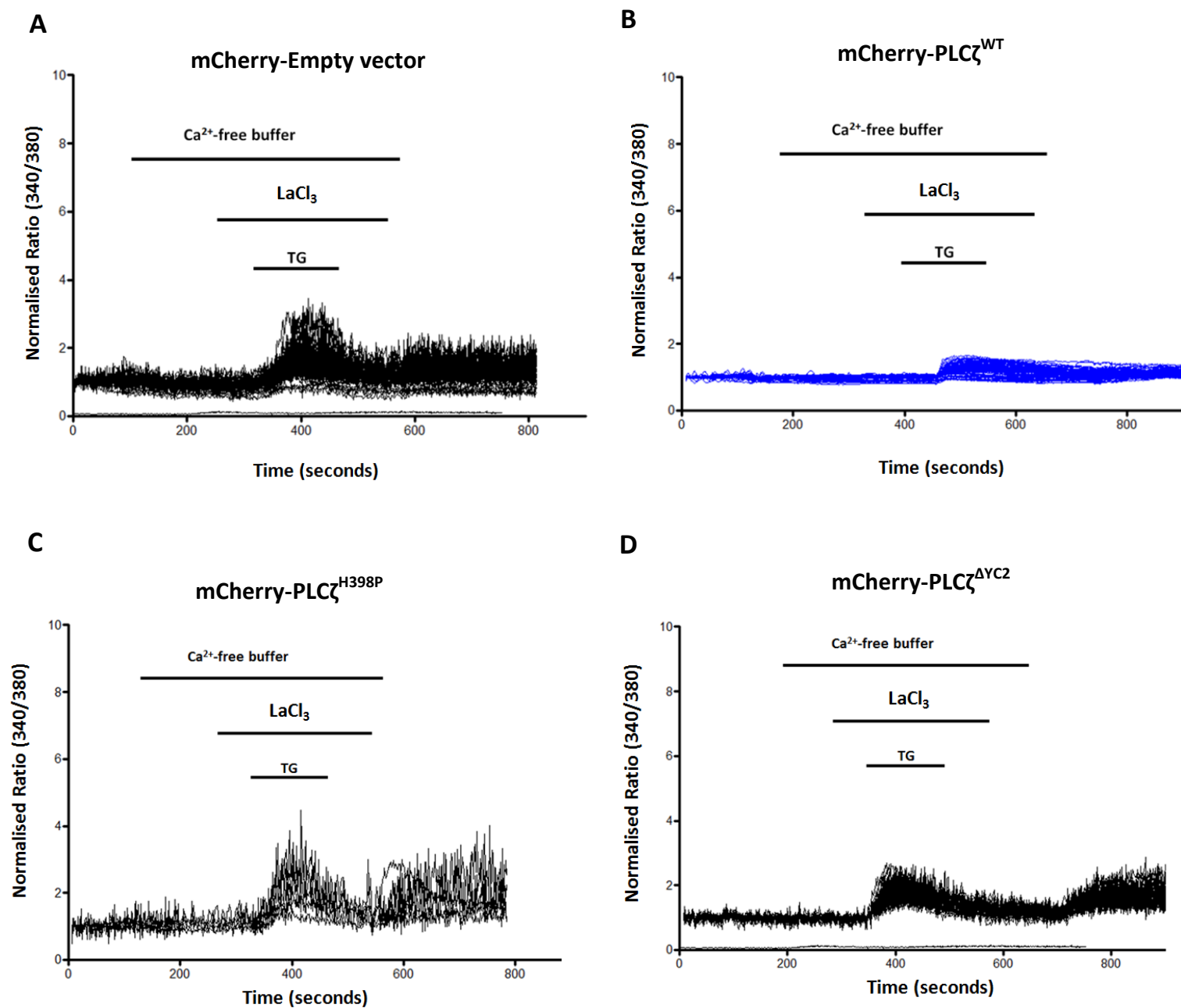


Figure 7.3: Representative Ca²⁺ responses of individual HEK293T cells transfected with mCherry (A) -empty vector, (B) -PLC ζ^{WT} , (C) -PLC ζ^{H398P} and (D) -PLC $\zeta^{\Delta\text{YC2}}$ constructs stimulated with 5 μM Thapsigargin (TG). TG stimulation was performed under Ca²⁺ free conditions using LaCl₃, an inhibitor of Ca²⁺ transport across the plasma membrane, and a Ca²⁺ free buffer. TG: Thapsigargin

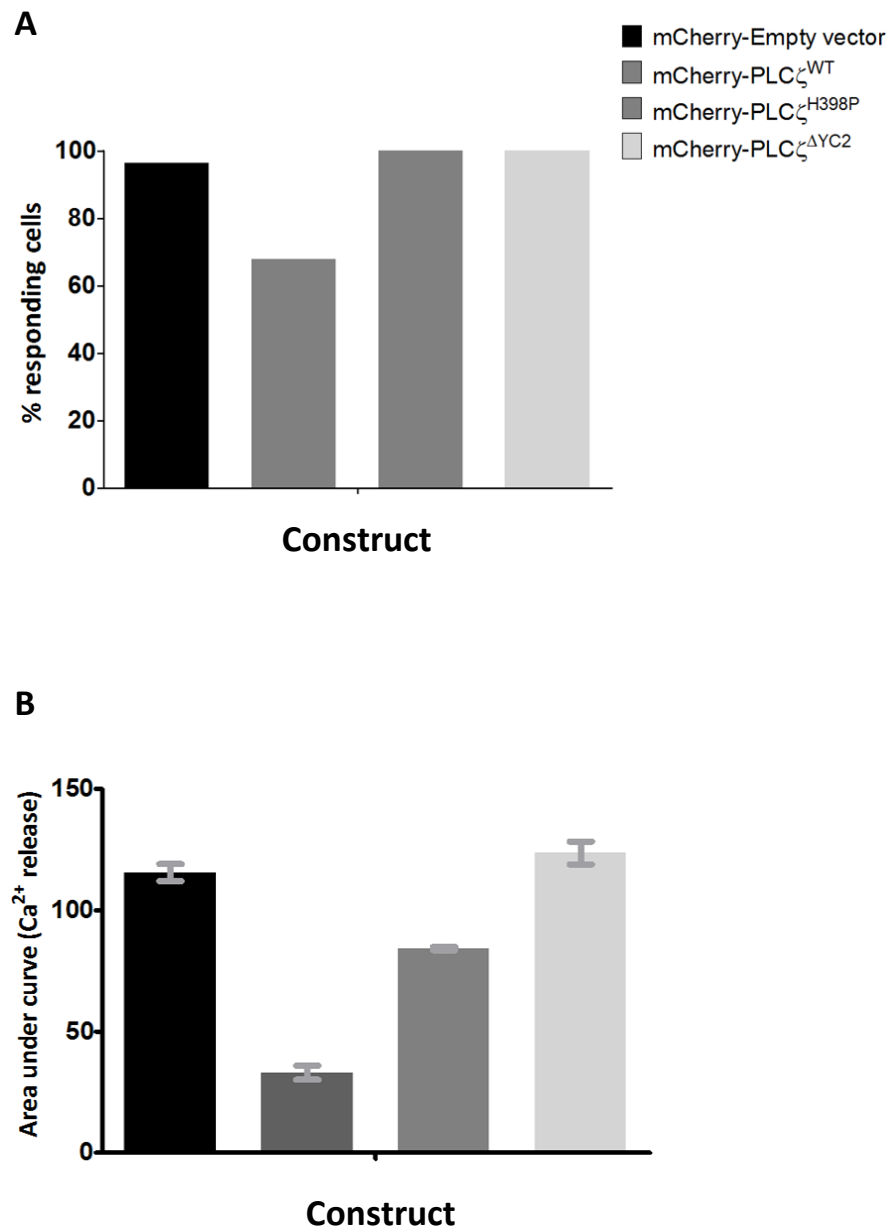


Figure 7.4: **A)** Mean data illustrating the proportion of cells responding to TG stimulation for cells transfected with mCherry-PLC ζ constructs. **B)** The amount of Ca²⁺ release by HEK293T cells transfected with mCherry-PLC ζ constructs in response to the different ATP concentrations, as calculated by the area under the curve (AUC) for TG stimulation. Mean AUC is shown for each PLC ζ isoform. Data represent mean $\pm$ SEM.

7.3.2 IP₃R-1 immunoblotting

Immunoblotting suggested that total IP₃R-1 levels of cells transfected with pTracer-PLC ζ^{WT} were marginally reduced compared to those transfected with pTracer-empty vector, while cells transfected with pTracer-PLC ζ^{H398P} exhibited a relatively larger reduction compared to those transfected with pTracer-empty vector or pTracer-PLC ζ^{WT} (Figure 7.5). Equality of loading was demonstrated with a β -actin loading control (refer to Supplementary Appendix 10).

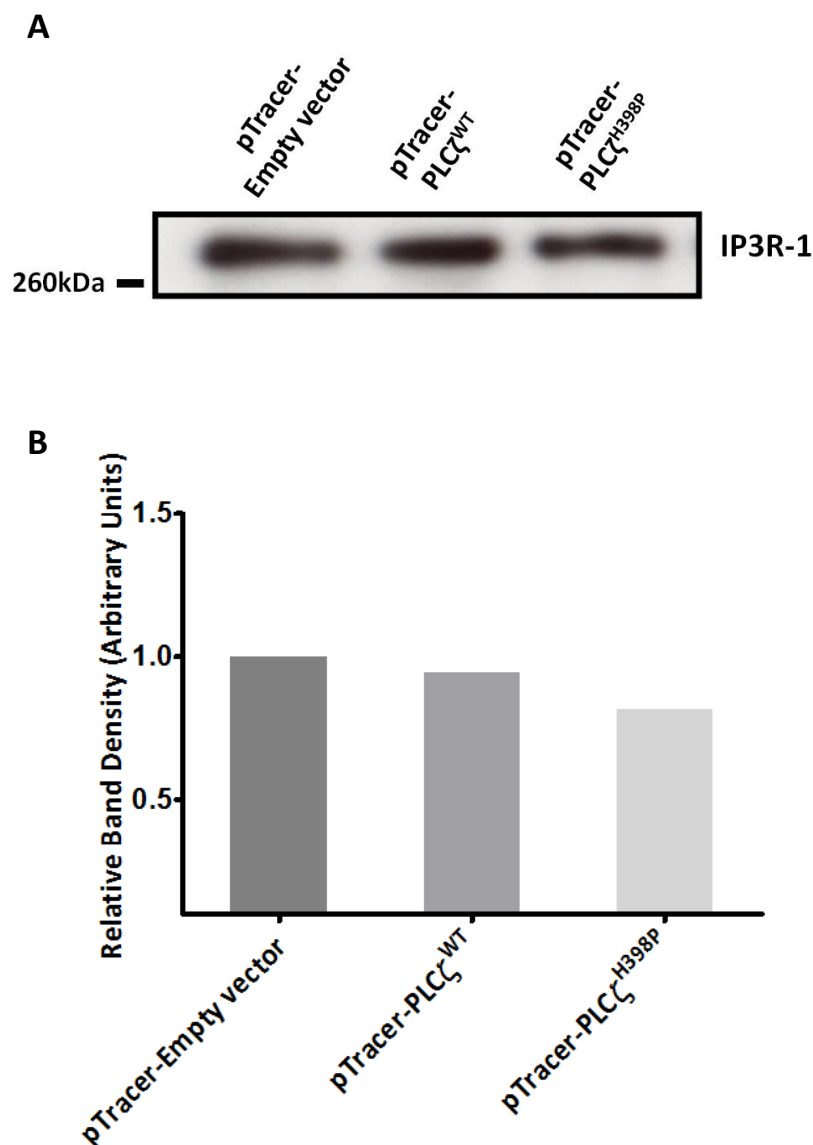


Figure 7.5: **A)** Representative immunoblotting using an IP₃R-1 antibody. **B)** Quantification of immunoblotting bands using relative densitometry

7.3.3 Fluorescence quantification and intracellular localisation of mCherry-PLC ζ isoforms in HEK293T cells

A reduced level of fluorescence was observed for HEK293T cells transfected with mCherry-PLC ζ^{H398P} compared to mCherry-PLC ζ^{WT} transfected cells (Figure 7.6). For both constructs, the fluorescent signal became visible from 24 hours post-transfection, reaching maximal levels at 48 and 56 hours. One-way ANOVA determined that all fluorescence values were significantly different ($p \leq 0.05$) (Figure 7.7). The Bonferroni post *hoc* multiple comparisons test indicated that fluorescence levels varied significantly between constructs, such that PLC ζ^{WT} > all mutant isoforms at both 48 and 56 hour time points ($p < 0.05$). Mutant isoforms did not exhibit any significant differences between each other apart from PLC ζ^{H398P} > PLC $\zeta^{\text{H233L+H398P}}$ at the 48 hours timepoint ($p \leq 0.05$).

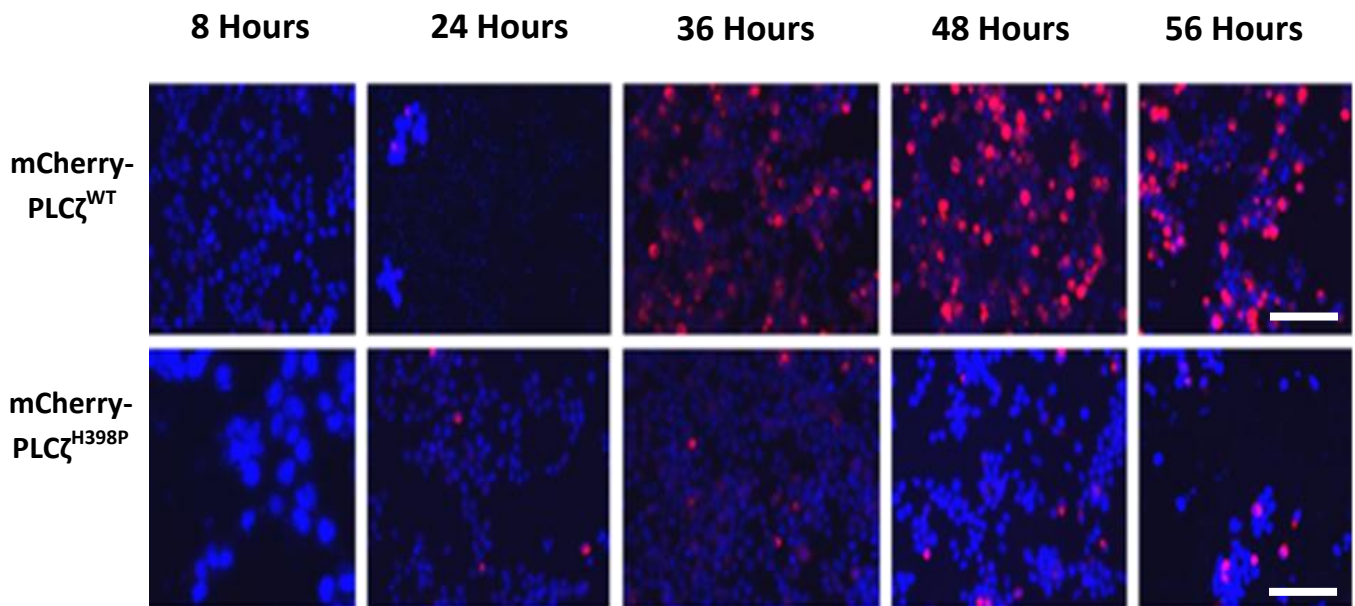


Figure 7.6: Representative fluorescence microscopy image taken across 8, 24, 36, 48, and 56 hour time points for HEK293T cells transfected with mCherry-PLC ζ constructs. Images were captured at a magnification of 10X, and were repeated 3x3 times. Images are overlays of DAPI and TRITC fluorescence. White scale bars indicate 50 μm .

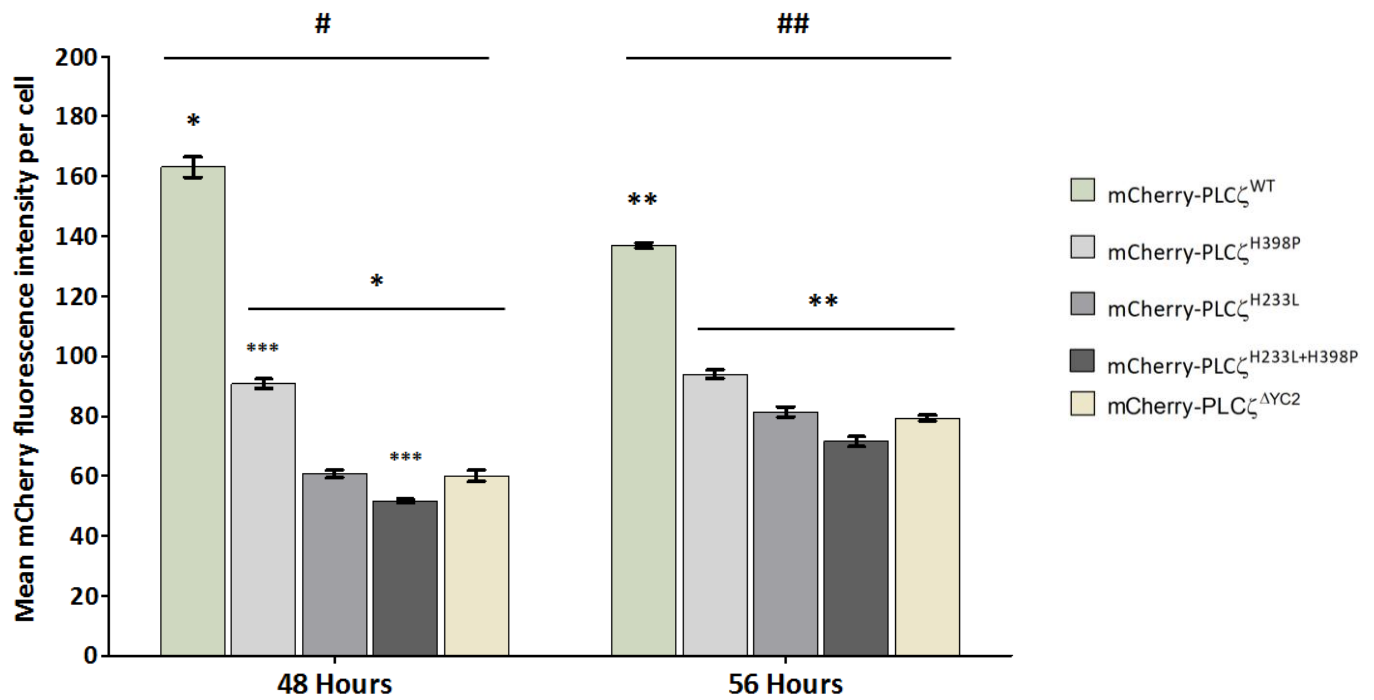


Figure 7.7: Mean mCherry fluorescence intensity per cell for HEK293T cells transfected with mCherry-PLC ζ^{WT} , -PLC ζ^{H398P} , -PLC ζ^{H233L} , -PLC $\zeta^{H233L+H398P}$, and -PLC $\zeta^{\Delta YC2}$ at 48 and 56 hour time points. 300 cells were analysed per construct. Data are shown as mean $\pm$ SEM. Asterisks (*, **, ***) and Hash (#, ##) indicate statistically significant differences ($p < 0.05$).

WGA staining of the plasma membrane indicated that all PLC ζ isoforms localised to discrete regions in the cell cytoplasm and not the plasma membrane (Figure 7.8; 7.10). Cytoplasmic localisation appeared to be concentrated to regions adjacent to the nuclear envelope with a reticular network, suggesting localisation to specific cytoplasmic organelles. mCherry-empty vector exhibited extensive expression throughout transfected cells (Figure 7.9).

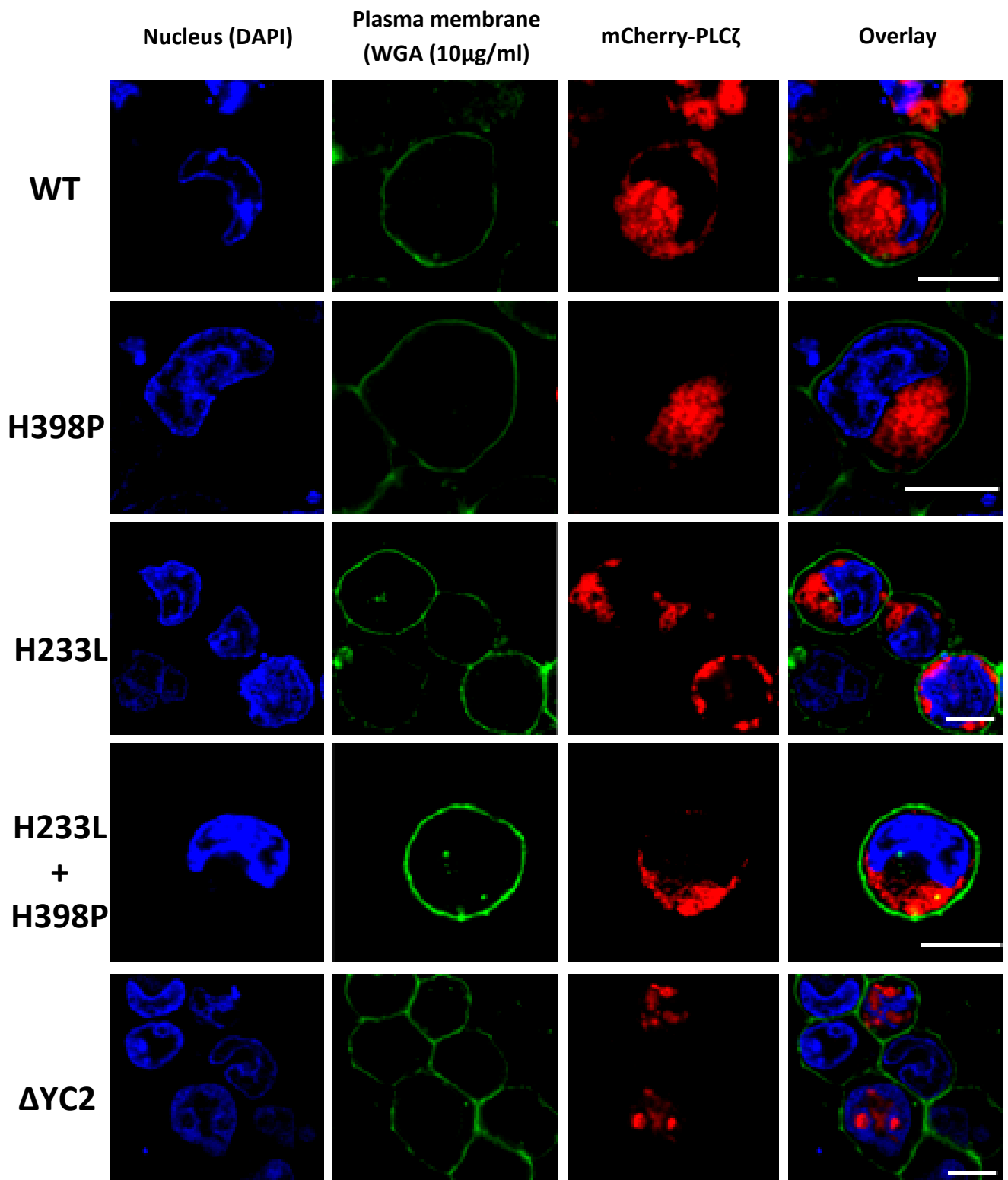


Figure 7.8: Representative single plane confocal microscopy images of 48 and 56 hour time points for HEK293T cells transfected with mCherry-PLC ζ constructs using a plasma membrane stain. Images are representative of 300 cells studied for each construct, and were captured at a magnification of 63x. White scale bars indicate 10 μ m.

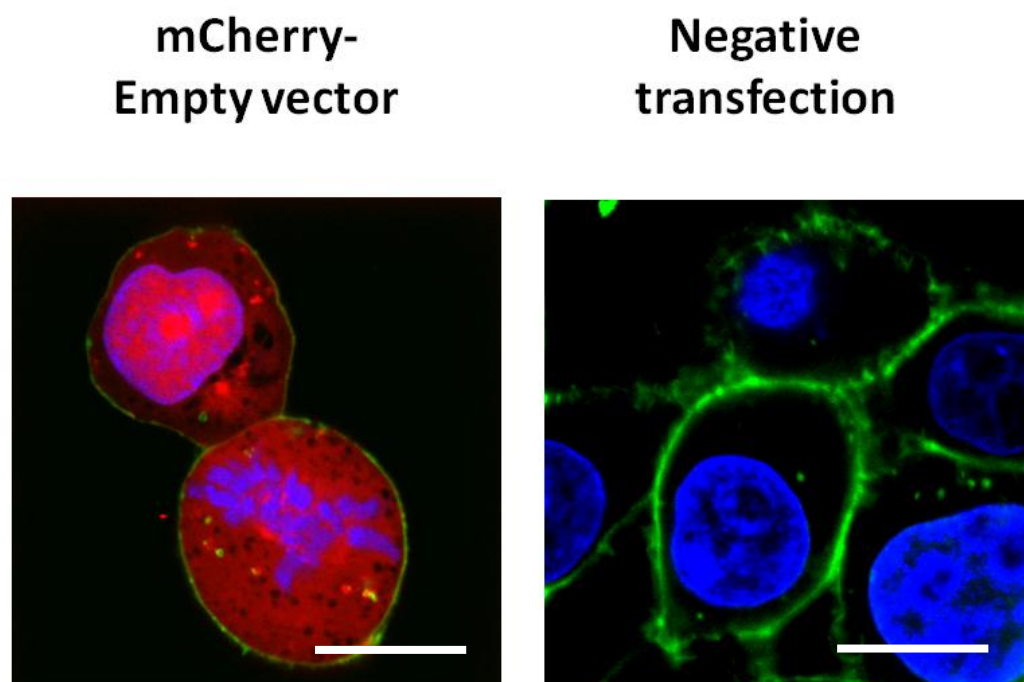


Figure 7.9: Representative single plane confocal microscopy images of cells transfected with mCherry-empty vector and negative transfected cells using a plasma membrane stain. Red: mCherry; Blue: Nucleus (DAPI); Green plasma membrane (WGA). Images are representative of 300 cells studied for each construct, and were captured at 63x optical zoom, and enlarged following image capture.

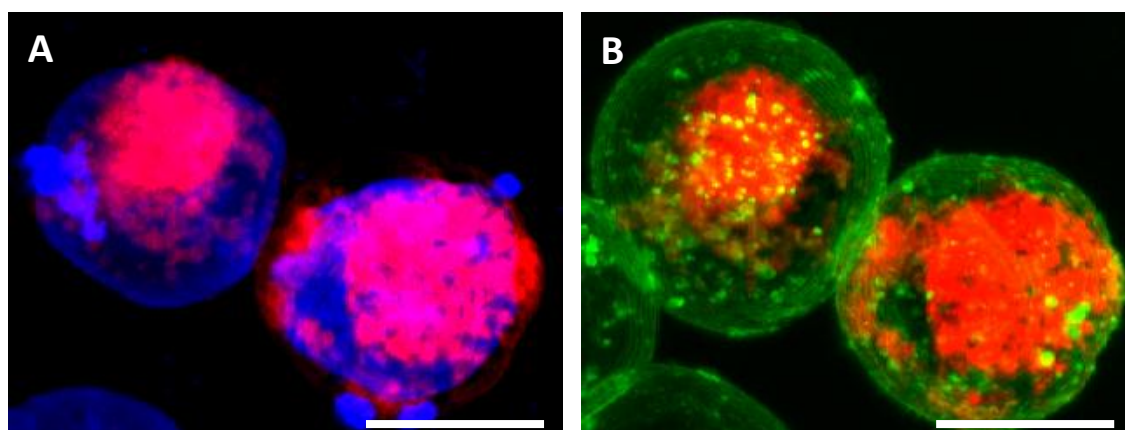


Figure 7.10: Representative 3D projections, obtained by reconstructing Z-stack images obtained by confocal microscopy, of HEK293T cells transfected with mCherry-PLC ζ^{WT} using a plasma membrane stain. **A)** Overlay image of nuclei (blue; DAPI) and PLC ζ^{WT} (red; mCherry), showing the peri-nuclear reticular localisation of PLC ζ . **B)** Overlay image of plasma membrane (green; WGA) and PLC ζ^{WT} (red; mCherry), indicating PLC ζ does not co-localise to the plasma membrane. Images are representative of 300 cells studied for each construct, and were captured at 63x optical zoom, and enlarged following image capture. Z-stack reconstructions were performed using the LSM image browser (Zeiss, Germany). White scale bars indicate 10 μm .

ER staining of HEK293T cells transfected with mCherry-PLC ζ^{WT} indicated that this cytoplasmic organelle localisation pattern was at the ER. mCherry-PLC ζ^{EF} exhibited a similar localisation pattern at the ER to all other isoforms of mCherry-PLC ζ (Figure 7.11; 7.12). Intriguingly, however, while some marginal localisation at the ER could be observed by mCherry-PLC $\zeta^{\Delta\text{EF}}$, some mCherry fluorescence could also be observed within the nuclei of transfected HEK293T cells. Interestingly, the nuclear localisation populations of mCherry-PLC $\zeta^{\Delta\text{EF}}$ also exhibited some green fluorescence from the ER-Tracker staining, indicating that some ER was also present with this population (Figure 7.13).

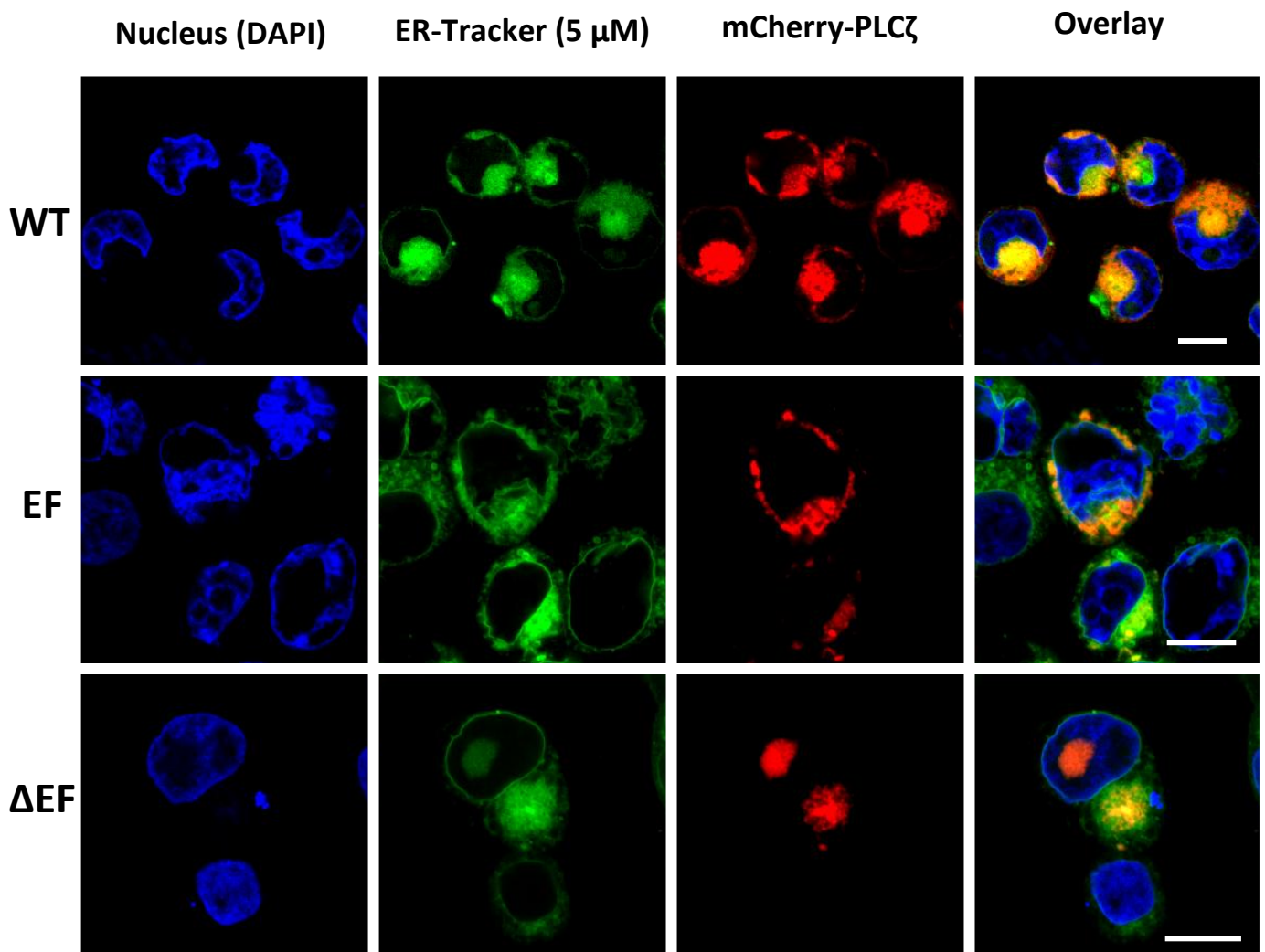
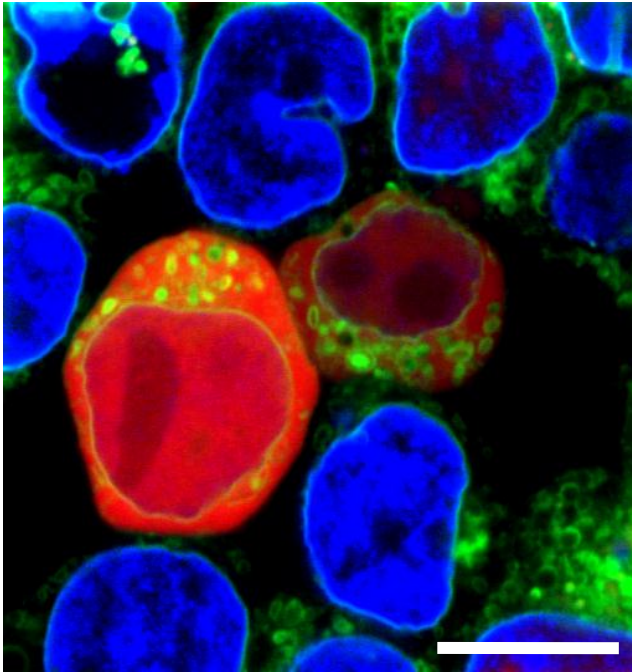


Figure 7.11: Representative single plane confocal microscopy images of 48 and 56 hour time points for HEK293T cells transfected with mCherry-PLC ζ^{WT} , - PLC ζ^{EF} , and -PLC $\zeta^{\Delta\text{EF}}$ constructs using a ET stain. Images are representative of 300 cells studied for each construct, and were captured at 63x optical zoom. White scale bars indicate 10 μm . WT: wild type PLC ζ construct; EF: construct consisting of only the EF hands of PLC ζ ; ΔEF : construct consisting of PLC ζ without its EF hands.

**mCherry-
Empty vector**



**Negative
transfection**

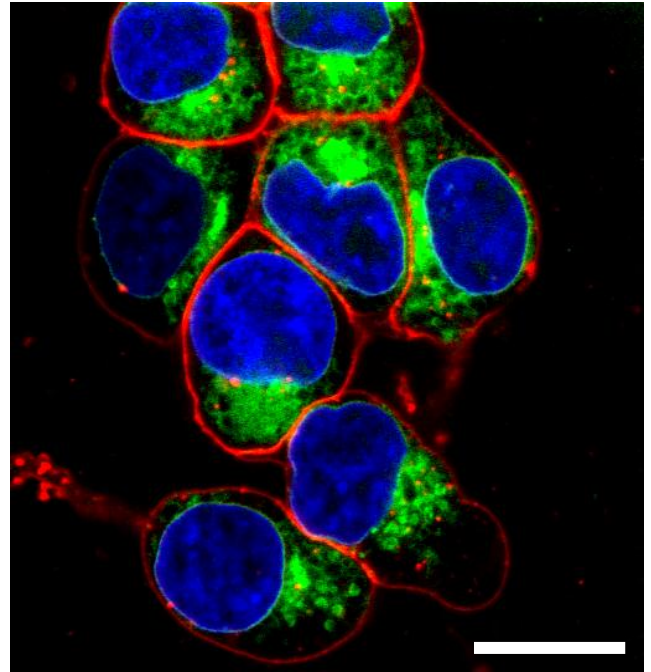


Figure 7.12: Representative single plane confocal microscopy images of cells transfected with mCherry-empty vector and negative transfected cells using a ER stain. Red: mCherry; Blue: Nucleus (DAPI); Green: Endoplasmic reticulum (ER-Tracker). Images are representative of 300 cells studied for each construct, and were captured at 63x optical zoom, and enlarged following image capture. White scale bars indicate 10 μ m.

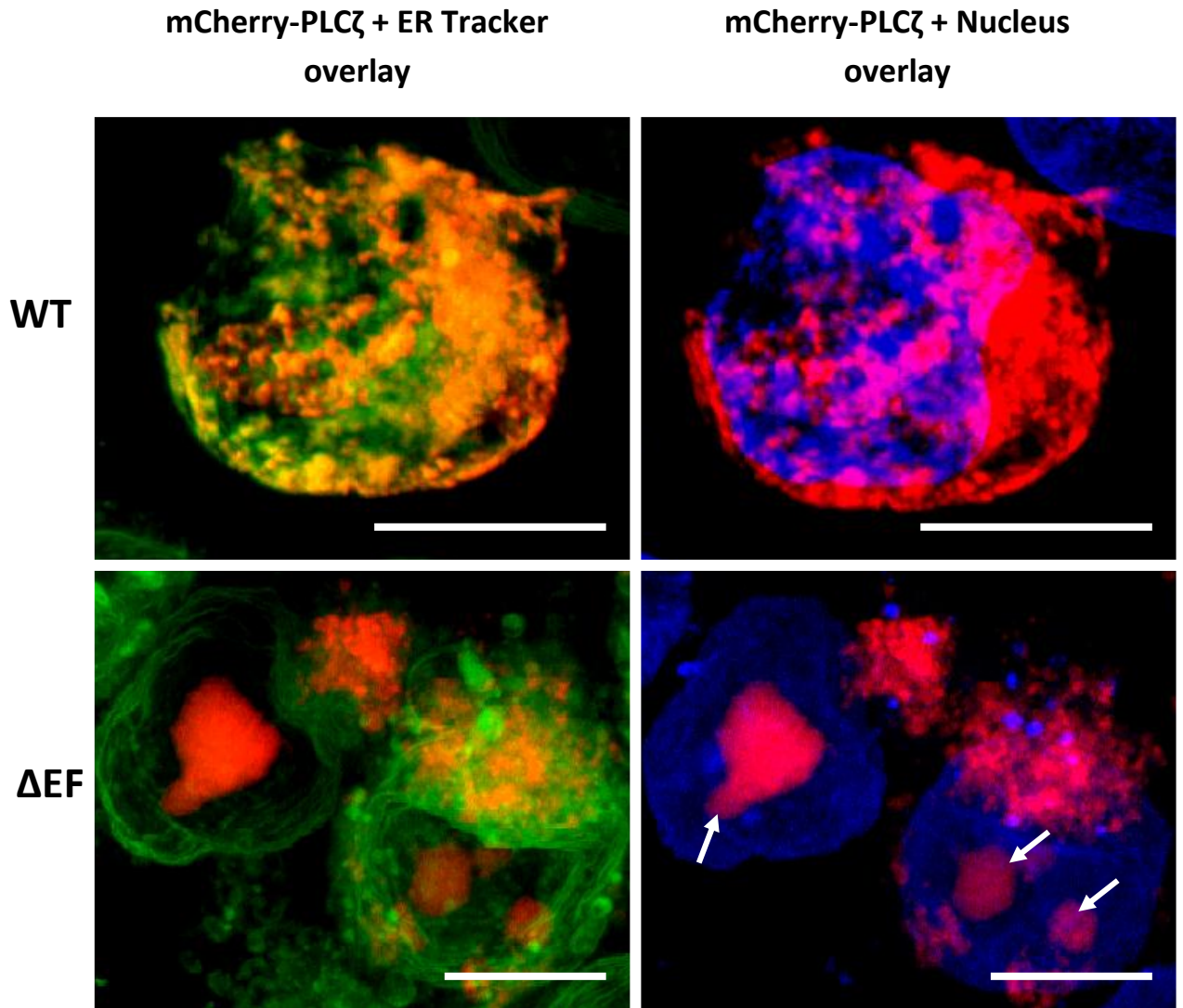


Figure 7.13: Representative 3D projections, obtained by reconstructing Z-stack images obtained by confocal microscopy, of HEK293T cells transfected with mCherry-PLC ζ^{WT} and mCherry-PLC $\zeta^{\Delta\text{EF}}$ using a ER stain. Blue: DAPI; PLC ζ : red; Green: ER-tracker. Z-stacks indicate peri-nuclear ER localisation of PLC ζ^{WT} , while also indicating a nuclear population of PLC $\zeta^{\Delta\text{EF}}$ (white arrows). Images are representative of 300 cells studied for each construct, and were captured at 63x optical zoom, and enlarged following image capture. Z-stack reconstructions were performed using the LSM image browser (Zeiss, Germany). White scale bars indicate 5 μm . WT: wild type PLC ζ construct; ΔEF : construct consisting of PLC ζ without its EF hands.

7.4 Discussion

PLC ζ is now regarded as the best current candidate for the endogenous mammalian sperm-specific oocyte activation factor, responsible for eliciting the characteristic Ca²⁺ oscillations observed in activating oocytes at fertilisation (Saunders *et al.*, 2002; Fujimoto *et al.*, 2004; Knott *et al.*, 2005; Kouchi *et al.*, 2005; Kurokawa *et al.*, 2005; Swann *et al.*, 2006; Parrington *et al.*, 2007; Kashir *et al.*, 2010). However, many questions regarding PLC ζ mechanism of action persist, particularly with regards to intracellular localisation within activating oocytes, as well as the potential role for multiple PLC ζ isoforms (Grasa *et al.*, 2008; Bi *et al.*, 2009; Young *et al.*, 2009), and whether oocyte activation consists of the action of multiple isoforms of sperm factor as opposed to one sole factor. Intriguingly, Bi *et al.* (2009) indicated the presence of a novel isoform of PLC ζ , which they named NYD-SP27, within the acrosome of mouse and human sperm, thought to be lacking the four EF hands from the N-terminus (PLC $\zeta^{\Delta EF}$). While numerous studies have confirmed multiple localisation patterns of PLC ζ (acrosomal and equatorial) from a range of species (Grasa *et al.*, 2008; Young *et al.*, 2009; Bedford-Guaus *et al.*, 2011), the truncated isoform has yet to be independently verified by other studies.

The work described in this Chapter aimed to utilise HEK293T cells, with which to investigate localisation patterns of several fluorescently tagged human PLC ζ isoforms, utilising PLC ζ^{WT} , PLC ζ^{H398P} , PLC ζ^{H233L} , PLC $\zeta^{H233L+H398P}$, and PLC $\zeta^{\Delta YC2}$, and to examine potential deleterious effects of loss-of-activity mutations on such patterns. In addition, the response of cells transfected with fluorescently tagged versions of PLC ζ^{WT} , PLC ζ^{H398P} , and PLC $\zeta^{\Delta YC2}$ to ATP-induced Ca²⁺ release was also investigated, in order to ascertain their respective activity upon over-expression in HEK293T cells. Localisation patterns were

also investigated with a fluorescently tagged version of PLC $\zeta^{\Delta EF}$, and a tagged version of the EF hands alone (PLC ζ^{EF}), to investigate the possible role of the EF hands within PLC ζ localisation. Collectively, these experiments aimed to further understanding of the molecular mechanisms of activity and regulation involved within the mechanism of action of PLC ζ , a necessary step in developing PLC ζ as a therapeutic agent for treatment of oocyte activation failure, as well as increasing understanding of the basic mechanism of oocyte activation

7.4.1 HEK293T cells transfected with PLC ζ^{WT} exhibit reduced responses to ATP

ATP is a potent agonist of IP₃-mediated Ca²⁺ release, and acts via the activation of P2Y purinergic receptors (Nezu *et al.*, 2010). However, the reduced response of HEK293T cells transfected with mCherry-PLC ζ^{WT} to ATP stimulation may indicate that over-expression of mCherry-PLC ζ^{WT} in these cells desensitises Ca²⁺ release via the IP₃ pathway. This may be related to an auto-inhibitory response to the over-expression of PLC ζ , mediated by diacylglycerol (DAG) production and the subsequent activation of protein kinase C (PKC) (Miyazaki *et al.*, 1993; Yoda *et al.*, 2004; Yu *et al.*, 2008a). It is also possible that cells transfected with PLC ζ^{WT} regulate Ca²⁺ homeostasis via mechanisms such as IRBIT (IP₃ receptor binding protein release with IP₃), presumably to reduce the sensitivity of intracellular Ca²⁺ stores (Brind *et al.*, 2000; Jellerette *et al.*, 2000; Vanderheyden *et al.*, 2009; Cooney *et al.*, 2010; Lee *et al.*, 2010).

In contrast, however, the normal ATP-induced Ca²⁺ release profiles of cells transfected with mCherry-PLC ζ^{H398P} and mCherry-PLC $\zeta^{\Delta YC2}$ may indicate that these isoforms are not active in HEK293T cells, in line with their inability to induce Ca²⁺ oscillations in oocytes, and thus do not cause such desensitisation of ATP-induced Ca²⁺

release. While such desensitisation may limit the usefulness of HEK293T cells as a system to study the functional properties of human PLC ζ , clues may be revealed regarding the mechanisms governing regulation of PLC ζ expression and packaging in the sperm, and its action within the oocyte. The preliminary data described in this Chapter regarding TG treatment of transfected cells indicates that cells transfected with PLC ζ^{WT} exhibit a low Ca $^{2+}$ content within intracellular stores, which may have led to an overall reduction in IP $_3$ R-1 sensitivity, preventing cell death caused by hyperactive IP $_3$ production. Indeed, the current data indicate that levels of IP $_3$ R-1 (the major receptor involved during Ca $^{2+}$ release at oocyte activation) do not decrease. However, these data require further repetition to ascertain the specific mechanism(s) involved.

Interestingly, Phillips *et al.* (2011) have recently shown that fluorescently-tagged mouse PLC ζ in CHO cells did not alter the ATP induced Ca $^{2+}$ response of such cells, suggesting that the mouse PLC ζ expressed in these cells were in an inactive state. However, injection of lysates obtained from such cells into mouse oocytes resulted in Ca $^{2+}$ oscillations, indicative of active PLC ζ , indicating that mouse PLC ζ may require oocyte-specific modifications and/or interactions to reach an active state. In contrast, however, results indicated that human PLC ζ^{WT} is transcribed in an active state within transfected HEK293T cells, considering the complete de-sensitisation of Ca $^{2+}$ release pathways within HEK293T cells transfected with mCherry-PLC ζ^{WT} .

It is possible that these differences are due to variances between species, or cell type used within experimentation. Indeed, human PLC ζ is thought to be much more potent in its PLC activity compared to mouse PLC ζ (Swann *et al.*, 2006; Parrington *et al.*, 2007; Kashir *et al.*, 2010), owing to which complete desensitisation of pathways in the cells used in the

present study were observed, while mouse PLC ζ was unable to elicit such a response in other studies. It also remains possible that HEK293T cells contained interactors and/or modulators not present within CHO cells, which ultimately led to the presence of active PLC ζ .

It is particularly important to ascertain the mechanisms underlying the observed desensitisation of ATP-induced Ca²⁺ release in HEK293T cells transfected with mCherry-PLC ζ^{WT} as little is known as to the state of PLC ζ protein within sperm, and how it may be kept in an active state without cell death. Further studies in this area could lead to a better understanding of the mechanism(s) underlying PLC ζ action, and the regulatory pathways involved. Particular emphasis must also be placed upon the investigation of possible interactors and/or modulators that may be present within the HEK293T cells, which may be leading to the activation of PLC ζ activity. Such studies are critical not only to establish fully the therapeutic potential of PLC ζ for infertility treatment, but investigate the possible implications these findings upon their understanding of fundamental signalling events within the human body.

7.4.2 Loss-of-activity mutations in highly conserved regions of human PLC ζ may lead to overall protein instability

HEK293T cells transfected with mCherry-mutant isoforms of PLC ζ exhibited markedly reduced levels of fluorescence compared to cells transfected with PLC ζ^{WT} . The histidine residue at position 398 of PLC ζ has been shown to possess high levels of conservation across different species (Heytens *et al.*, 2009). It is possible that this residue plays a critical role in promoting and/or maintaining stable expression of wild type PLC ζ , by contributing towards either stability or folding of the protein structure. In support of

this hypothesis, the present results indicate that the H398P and H233L mutations may have resulted in lower levels of PLC ζ in sperm from the H398P patient and in HEK293T cells. Expression of the artificially-induced mutation, PLC $\zeta^{\Delta YC2}$, a frameshift mutation causing a partial Y and complete C2 domain deletion creating a premature stop codon, was also highly compromised in HEK293T cells.

Intriguingly, levels of fluorescence observed in cells transfected with mCherry-PLC ζ^{H398P} were significantly higher than levels observed in cells transfected with mCherry-PLC $\zeta^{H233L+H398P}$, possibly indicating that the presence of both mutations together at the same time results in a larger reduction in protein levels, possibly due to a greater destabilisation of protein structure, further suggesting that both mutations may have a destabilising effect within sperm when translated from their respective alleles. A single destabilising mutation may result in a substantial or complete reduction, or even complete loss, of protein translation due to an array of factors including misfolding, aggregation or proteolytic clearance (Tokuriki & Tawfik, 2009; Soskine & Tawfik, 2010). Mutations which are destabilising tend to disrupt overall protein stability, as appears to be the case with both PLC ζ^{H398P} (Heytens *et al.*, 2009) and PLC ζ^{H233L} , and once a certain threshold is exceeded, levels of functional protein may decrease resulting in reduced protein fitness (DePristo *et al.*, 2005; Yue & Moulton, 2006; Camps *et al.*, 2007; Tokuriki & Tawfik, 2009; Soskine & Tawfik, 2010). Absent/reduced levels of PLC ζ in sperm have been implicated in forms of male infertility where such sperm is repeatedly unable to activate oocytes (Yoon *et al.*, 2008; Heytens *et al.*, 2009; Taylor *et al.*, 2010). Indeed, results obtained from Chapter 3 of this thesis also supported this where sperm from patient^{H398P} (all of which would contain mutant isoforms of PLC ζ) exhibited severely decreased total levels of PLC ζ compared to sperm from fertile controls.

While Heytens *et al.* (2009) previously observed that sperm from the infertile patient in which the H398P mutation had been identified appeared to exhibit reduced levels of PLC ζ immunofluorescence, this difference was quantified in comparison to fertile controls in the results described in Chapter 3 of this thesis, and revealed that sperm from the H398P patient exhibits a greatly reduced level of PLC ζ immunofluorescence compared to sperm from males with no previous history of infertility, while also demonstrating a significantly reduced proportion of sperm exhibiting PLC ζ immunofluorescence within a semen sample. Furthermore, the results corroborated with those observed in Chapter 6, whereby levels of PLC ζ^{H398P} were 6 times lower than that of PLC ζ^{WT} in equal amounts of lysates obtained from HEK293T cells transfected with pTracer constructs, despite similar transfection efficiencies. It is therefore possible that such cases where PLC ζ is absent or severely reduced in sperm may be due to a severely destabilising effect of mutations in highly conserved regions of PLC ζ .

However, studies examining the role of deletions in domains essential to mouse PLC ζ function did not reveal any differences in protein stability (Nomikos *et al.*, 2005; Kurokawa *et al.*, 2007). It should also be noted that Chapter 3 of this thesis only identified one novel mutation, out of the 12 patients diagnosed with oocyte activation deficiency screened. However, as discussed previously in Chapter 3, only the prevalence of loss-of-activity mutations in the active site of the PLC ζ ORF were investigated. The current data indicate that it would be necessary to expand screening for mutations to highly conserved regions of the PLC ζ ORF, which may lead to overall protein instability. Furthermore, considering that sperm and HEK293T cells are very different systems, it is important that future studies examine the effect of mutant PLC ζ in testicular germ cells to investigate whether such a trend is repeated, and to determine whether such loss-of-activity mutations

are potentially disruptive to the overall protein fold, utilising mutagenesis studies on highly conserved regions of PLC ζ , and investigating protein stability *in-vitro* and possibly within knock-in mouse models. Knock-in models would involve the mouse equivalents of H398P and H233L replacing WT mouse PLC ζ , which would then lead to mouse sperm expressing these versions, enhancing understanding of not only the roles of these two mutations in leading to infertility, but the role of PLC ζ in normal fertility.

7.4.3 Human PLC ζ localises to the endoplasmic reticulum and not the plasma membrane in HEK293T cells

Confocal microscopy indicated that mCherry-tagged human PLC ζ isoforms expressed in HEK293T cells were not localised to the plasma membrane, but instead appeared contained in discrete cytoplasmic regions close to the nuclear envelope, which were identified as the ER, though the relevance of this finding for the pattern of localisation of PLC ζ in the oocyte and the mechanism whereby it accesses PIP₂ requires further investigation. PLC ζ is known to diffuse throughout the oocyte cytoplasm, suggesting that its substrate PIP₂ may be targeted to intracellular membranes (Yoda *et al.*, 2004; Sone *et al.*, 2005; Kuroda *et al.*, 2006; Ito *et al.*, 2008; Yu *et al.*, 2008a; 2008b).

Phillips *et al.* (2011) recently suggested that fluorescently-tagged mouse PLC ζ does not exhibit activity upon over-expression in CHO cells, and initially exhibited a diffuse cytoplasmic localisation within these cells, which changed to a predominantly nuclear localisation 24- and 48- hours post-transfection. It is also worth noting that Ito *et al.* (2008) demonstrated nuclear localisation of human and mouse PLC ζ in COS-7 cells. However, Coward *et al.* (2006) found that in COS cells, mouse PLC ζ could be localised to either the nucleus or cytoplasm. While there seems to be conflicting evidence in this regard, the lack

of nuclear localisation could reflect either the cell type used, or reduced functional ability of human PLC ζ compared to mouse. It may also be the case that functional disparities may exist between the mouse NLS as well as any potential human NLS. However, the existence, and functional significance if any, of the human PLC ζ NLS must be confirmed before any such comparison can be made.

While general consensus agrees that PIP₂ is generally located at the plasma membrane in most cell types, this may not be the same for oocytes, especially given that oocytes contain larger numbers of intracellular organelles such as ER clusters, lysosomes, and yolk granules (Helms *et al.*, 1991; Godi *et al.*, 1999; Rice *et al.*, 2000; Halet *et al.*, 2002; Irvine, 2003; Saunders *et al.*, 2007). In summary, with the caveat that these findings relate to the expression of a testis-specific protein within a cell of somatic origin, these observations may indicate that human PLC ζ has a higher preference for intracellular organelle localisation as opposed to localisation at the plasma membrane, which could be highly relevant to its mechanism of action in the oocyte.

7.4.4 The regulatory EF hands may play a novel role in intracellular and potential nuclear localisation of human PLC ζ

Confocal microscopy indicated that deletion of the EF hands of PLC ζ (PLC $\zeta^{\Delta EF}$) resulted in an alteration of the ER localisation pattern observed with PLC ζ^{WT} . While some marginal localisation was observed at the ER, HEK293T cells transfected with mCherry-PLC $\zeta^{\Delta EF}$ also exhibited nuclear populations of mCherry fluorescence. Fluorescently tagged EF hands (PLC ζ^{EF}) retained the same ER localisation exhibited by PLC ζ^{WT} , indicating that the EF hands may play a role in targeting PLC ζ to organelle bound PIP₂ to perform its PLC activity. Indeed, the EF hands are thought to play an integral role in the Ca²⁺

sensitivity of PLC ζ , deletion of which significantly decreases PIP₂ hydrolysis *in-vitro* and results in a depletion of Ca²⁺ oscillations upon injection into mouse oocytes (Kouchi *et al.*, 2005; Nomikos *et al.*, 2005). Furthermore, Waldo *et al.* (2010) indicated that the third and fourth EF hands of PLC β isoforms played an integral role in its localisation at the plasma membrane. It is, therefore, possible that the EF hands play a similar role in aiding PLC ζ localisation to its target substrate.

Furthermore, results indicated that the EF hands of human PLC ζ may contribute towards the inhibition of nuclear localisation within HEK293T cells. Confocal microscopy indicated that deletion of the EF hands (PLC $\zeta^{\Delta EF}$) resulted in the presence of a population of PLC ζ in discrete regions of the nuclei of transfected HEK293T cells, possibly within nucleoli. Despite the fact that PLC ζ of various species from rats and mice (Larman *et al.*, 2004; Ito *et al.*, 2008b), chicken (Coward *et al.*, 2005) and medaka fish (Ito *et al.*, 2008b), to porcine (Yoneda *et al.*, 2006), bovine (Cooney *et al.*, 2010), and humans (Cox *et al.*, 2002), are thought to contain a nuclear localisation signal (NLS) in the linker region between the X and Y domains, nuclear localisation has only been observed in mouse zygotes (Larman *et al.*, 2004). Remarkably, however, mutation of several conserved residues within the EF hands of mouse PLC ζ abolished the nuclear localisation ability of mouse PLC ζ (Kuroda *et al.*, 2006). It would, therefore, appear that EF-hand domains may be essential for nuclear translocation in the mouse, presumably due to enzyme folding (Ito *et al.*, 2011).

However, the present results indicate that the opposite may be true for human PLC ζ , where the presence of the EF hands prevented PLC ζ from entering the nuclei of transfected cells. It is possible that this regulatory domain exerts an inhibitory effect upon the NLS in

the human PLC ζ sequence. While the EF hands are thought to play an important role in the regulation of Ca²⁺ sensitivity, it is worth noting that mice endogenously express a truncated version of PLC ζ , lacking the first three EF hand regions (Fujimoto *et al.*, 2004; Kurokawa *et al.*, 2005). While eliciting lower frequencies of Ca²⁺ oscillations and delayed pronuclear formation, oocytes injected with this truncated isoform resulted in normal full-term offspring, suggesting that this isoform may be active in this species (Nakanishi *et al.*, 2008; Ito *et al.*, 2011). Intriguingly, some ER staining was also observed within the nuclear populations of PLC $\zeta^{\Delta EF}$, possibly indicating that PLC $\zeta^{\Delta EF}$ first localises to the ER, and is then actively shuttled into the nucleus. Furthermore, considering that Bi *et al.* (2009) indicated the presence of PLC $\zeta^{\Delta EF}$ within acrosomal populations in human and mouse sperm, it is possible that this isoform of PLC ζ , may play a role in the overall PLC ζ -mediated Ca²⁺ oscillation profile at oocyte activation.

While it would seem that a large degree of variance is present in terms of mechanism and mode of PLC ζ action, which seems largely dependent upon the species studied, it would be intriguing for future studies to investigate the roles of other catalytically important domains of human PLC ζ in its localisation to its intracellular substrate. Indeed, the X-Y linker of mouse PLC ζ may play a role in PIP₂ binding of PLC ζ , while the C2 domain did not perform such a function (Nomikos *et al.*, 2011b; 2011c). It would be interesting to investigate whether this holds true for human PLC ζ ; an important step in determining the feasibility of utilising PLC ζ protein as a clinical therapeutic within the clinic. Furthermore, answering questions regarding these signalling events would not only increase understanding of fundamental systems within the human body, but clarifying how PLC ζ differs in its mechanism of action from other PLCs may aid in designing a more efficient and more effective therapeutic to use within the clinic.

7.5 Conclusions and future work

In summary, this Chapter demonstrated that cells transfected with PLC ζ^{WT} are less responsive to ATP-induced Ca²⁺ release, possibly due to desensitisation of IP₃ signalling to maintain Ca²⁺ homeostasis. Loss-of-activity mutations in highly conserved regions of PLC ζ may contribute to overall protein instability, possibly resulting in an increased rate of protein degradation. Future studies should attempt to investigate the significance of these findings by examining whether such a pattern is still observed in a more applicable cell expression system such as testicular germ cells. Fluorescently tagged PLC ζ isoforms exhibited localisation to a cytoplasmic organelle, which was determined to be the ER. However, deletion of the PLC ζ EF hands caused a change in localisation pattern from predominantly at the ER, to the ER as well as within the nuclei of transfected cells, possibly localising to nucleoli. While indicating that the EF hands of PLC ζ may play a role in determining the localisation of the enzyme to its membrane-bound substrate, this regulatory domain may also exert a negative influence over the nuclear localisation signal of human PLC ζ . It would be interesting for future studies to investigate the precise localisation of the nuclear population, which may potentially be the nucleoli, as well as to investigate the potential physiological significance of such a localisation.

It is possible that PLC $\zeta^{\Delta\text{EF}}$ first requires binding to the ER before it is shuttled into the nuclei, along with potential interactors. This would be of extreme interest for future studies to investigate, since it implies the presence of modulators or activators of PLC ζ activity within the ER. Determining such factors would enable further understanding of PLC ζ mode and mechanism of action, something which is scarcely understood at present. Future studies should also investigate the precise localisation of PLC $\zeta^{\Delta\text{EF}}$ within the

nucleus to better establish the function and purpose of such shuttling within HEK293T cells and possibly oocytes. While PLC ζ isoforms seem to have a preference for ER binding as opposed to the plasma membrane within HEK293T cells, the applicability of these findings should be investigated within activating oocytes, possibly utilising microinjection of cRNA corresponding to the fluorescently tagged isoforms of PLC ζ utilised in this study. Of utmost importance though, is to investigate the functional differences between PLC $\zeta^{\Delta EF}$ and PLC ζ^{WT} , to further indicate whether PLC $\zeta^{\Delta EF}$ has a role to play within Ca²⁺ signalling at oocyte activation.

Finally, with the caveat that the heterologous mammalian expression system may be different from the sperm cells in which PLC ζ is endogenously expressed, human PLC ζ is transcribed in an active state within HEK293T cells, which differs greatly from the case of mouse PLC ζ which appears to require the action of oocyte based activators/interactors to become active (Phillips *et al.*, 2011). Future studies should attempt to investigate the reasons underlying the desensitisation of HEK293T cells transfected with PLC ζ^{WT} to ATP treatment and the mechanisms by which Ca²⁺ homeostasis is maintained. This is particularly prudent considering that there is little knowledge in the published literature as to how PLC ζ is held inactive within the sperm, but converts to an active state within the oocyte following gamete fusion. Collectively, these experiments would play an integral role in determining the mode and mechanism of PLC ζ action and regulation within the sperm and activating oocyte, and would ultimately provide novel information with regards to the role played by PLC ζ in forms of male infertility where oocyte activation is deficient, and its potential therapeutic viability within the clinic.



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CHAPTER 8

General discussion

In all species studied to date, activation of eggs or oocytes requires an increase in the intracellular concentration of Ca^{2+} (Stricker, 1999). Numerous studies indicate that the sperm delivers an endogenous oocyte activation factor to the oocyte either during or directly following gamete fusion (Swann, 1990; Kyoizuka *et al.*, 1998; Stricker, 1999; *et al.*, 2000; Coward *et al.*, 2003; 2005; Swann *et al.*, 2006; Parrington *et al.*, 2007), with a considerable body of research suggesting that the IP_3 -dependant nature of oocyte activation depended upon a phospholipase C (PLC) -mediated mechanism (Miyazaki *et al.*, 1993; Saunders *et al.*, 2002; Parrington *et al.*, 2007; Swann & Yu, 2008), subsequently identified as PLC ζ (Cox *et al.*, 2002; Saunders *et al.*, 2002).

Given the apparent fundamental role for PLC ζ at fertilisation, it follows that genetic or molecular defects in PLC ζ may underlie forms of male infertility where oocyte activation is deficient. Sperm from infertile men which consistently fail routine clinical procedures such as IVF and ICSI are either unable to elicit Ca^{2+} oscillations, or do so, but with reduced frequency and amplitude (Yoon *et al.*, 2008; Heytens *et al.*, 2009). Furthermore, sperm from such patients exhibited reduced/absent levels of PLC ζ within the sperm head (Yoon *et al.*, 2008; Heytens *et al.*, 2009). By screening the PLC ζ gene in genomic DNA from patients whose sperm routinely failed IVF and ICSI, Heytens *et al.* (2009) identified a heterozygous substitution mutation in the coding sequence of PLC ζ in an infertile male with morphologically normal sperm, occurring within the Y domain of the PLC ζ open reading frame (ORF) at position 398 resulting in histidine (H398) being changed to proline. 3D modelling of this mutation predicted disruption of an alpha helix structure in the catalytic region and affect interactions with neighbouring amino acids (Heytens *et al.*, 2009).

Considering the importance of PLC ζ in mammalian fertilisation, and the compelling implications regarding its involvement in forms of human male infertility where oocyte activation is deficient, PLC ζ holds great therapeutic and diagnostic potential for use within the fertility clinic as a prognostic marker of male fertility, and a therapeutic pharmacological agent with which to replace current assisted oocyte activation (AOA) protocols. However, there are many outstanding queries with regards to the precise mechanisms of PLC ζ action, the regulatory pathways involved, and the relative roles of various localisation patterns in human sperm reported in the literature. Little is known as to the mechanism employed by PLC ζ in targeting PIP₂, the potential roles of post-translational modification, or the involvement of multiple PLC ζ isoforms at fertilisation and oocyte activation. Furthermore, only one mutation in PLC ζ from an infertile male had been identified prior to the start of this project, raising questions as to the prevalence of loss-of-activity PLC ζ mutations within forms of male factor infertility where oocyte activation is deficient.

This thesis, therefore, aimed to address the questions regarding PLC ζ mechanism and mode of action, and the potential diagnostic and therapeutic applicability of PLC ζ within the clinic. A PCR-based approach was employed to screen exons coding for the active site open reading frame (ORF; exons 5-12) of the PLC ζ gene of multiple infertile patients diagnosed with oocyte activation deficiency from various bio-geographical regions to establish the role of mutations in PLC ζ in such forms of human male factor infertility. The current study also examined total PLC ζ immunofluorescence and performed proportional analysis of the various populations of PLC ζ localisation within the sperm head using fluorescence and confocal microscopy from patient and control sperm, to examine the scope for employing PLC ζ as a prognostic marker with which to create a

diagnostic test for oocyte activation deficiency. The potential for routine clinical ART methodology such as density gradient washing (a method used to obtain the best quality sperm for IVF and ICSI), and cryopreservation to induce iatrogenic damage upon PLC ζ within fertile human sperm were also examined. Furthermore, the previously identified PLC ζ^{H398P} , and the novel H233L mutation identified in Chapter 3, were used to investigate PLC ζ 's mechanism of action within mammalian somatic cells and oocytes. Finally, a major aim of this project was to produce an active recombinant human PLC ζ protein with which to examine the therapeutic prospect of using such a compound within the clinic as a replacement for current AOA protocols.

8.1 The role of PLC ζ in human male factor infertility

While Yoon *et al.* (2008) provided the first crucial link between absent/reduced levels of PLC ζ in human sperm and forms of male infertility where oocyte activation is deficient, Heytens *et al.* (2009) established the first association between such forms of male infertility and genetic defects in the PLC ζ gene of such infertile males, identifying a point mutation in the active site of the PLC ζ ORF from an infertile male; sperm from whom routinely failed IVF and ICSI (PLC ζ^{H398P}). While groundbreaking, these studies did not establish the prevalence of genetic defects in PLC ζ in oocyte activation deficiency within the infertile male population.

In the present study, a novel mutation in the active site of the PLC ζ ORF (PLC ζ^{H233L}) was identified. However, this was from the same patient from whom PLC ζ^{H398P} had previously been identified. The patient screen failed to identify any further novel mutations in a further 11 patients, although the rigour of the analysis was limited by problems encountered with the screening protocol. Intriguingly, the patient who possessed these two

mutations inherited them from separate parents; PLC ζ^{H398P} from his father, and PLC ζ^{H233L} from his mother, providing, to the best of one's knowledge, the first description of a maternally inherited non-autosomal point mutation resulting in a deficit of sperm function. Indeed, PLC ζ^{H233L} was unable to elicit the characteristic Ca²⁺ oscillations usually observed at fertilisation upon injection of the corresponding cRNA into mouse oocytes.

The heterozygous nature of both PLC ζ^{H233L} and PLC ζ^{H398P} provides us with new understanding of the possible first point of human PLC ζ expression within the testes, given that the father of the patient was heterozygous for PLC ζ^{H398P} , but still conceived the patient naturally, indicating presence of mutant DNA alongside PLC ζ^{WT} protein. The present study also established that mutations in PLC ζ may be functionally recessive, requiring loss-of-activity mutations on both inherited alleles within a patient, or requiring a single homozygous mutation in PLC ζ , for complete infertility to occur. Furthermore, these results provide new insights into understanding of how similar genetic deficiencies may lead to male infertility. It is possible that similar heterozygous mutations may be present within the male population, which instead of causing complete deficit of sperm function, may lead to sub-fertility owing to heterogeneous populations of sperm exhibiting normal and abnormal populations of PLC ζ . Indeed, sperm from the patient from whom the two mutations were identified only exhibited one mutation at a time within individual sperm, and never both at once. While this may explain the complete deficit of sperm function in this patient (since all sperm would have mutant PLC ζ protein), populations of males exhibiting only one loss-of-activity mutation on a single allele may exhibit sub-fertility, rather than complete infertility. Furthermore, these studies may be particularly relevant for further autosomally-located genes coding for sperm proteins. This would be particularly important to investigate, given that the incidence of sub-fertility affects 1 in 15 men within

the general population, with sperm defects thought to be the most common cause (Publicover *et al.*, 2007). Furthermore, the current results indicate the importance of understanding the impact of maternal modes of inheritance of autosomal factors which may lead to male infertility, providing a novel starting point with which to understand, and one day combat, complex genetic syndromes which lead to male infertility.

Unfortunately, it was not possible to identify any further mutations from the patient population examined by the current study. While this may, in part, be due to inefficiencies inherent within the screening protocol employed herein, the current study was able to successfully screen most of the active site coding exons (5-12) for most patients. These results may indicate that such mutations are not a common occurrence within the infertile male population where oocyte activation is deficient. However, the current patient screen only encompassed 8 out of 15 coding exons for the PLC ζ gene, without addressing regulatory domain coding exons since it was felt that the best chance of discovering novel loss-of-activity mutations would be via screening exons coding for the active site. Coupled with restrictions on funds as well as time, focusing the screening target was a necessary step. Numerous studies have indicated that the regulatory regions of PLC ζ (the EF hands and C2 domains) play a crucial role within substrate binding, Ca²⁺ sensitivity, and the overall PLC activity of PLC ζ (Fujimoto *et al.*, 2004; Kouchi *et al.*, 2004; Yoda *et al.*, 2004; Kurokawa *et al.*, 2005; Nomikos *et al.*, 2005; Swann *et al.*, 2006; Nakanishi *et al.*, 2008; Nomikos *et al.*, 2011b). It is, therefore, possible that mutations within these regions, may lead to a deficit of PLC ζ activity, thus resulting in a deficiency of oocyte activation capability. Therefore, it is imperative that future studies incorporate areas such as the promoter region within any screen of the PLC ζ gene for novel mutations. Further sites of interest include intronic regions of the PLC ζ gene, considering that recent studies indicate

a potentially fundamental role for these regions in determining gene expression by influencing transcriptional regulation (Kasowski *et al.*, 2010; Oswald & Oates, 2011). It is also important that future studies incorporate improved screening methodologies, such as TGCE, to enable the rapid screening of multiple patients presented within a clinical scenario.

While the discovery of novel mutations in PLC ζ have thus far been a rare event, a consistent observation seems to be that sperm from infertile males who routinely fail IVF and ICSI exhibit absent/reduced levels of PLC ζ protein following immunofluorescence (Yoon *et al.*, 2008; Heytens *et al.*, 2009; Taylor *et al.*, 2010). Indeed, the current patient screen was consistent with these observations, with all infertile patients exhibiting sperm with either severely reduced, or a complete absence of PLC ζ . Intriguingly, data indicate that loss-of-activity mutations within highly conserved regions of the PLC ζ ORF may be contributing towards these absent/reduced levels in sperm. The transfection of fluorescently tagged PLC ζ isoforms into HEK293T cells led to these cells exhibiting fluctuations in levels of fluorescence such that isoforms of PLC ζ exhibiting loss-of-activity (H233L; H398P; H233L+H398P, and Δ YC2) exhibited significantly lower levels of fluorescence compared to PLC ζ^{WT} . This was further supported by the finding that levels of PLC ζ^{H398P} protein present in cell lysates obtained from HEK293T cells transfected with pTracer-PLC ζ^{H398P} were ~6 times lower than levels of PLC ζ^{WT} protein in cell lysates obtained from HEK293T cells transfected with pTracer- PLC ζ^{WT} , despite similar transfection efficiencies. While these results may represent an artefact of the *in vitro* expression system, it may be possible that loss-of-activity mutations in highly conserved residues of the PLC ζ ORF lead to overall protein instability. Of course, other scenarios, such as the possibility that mutant PLC ζ protein was transcribed less efficiently in

HEK293T cells compared to wild type PLC ζ protein, cannot be excluded. However, to distinguish between these different possibilities would require the use of purified isoforms of PLC ζ to allow firm conclusions to be drawn about the effects of such mutations upon the stability of the protein.

With the caveat that the somatic HEK293T cell expression system used in this project is very different from the haploid sperm cells which normally express this enzyme, the results indicate that loss-of-activity mutations in highly conserved regions of PLC ζ may lead to overall protein instability, and thus degradation of mutant protein, leading to absent/reduced levels within the sperm. However, one should also note that sperm from control males exhibited a large variance of levels of total PLC ζ fluorescence, indicating that levels of PLC ζ may vary to a large degree between males and possibly between different semen samples from the same male. It is therefore essential that efforts are made in future to establish whether loss-of-activity mutations such as PLC ζ^{H233L} and PLC ζ^{H398P} do indeed lead to an increase in protein instability, and whether similar mutations underlie the absent/reduced levels of PLC ζ in sperm from males who repeatedly fail IVF and ICSI. A particularly suitable route with which to investigate this would involve the utilisation of knock-in mouse models, to study the *in-vivo* effects of mutation in PLC ζ in sperm and fertilisation.

8.2 PLC ζ as a prognostic marker of male infertility

Considering the apparent major role that PLC ζ plays within fertilisation and its implications for male infertility where oocyte activation is deficient, it is of great interest to examine whether PLC ζ may serve a role as a predictive biomarker of sperm fertility within the research laboratory and the clinic. Several studies now implicate absent/reduced levels of PLC ζ within sperm which repeatedly fail IVF and ICSI. The viability of utilising quantitative PLC ζ immunofluorescence to measure comparative levels of PLC ζ between fertile control and patient sperm was investigated. Indeed, Kaewmala *et al.* (2011) and Aghajanpour *et al.* (2011) recently demonstrated that levels of PLC ζ mRNA may be used as such a predictor in boar and human sperm respectively. However, while it has been suggested that PLC ζ RNA transcripts present in sperm could be transcribed during fertilisation and sustain a 'long-lived' Ca²⁺ response (Lalancette *et al.*, 2008), numerous studies indicate that the majority of protein transcription and translation occurs within the meiotic stages of spermatogenesis, and starts to decline upon passage into the spermiogenesis stage (Bettegowda & Wilkinson, 2010). Whilst indicating that the functional significance of such RNA transcripts in human sperm remains to be further investigated, this casts doubts as to the efficacy of using RNA transcripts in sperm as an index with which to assess male fertility.

While sperm from infertile patients exhibited low levels of total PLC ζ immunofluorescence compared to the majority of controls, some controls exhibited levels of total immunofluorescence similar to the patient population. Furthermore, levels of PLC ζ immunofluorescence varied widely between sperm from different controls. Collectively, while indicating that levels of total PLC ζ immunofluorescence were significantly reduced compared to fertile controls, there was variability between total levels of PLC ζ

immunofluorescence in sperm from fertile controls. These findings different from that of Aghajanzpour *et al.* (2011), and may indicate that utilising quantitative PLC ζ immunofluorescence may not be a viable method of predicting the fertility of human sperm.

This is of particular concern when one considers that this would have to be performed on an individual (and not a collective) basis within a clinical setting, possibly leading to potential misdiagnosis. It is, however, possible that levels of PLC ζ protein in sperm may be supplemented by RNA transcripts, which following translation within the oocyte, contribute towards oocyte activation. This may account for why some control sperm, exhibiting low levels of immunoreactive PLC ζ similar to patient sperm, were still fertile. However, it should be noted that a functional role for PLC ζ RNA, as with all sperm RNA transcripts, has yet to be established (Kashir *et al.*, 2010). It would be very interesting to examine whether there is a correlation between low levels of immunoreactive PLC ζ and levels of PLC ζ RNA transcripts within sperm, and whether RNA transcripts within sperm, following translation in the oocyte, contribute towards the overall Ca²⁺ oscillation profile observed at oocyte activation, and their functional effect upon developmental competence and gene expression.

Intriguingly, the analyses indicated that there was a difference between control and patient sperm in terms of the proportion exhibiting PLC ζ immunoreactivity, with control samples containing a significantly larger proportion of sperm exhibiting PLC ζ immunofluorescence compared to patient sperm. It may, therefore, be argued that proportional analysis of PLC ζ immunoreactivity within sperm from patients seeking fertility treatment may be used successfully as a prognostic predictor of male fertility.

Indeed, the findings also indicated that the proportion of sperm from fertile males exhibiting PLC ζ immunoreactivity increased dramatically following selection by density gradient washing (DGW), which selects the best quality sperm from an ejaculate for fertility treatment (Allamaneni *et al.*, 2005).

Collectively, these results indicate that PLC ζ still has potential for use as a biomarker of sperm fertility. Sperm from infertile patients did not exhibit a predominant pattern of PLC ζ localisation within the sperm head, while fertile controls did, although even this was variable between males. This is further supported by the finding that levels of total PLC ζ declined following cryopreservation of sperm from the majority of fertile males used in the present study. Considering that cryopreservation causes a reduction in many sperm parameters (Ozkavukcu *et al.*, 2008), and may induce DNA fragmentation and oxidative damage (Zribi *et al.*, 2010), these various findings represent a worrying trend of evidence indicating that cryopreservation may detrimentally affect sperm fertility, and should be expanded by studies to include other routine ART methodology such as overnight incubation and sperm swim-up selection on PLC ζ .

Of particular interest is the potential variability in levels of PLC ζ within control sperm, along with the differential predominant patterns of PLC ζ localisation. Considering that, in contrast to the findings of Grasa *et al.* (2008), no overall predominant localisation pattern of PLC ζ was observed in the entire data set, the precise localisation/functional relationship of PLC ζ in human sperm remains inconclusive. The discrepancies between the findings of Grasa *et al.* (2008) and the current study may be due to the fact that sperm from each individual fertile control were analysed separately (and utilising a higher control population), and collectively, as well as including infertile patients in the analysis. This

may explain the difference in the present findings, even though the same antibody was utilised by both studies.

The data may indicate that the presence of PLC ζ in sperm is all that is required for competent oocyte activation to proceed, with the different localisation patterns serving an as yet unidentified role within either oocyte activation, or early embryogenesis. It would therefore be interesting for future studies to examine the Ca²⁺ oscillation profile elicited by each structural feature of sperm exhibiting a particular localisation pattern of PLC ζ (i.e. acrosome, equatorial segment, etc.). Indeed, the varying localisation patterns have previously been proposed to correspond either to different isoforms of PLC ζ , or be responsible for functions other than eliciting Ca²⁺ oscillations at fertilisation (Grasa *et al.*, 2008; Bi *et al.*, 2009). However, such postulates have yet to be proven. It would be interesting to investigate such correlations utilising micro-dissection techniques to isolate the different populations of PLC ζ localisation and investigate their identity (by immunoblotting or LC/MS) and function (by oocyte microinjection and resultant Ca²⁺ release profile).

Crucially, however, the wide variability observed with regards to levels and localisation patterns of PLC ζ within human sperm may indicate that these factors have an important role within early embryogenesis. Indeed, prior research indicates that levels of PLC ζ directly impact upon the frequency and amplitude of the resulting Ca²⁺ oscillations, which in turn may lead to an altered profile of gene expression during early embryogenesis (Saunders *et al.*, 2002; Yu *et al.*, 2008). Furthermore, considering that oocyte activation is directly followed by a series of successive waves of embryonic transcriptional initiation, beginning as early as the 2-cell stage (Vassena *et al.*, 2011), levels and localisation patterns

of PLC ζ may be asserting hitherto unknown mechanisms regulating genetic and epigenetic diversity within reproduction.

It would also be interesting to examine the implications of levels and localisation patterns of PLC ζ in human sperm on the ‘quiet embryo’ hypothesis, particularly considering that levels of PLC ζ may directly impact rates of cell cycle progression and altered gene expression profiles during early embryogenesis (Ducibella *et al.*, 2002; Saunders *et al.*, 2002; Yu *et al.*, 2008). The ‘quiet embryo’ hypothesis proposes that viable embryos may have lower rates of metabolism compared to oocytes which undergo developmental arrest, theorising that stochastic events and environmental stresses may determine embryonic outcome (Leese, 2002; Baumann *et al.*, 2007; Leese *et al.*, 2008). Indeed, it is thought that the genome, transcriptome and proteome of viable embryos are not overly compromised, thus supporting viable development and leading to a decreased level of oxygen and nutrient consumption. In contrast, however, less viable embryos may have more molecular/cellular damage, or their transcriptome and proteome levels may not be able to cope with any damage present, resulting in such cells attempting to repair themselves or undergo apoptosis with the consumption of a greater quantity of nutrients, reflecting an elevated level of metabolism (Leese *et al.*, 2007; 2008). Indeed, Wong *et al.* (2010) demonstrated that the rate of the first cell divisions within human zygotes may be a predictive indicator of embryogenesis, with zygotes that undergo rapid or delayed division arresting before reaching the blastocyst stage. It would be intriguing to examine the metabolic rates of embryos activated by varying degrees of Ca²⁺ oscillation profiles elicited by different levels of PLC ζ , reflecting the inherent variability of levels of PLC ζ within human sperm. Considering that most species require a specific ‘window’ of PLC ζ for activation to occur, with other levels leading to developmental arrest, it may be that not

all levels or localisation patterns of PLC ζ within a fertilising sperm lead to competent oocyte activation and healthy embryogenesis.

It is also essential that future studies investigate the implications of variable levels and localisation patterns of PLC ζ in human sperm to understand the implications these findings may have upon ART. Indeed, recent studies implicate an iatrogenic alteration in the signalling pathways involved at fertilisation and oocyte activation, which may result in altered embryonic gene expression, as well as increased epigenetic risk, due to differential Ca²⁺ oscillations profiles resulting from different techniques, such as ICSI or IVF, which may manifest as disease, not only in young children but in adults as well (Bridges *et al.*, 2011; Ciapa & Arnoult, 2011).

8.3 The clinical use of PLC ζ as a therapeutic agent within ART

Yoon *et al.* (2008) were the first to show, in principle, the potential use of PLC ζ within the clinic to ‘rescue’ the activating ability of ICSI failed sperm by its co-injection with PLC ζ mRNA. However, little progress has been made with regards to the clinical utilisation of PLC ζ , most likely due to the failure to purify an active recombinant human version of the enzyme, coupled with large gaps in knowledge regarding PLC ζ mechanism and mode of action within sperm and during oocyte activation. Furthermore, a large-scale screen of the PLC ζ gene of infertile males diagnosed with oocyte activation deficiency has been lacking, raising questions as to the relative importance of the role of mutations in PLC ζ in human infertility.

Chapter 6 of this thesis describes, for the first time, the successful production of whole cell lysates containing active recombinant human PLC ζ , which were able to elicit Ca²⁺ oscillations characteristic of fertilisation when injected into mouse oocytes. The results may serve to illuminate the reasons as to why the literature has been less than forthcoming with regards to a report of the purification of active recombinant human PLC ζ . Indeed, human PLC ζ may require post-translational modifications to reach an active state. While recombinant human protein was successfully produced from a prokaryotic system, these preparations were unable to elicit Ca²⁺ oscillations upon microinjection into mouse oocytes. However, cell lysates obtained from mammalian HEK293T cells (which would permit for any PTMs that PLC ζ may require for functional activity) transfected with PLC ζ isoforms were able to perform this function successfully. The PLC ζ being produced by this system seemed to possess a C-terminal truncation, resulting in the cleavage of ~100bp of non-domain regions of PLC ζ , possibly explaining why a C-terminal tag purification strategy was unsuccessful in yielding a purified product.

However, it remains unclear as to whether this truncated version of PLC ζ is the active component required for oocyte activation, or whether it is an artefact of the HEK293T cells utilised in this study. It is vital that future studies examine whether the same truncation occurs using different mammalian cell lines, to establish the importance of such modification. It would also be interesting to examine whether the use of N-terminal purification tags leads to the successful purification of active human PLC ζ protein. Considering the potential therapeutic applications of successfully purified active human PLC ζ protein, it would be necessary to test any such protein within human oocytes to confirm its efficacy as a potential replacement for current AOA protocols.

While the current results represent a significant advance in the development of such a protocol, and represent a crucial first step in the clinical application of PLC ζ , other results presented in this thesis concerning PLC ζ mechanism and mode of action indicate that more study is required before PLC ζ protein may be used as a replacement for current AOA protocols. While there seems to be a large degree of variance with regards to the expression of mouse or human PLC ζ and their localisation patterns within somatic cells lines (Coward *et al.*, 2006; Ito *et al.*, 2008a; Phillips *et al.*, 2011), this may reflect the cell type used, or differential functional ability of human and mouse PLC ζ . However, human PLC ζ may exhibit increased preference for organelle membrane-bound PIP₂ as opposed to plasma membrane bound substrate within HEK293T cells.

Furthermore, results indicated that the EF hands appear to play a significant role for PLC ζ localisation, as well as a possible inhibitory role for the potential nuclear localisation of human PLC ζ . Indeed, the EF hands of mouse PLC ζ are thought to facilitate its nuclear localisation (Ito *et al.*, 2011). Furthermore, co-localisation of the nuclear populations of PLC $\zeta^{\Delta EF}$ with ER described in this thesis may imply that nuclear localisation occurs following organelle binding, possibly indicating the presence of a potential interactor(s) and/or modulator(s) of PLC ζ activity. However, it is also possible that the current results relate to the disruption of localisation mechanisms present *in vivo*, and therefore further investigation is required to determine the specific role of the EF hands in mechanism of action and localisation of PLC ζ . It is essential that future studies elucidate the exact localisation of the nuclear populations of PLC $\zeta^{\Delta EF}$, as well as identifying potential interactors that may be involved in ER localisation. Such studies are essential to further understand PLC ζ mechanism and mode of action, which currently restricts the clinical viability of PLC ζ within ART.

8.4 PLC ζ : the chosen one, or one of many?

An overwhelming body of evidence now indicates that the sperm factor theory of oocyte activation is the most likely scenario by which mammalian oocytes alleviate MII arrest, and proceed with embryogenesis following fertilisation. PLC ζ is currently the best candidate for this endogenous sperm factor, which is responsible for eliciting the characteristic Ca^{2+} oscillations which accompany fertilisation (Swann *et al.*, 2006; Parrington *et al.*, 2007; Kashir *et al.*, 2010; Ito *et al.*, 2011). Numerous studies relating to PLC ζ structure/function and prevalence in a variety of species (Saunders *et al.*, 2002; Fujimoto *et al.*, 2004; Coward *et al.*, 2005; Kouchi *et al.*, 2005; Kurokawa *et al.*, 2005; Ito *et al.*, 2008a; 2008b; Mizushima *et al.*, 2008; Nomikos *et al.*, 2011b; 2011c), as well as mounting clinical evidence (Yoon *et al.*, 2008; Heytens *et al.*, 2009; Taylor *et al.*, 2010) support the assertion that PLC ζ is indeed the oocyte activation factor. However, conclusive evidence is currently lacking in the form of a knock-out mouse, which demonstrates infertility, although one may argue that absent/reduced levels of PLC ζ in mouse and human sperm (Yoon *et al.*, 2008; Heytens *et al.*, 2009; 2010) which are unable to activate oocytes already serve this purpose. Furthermore, considering that PLC ζ shares its bi-directional promoter with the *CAPZA3* gene, knocking out this promoter may result in defects in sperm structure.

However, numerous studies have suggested evidence for alternative sperm-borne activating factors, ranging from Ca^{2+} ions, IP_3 , and cyclic guanosine monophosphate (Whitaker & Crossley, 1990) to a truncated c-kit tyrosine kinase (Sette *et al.*, 1997; 1998), as well as citrate synthase (Harada *et al.*, 2007) and PAWP (Wu *et al.*, 2007; Aarabi *et al.*, 2010). Indeed, several researchers maintain that the G-protein hypothesis for oocyte activation, where activation occurs externally on the oocyte plasma membrane, similar to a

ligand attaching to a membrane receptor remains the correct theory (Gould & Stephano, 1991; Kline *et al.*, 1991; Bonilla *et al.*, 2008; Dale *et al.*, 2010; White *et al.*, 2010).

Furthermore, Src-family kinases are known to play a critical role in several events during oocyte development and fertilisation, relating to meiotic spindle control and contributing towards cortical actin cytoskeletal dynamics. Src-family kinases also play a role in IP₃-mediated Ca²⁺ signalling through the activation of PLCγ particularly within externally fertilising species such as sea urchins, contributing, or even playing a major role, in egg activation (McGinnis *et al.*, 2011). It is thought that the abnormal function of SRC-family kinase within these species may lead to significant loss of oocyte quality. It is possible that such defects may manifest in mammalian species as failure to complete meiosis, abnormal polar body/cytoplasmic volume, or failure of pronuclear congression (McGinnis *et al.*, 2011).

Considering that changes in plasma membrane conductance, Ca²⁺ ion release and MPF inactivation are features common to all animal eggs and oocytes during activation (Dale *et al.*, 2010), one would also expect the sperm-borne trigger to be common, as well as targeting the same substrate. Indeed, PLCζ has been identified from a variety of mammalian, as well as non-mammalian species (Coward *et al.*, 2005; Ito *et al.*, 2008a; 2008b; Mizushima *et al.*, 2008). However, the identification of ovarian and brain forms of PLCζ in puffer-fish (*Takifugu rubripes*), the microinjection of which failed to elicit Ca²⁺ oscillations in mouse oocytes (Coward *et al.*, 2011), illustrates that while substantial evidence implicates PLCζ as the mammalian sperm factor, further characterisation of fertilisation is required for other species. The discovery of this oocyte-specific PLCζ orthologue raises questions of how a sperm stimulus may activate the oocyte within this

species. While this may be an evolutionary adaption related to the reproductive strategy of the species, it is possible that PLC ζ from this species requires oocyte/sperm-based mechanisms or modifications to become active, in a manner similar to mouse oocytes (Phillips *et al.*, 2011).

It also remains possible that PLC ζ , while a major contributor towards the Ca²⁺ oscillation profile elicited following gamete fusion, is not the sole contributor towards oocyte activation. It is plausible that oocyte activation requires Ca²⁺ signalling input from a variety of sources, ranging from sperm ligand/oocyte receptor interaction, to multiple sperm factors released into the oocyte, each responsible for their own particular function within activation and subsequent embryogenesis (Ciapa & Arnoult, 2011). Indeed, McGinnis *et al.* (2007) demonstrated that the activation of Src-family protein tyrosine kinases within mouse oocytes was limited to distinct regions, and occurred at specific times following fertilisation. While these were shown not to be required for the fertilisation-induced Ca²⁺ oscillations in mouse oocytes, they were theorised to play a role in zygote development.

The greatest support for the sperm factor theory of oocyte activation has always been a combination of successful Ca²⁺ release following microinjection of sperm extracts, and the successful clinical application of ICSI, which has resulted in high birth rates around the world. However, ICSI results in Ca²⁺ oscillation profiles which are slightly different from those described after normal fertilisation (Ciapa & Arnoult, 2011), with gene expression profiles also differing markedly (Bridges *et al.*, 2011). The spatial distribution and duration of Ca²⁺ oscillations were not equivalent in mouse oocytes subjected to ICSI compared to those subjected to IVF (Nakano *et al.*, 1997; Kurokawa &

Fissore, 2003). However, this discrepancy in Ca^{2+} oscillation profiles resulting from different treatments may be due either to differential levels or patterns of localisation of PLC ζ within sperm. Indeed, the results within this thesis indicate that levels and localisation patterns of PLC ζ within sperm differ widely between fertile individuals.

Regardless of the reasons, ART has been associated with an increase in the frequency of Prader-Willi or d'Angelman syndrome, which results from genetic imprinting defects on chromosome 15, and of the Beckwith Wiedemann syndrome, which arises as a result of epigenetic abnormalities in a cluster of genes that are subject to imprinting on chromosome 11 (Manipalviratn *et al.*, 2009). However, it seems that such cases are rare, with the number of reported cases insufficient for deducing any firm conclusion (Ciapa & Arnoult, 2011). Furthermore, methylation reprogramming defects in particular genes and imprinting control regions have been reported to occur following ART in humans (Fauque *et al.*, 2007; Zechner *et al.*, 2010; Ciapa & Arnoult, 2011). Histone methylation patterns seemingly differed between IVF and ICSI in mice (Van der Heijden *et al.*, 2009) and in humans (Qiao *et al.*, 2010). In rats, the demethylation dynamics of the paternal genome at the pronuclear-stage is impaired when ART is used (Yoshizawa *et al.*, 2010). It is important for future research to focus on the reasons underlying these differences between Ca^{2+} oscillations profiles and in turn, epigenetic modifications (Ciapa & Arnoult, 2011). Are they due to the differential levels and localisation patterns of PLC ζ which are largely variable between fertile individuals, or are these modifications due to missing components in a much larger oocyte activation mechanism of action than currently understood? The answer to such questions is essential not only for the therapeutic and clinical use of PLC ζ , but to determine the efficacy of ART, and understand whether molecular mechanisms beyond the current understanding have inadvertently been altered.

8.5 General conclusions

In summary, this thesis demonstrates that a PCR-based screening approach to identify novel loss-of-activity mutations in the PLC ζ ORF may not be sufficient when confronted with multiple patient samples, and that more efficient and high throughput technology is required. Out of 12 patients and 2 fertile controls screened, only one novel mutation was identified, which resulted in a change of a histidine to a leucine at position 233 of the PLC ζ amino acid ORF, indicating that such loss-of-activity mutations may be rare occurrences. However, it is essential that future studies continue genomic screening while expanding the size and bio-geographical range of patients examined. It was also shown that the novel H233L mutation severely reduced PLC ζ enzymatic activity, and that the maternal inheritance of autosomal point mutations in male-specific proteins may result in certain forms of male factor infertility, highlighting the importance of studying maternal inheritance in male infertility.

Quantitative immunofluorescence studies indicated that levels of total PLC ζ varied dramatically, with some fertile controls exhibiting similar levels of PLC ζ fluorescence as sperm from infertile patients. The equatorial pattern of PLC ζ localisation, previously identified by Grasa *et al.* (2008) to be predominant, was only prevalent in one control, with the rest exhibiting post-acrosomal and equatorial + post-acrosomal patterns of localisation. Furthermore, the usefulness of PLC ζ as a biomarker of sperm fertility has been highlighted by demonstrating that density gradient washing dramatically increased the proportion of sperm exhibiting PLC ζ , while cryopreservation may reduce levels of PLC ζ in fertile sperm. However, this effect was shown to differentially affect sperm from different males. While possibly indicating a contributory role for PLC ζ in ensuring genetic and epigenetic diversity, these results imply that quantitative immunofluorescence may not be a useful

prognostic indicator of oocyte activation capability, but rather that analysis of the proportions of sperm exhibiting PLC ζ immunoreactivity may be more representative of male fertility.

Crucially, this thesis describes, for the first time, the successful production of active recombinant human PLC ζ protein within lysates obtained from transfected eukaryotic cells, which induced characteristic patterns of Ca²⁺ release upon microinjection in mouse oocytes. It was shown that the H398P mutation abolished these oscillations, indicating that this mutation causes loss of PLC activity of PLC ζ . It is possible that PLC ζ requires post translational modifications to reach an active state. Indeed, a non-domain C-terminal truncation was described, which may have led to the absence of C-terminal tags within PLC ζ expressed in HEK293T cells. Furthermore, human PLC ζ is expressed in an active state in transfected HEK293T cells, and may exhibit a preference towards organelle membrane bound PIP₂ as opposed to plasma membrane bound substrate. The EF hands of PLC ζ may also play a role in its localisation, while also possibly playing a role in the inhibition of the nuclear localisation signal of human PLC ζ .

The results described in this thesis further understanding not only of the mode and mechanisms of PLC ζ action and regulation within the sperm and the activating oocyte, but illustrate the potentially complex role that PLC ζ plays within human male fertility and possibly early embryogenesis. Collectively, the thesis describes the completion of the first crucial steps required to translate PLC ζ research into its potential therapeutic and diagnostic applications within the clinic, as well as furthering understanding of a fundamental biological event.



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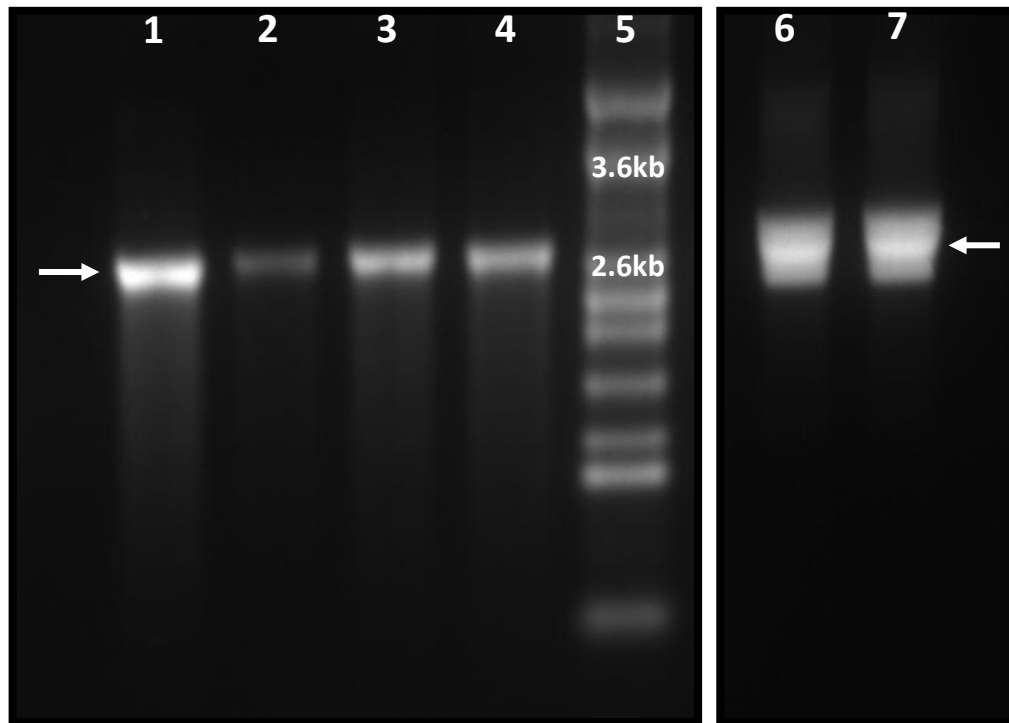
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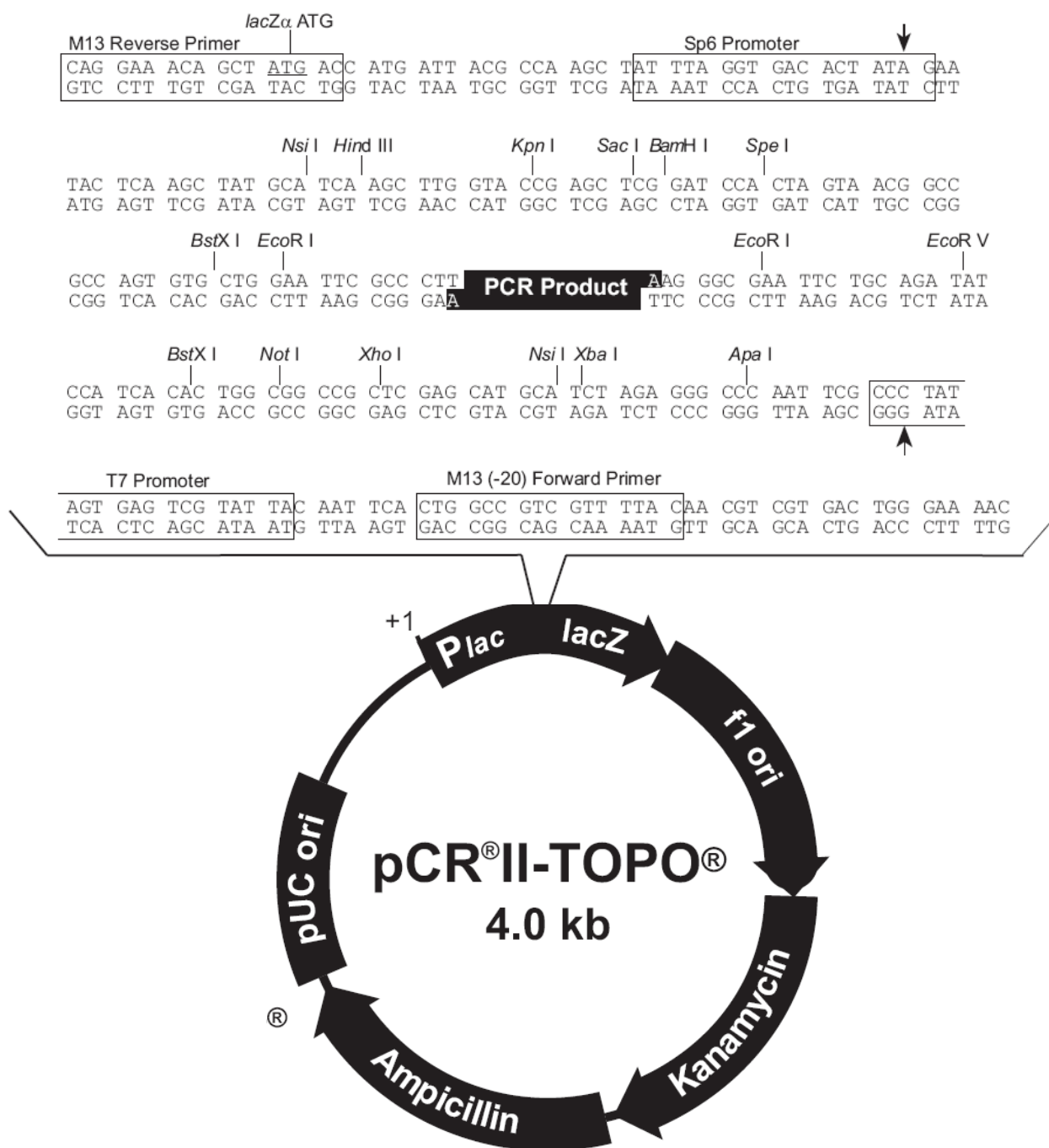
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Supplementary Appendices



Supplementary Appendix 1: Representative 1% RNA gel exhibiting synthesized cRNA size and integrity. All bands (lanes 1-4 and 6-7) represent mCherry-PLC ζ^{WT} cRNA bands (white arrows; ~2.7kb). Lane 5 represents a RNA standard. Lanes 1-4 represent un-degraded cRNA which would be of good quality to utilize for mouse oocyte injection, while lanes 6 and 7 represent cRNA which has degraded to a large extent as signified by the smearing present on either side of the bands.



Supplementary Appendix 2: Representative vector map of the pCR[®] II TOPO, and the sequence surrounding the TOPO[®] Cloning site. Restriction sites are labelled to indicate the actual cleavage site. The arrows indicate the start of transcription for Sp6 and T7 polymerases. Figure modified from the pCR[®] II-TOPO manual (http://tools.invitrogen.com/content/sfs/manuals/topota_man.pdf).

A

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 TGTTATGTTTGGTACGTGAGA

Exon 3: 14-134 Exon 4: 15-367
 Exon 5: 368-569 Exon 6: 570-714
 Exon 7: 715-864 Exon 8: 865-949
 Exon 9: 950-1017 Exon 10: 1018-1174
 Exon 11: 1175-1291 Exon 12: 1292-1461
 Exon 13: 1462-1591 Exon 14: 1592-1741
 Exon 15: 1742-1824

B

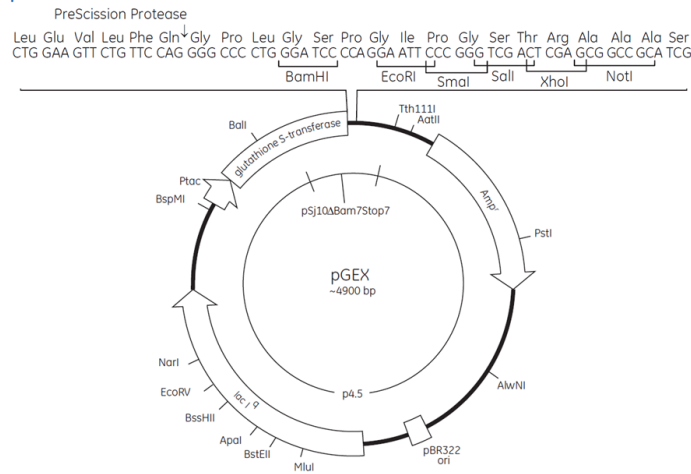
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 GTCAGA

EF HANDS X-DOMAIN X-Y LINKER
 Y-DOMAIN C2 DOMAIN
 NUCLEAR LOCALISATION SIGNAL
 CATALYTICALLY IMPORTANT RESIDUES
 RESIDUES VITAL FOR Ca²⁺ BINDING
 PUTATIVE PHOSPHOINOSITIDE BINDING SITE
 Predicted Splicing Sites: Donor sites
 Acceptor sites

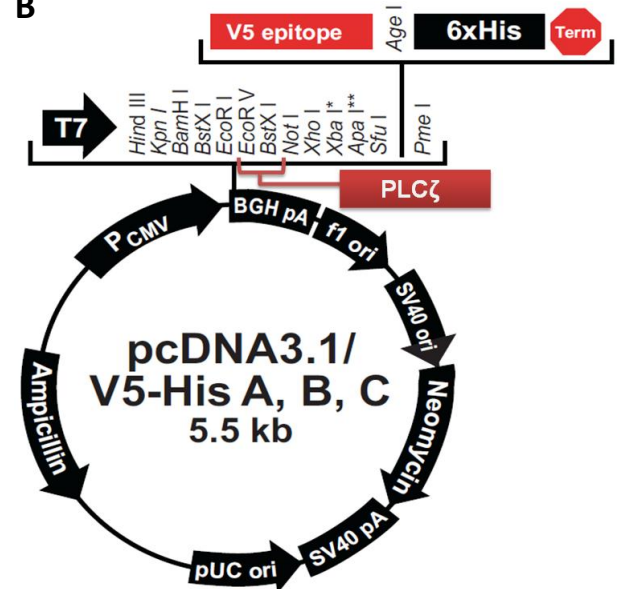
Supplementary Appendix 3: PLC ζ open reading frame (ORF) highlighted to represent (A) coding exons of the PLC ζ gene, and (B) sections of the ORF corresponding to the various domains of PLC ζ .

A

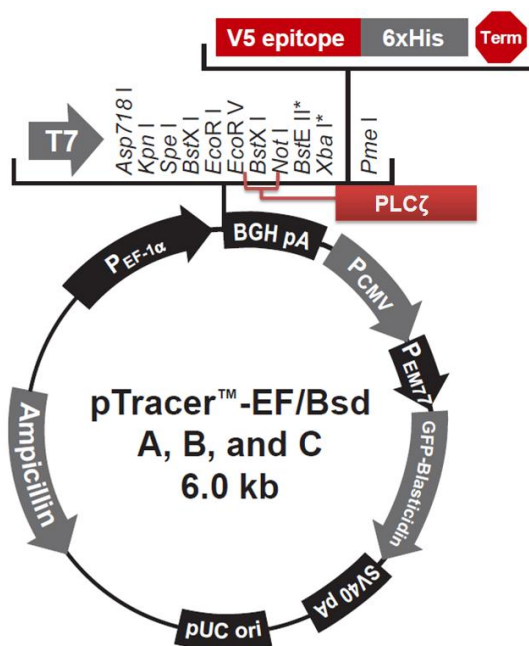
pGEX-6P-2



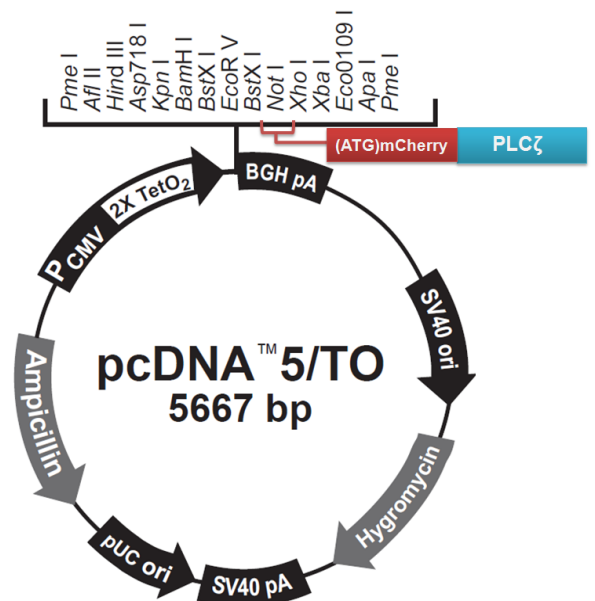
B



C

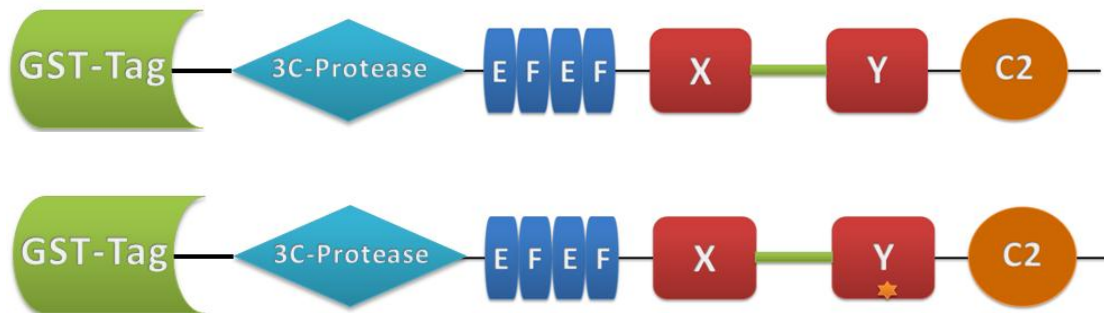


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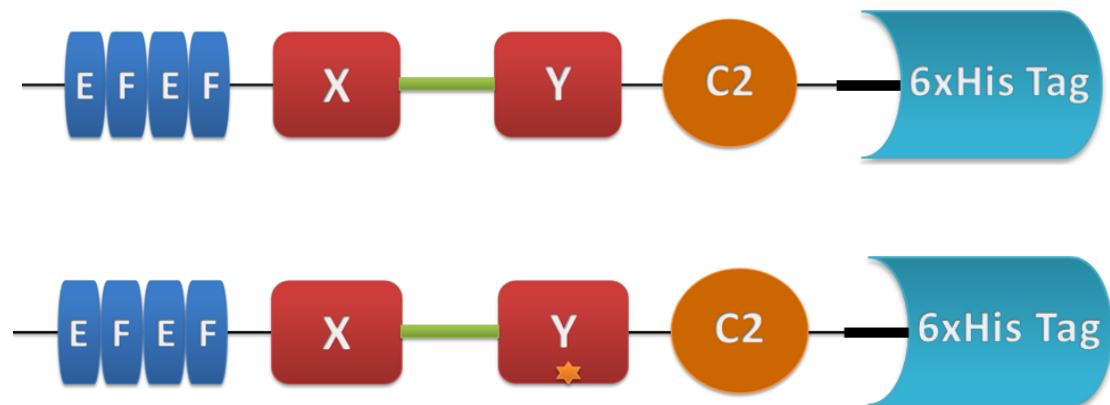


Supplementary Appendix 4: Schematic representations of; **A)** pGEX-6P-2 vector which confers an N-Terminal Glutathione S-transferase (GST) tag to the insert to aid in purification, along with a PreScission Protease site to aid in removal of the GST following purification. Also consists of an Ampicillin resistance gene to confer selectivity. **B)** pcDNA3.1 vector which adds a C-Terminal 6xHis tag to the required insert to aid in purification. Also consists of an ampicillin resistance gene to confer selectivity. **C)** pTracer vector, which adds a C-Terminal 6xHis tag to aid in purification. Also consists of an ampicillin resistance gene to confer selectivity. Consists of GFP and Blasticidin resistance genes with separate promoters to aid in the creation of stable cell lines. Figures adapted from vector maps available from Invitrogen and GE Healthcare (UK).

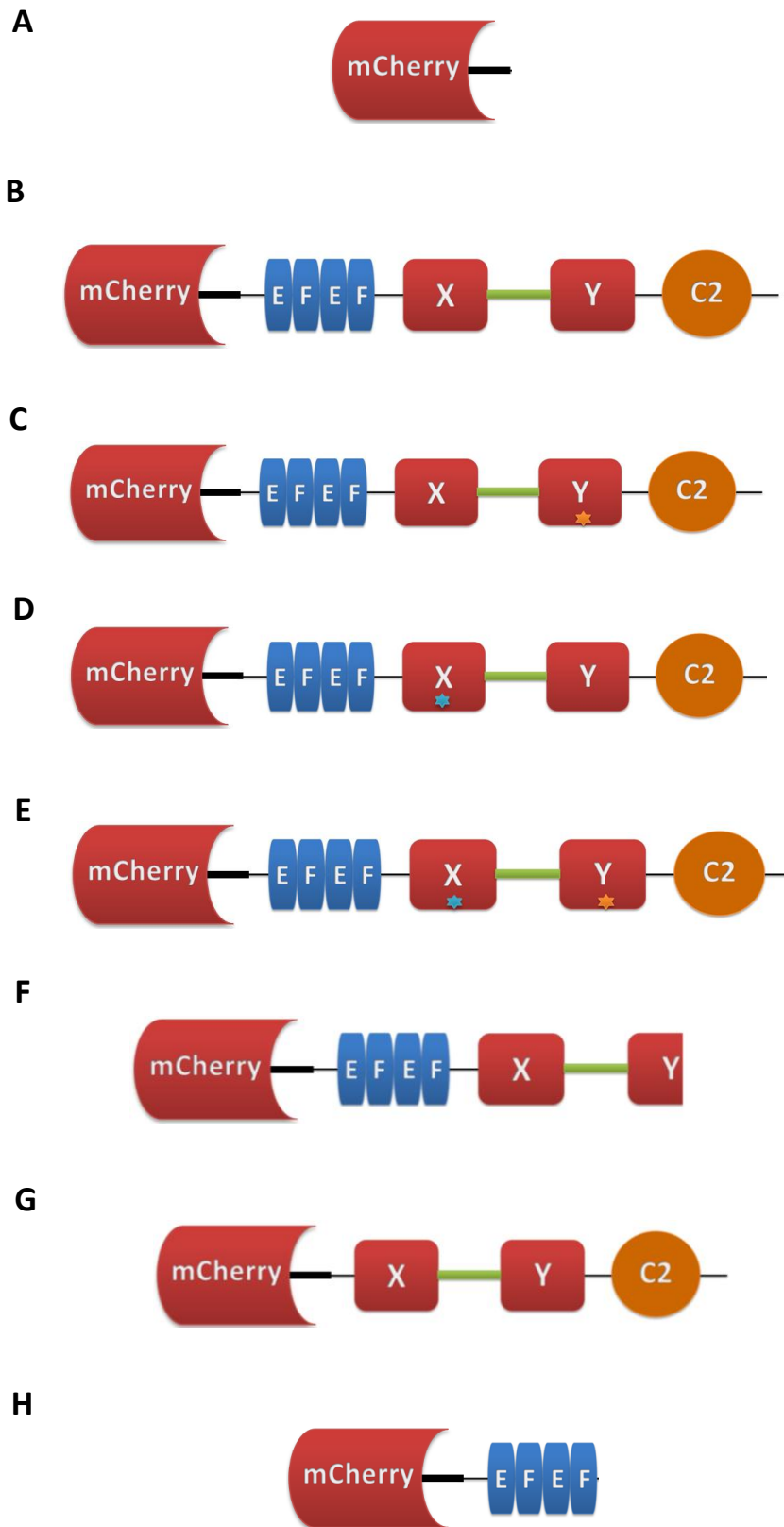
A



B

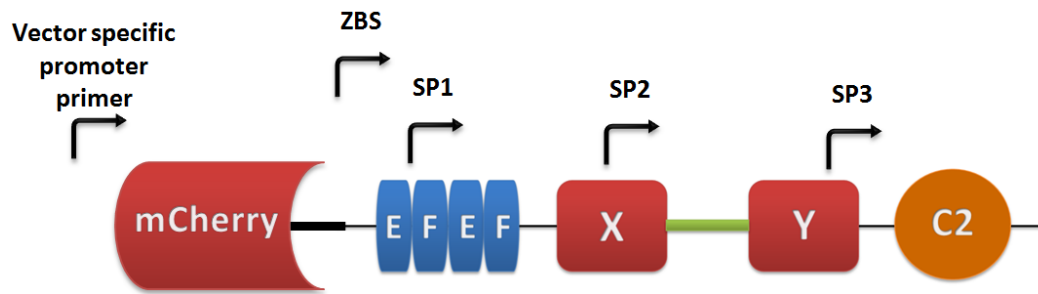


Supplementary Appendix 5: Schematic representations of **A)** PLCζ isoforms produced using the pGEX-6P-2 vector which confers an N-Terminal Glutathione S-transferase (GST) tag to the insert to aid in purification, along with a PreScission Protease site to aid in removal of the GST following purification. Also consists of an Ampicillin resistance gene to confer selectivity. **B)** PLCζ isoforms produced using the pcDNA3.1 and pTracer vectors which add a C-Terminal 6xHis tag to the required insert to aid in purification. The orange star in the Y domain refers to the H398P mutation.

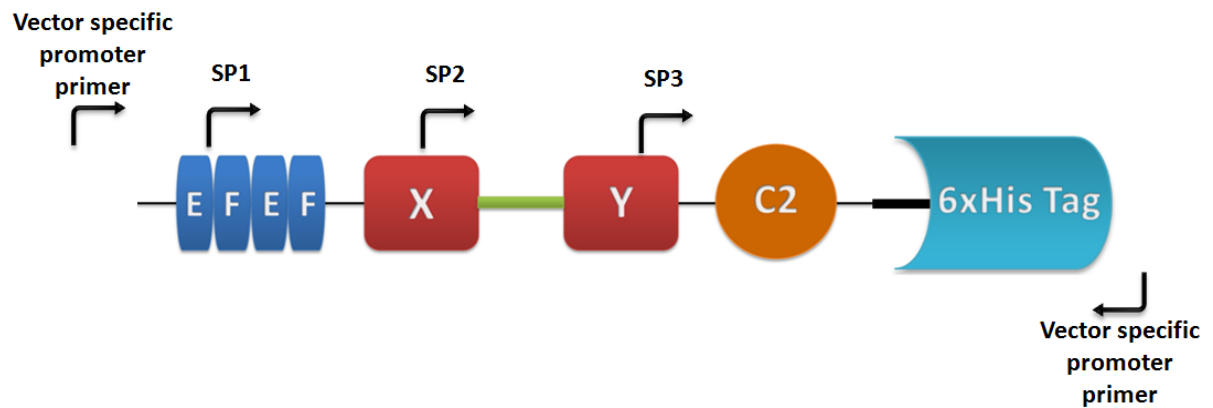


Supplementary Appendix 6: Schematic representations of mCherry-tagged PLCζ isoforms produced and used in this thesis. These schematics describe the products of **A)** mCherry-Empty vector; **B)** mCherry-PLCζ^{WT}; **C)** mCherry-PLCζ^{H398P}; **D)** mCherry-PLCζ^{H233L}; **E)** mCherry-PLCζ^{H233L+H398P}; **F)** mCherry-PLCζ^{ΔYC2}; **G)** mCherry-PLCζ^{ΔEF}; and **H)** mCherry-PLCζ^{EF}. The aqua and orange stars denote the H233L and the H398P mutations in the PLCζ ORF respectively.

A



B



C

Primer	PLCζ ORF binding site	Primer sequence (5' → 3')
Vector specific promoter primer	Either forward or reverse promoter	Variable
ZBS	End of mCherry sequence (to sequence first 100 bases of PLCζ)	ATGGACGAGCTGTACAAGTAA
SP1	Position 10 (to sequence up to position 800)	AGATGGTTTTTGTCAAAGATTCAG
SP2	Position 650 (to sequence up to position 1400)	ACACACTCACAAGCAAACCTCTG
SP3	Position 1300 (to sequence up to position 1824)	AATTTCAGACCCCTGGTCT

Supplementary Appendix 7: Schematic representations of the overlapping sequencing primer strategy used to sequence PLCζ isoforms following cloning into **A)** pcDNA 5/TO (mCherry), and **B)** pcDNA 3.1 and pTracer vectors. **C)** Table representing the details of the primers designed used for the overlapping sequencing strategy.

Score = 1013 bits (548), Expect = 0.0
 Identities = 550/551 (99%), Gaps = 0/551 (0%)
 Strand=Plus/Plus

```

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Query  61     ATAGAAGAATTTAGAGCAATTTATCGAATTATCACGCACAGAGAAGAAATTATTGAGATT  120
          |||
Sbjct  169     ATAGAAGAATTTAGAGCAATTTATCGAATTATCACGCACAGAGAAGAAATTATTGAGATT  228

Query  121    TTCAACACATATTCTGAAAACCGGAAAATTCTTTTAGCAAGTAATCTGGCTCAATTTCTG  180
          |||
Sbjct  229     TTCAACACATATTCTGAAAACCGGAAAATTCTTTTAGCAAGTAATCTGGCTCAATTTCTG  288

Query  181    ACACAAGAACAATATGCAGCTGAGATGAGTAAAGCTATTGCTTTTGAGATCATTAGAAA  240
          |||
Sbjct  289     ACACAAGAACAATATGCAGCTGAGATGAGTAAAGCTATTGCTTTTGAGATCATTAGAAA  348
          ↓
Query  241    TACGAGCCTATCAAAGAAGTTAGGAAAGCACACCAAATGTCATTAGAAGGTTTACAAGA  300
          |||
Sbjct  349     TACGAGCCTATCGAAGAAGTTAGGAAAGCACACCAAATGTCATTAGAAGGTTTACAAGA  408

Query  301    TACATGGATTACCGTGAATGTCTACTGTTTAAAAATGAATGTAGAAAAGTTTATCAAGAT  360
          |||
Sbjct  409     TACATGGATTACCGTGAATGTCTACTGTTTAAAAATGAATGTAGAAAAGTTTATCAAGAT  468

Query  361    ATGACTCATCCATTAAATGATTATTTTATTTTCATCTTCACATAACACATATTTGGTATCT  420
          |||
Sbjct  469     ATGACTCATCCATTAAATGATTATTTTATTTTCATCTTCACATAACACATATTTGGTATCT  528

Query  421    GATCAATTATTGGGACCAAGTGACCTTTGGGGATATGTAAGTGCCCTTGTGAAAGGATGC  480
          |||
Sbjct  529     GATCAATTATTGGGACCAAGTGACCTTTGGGGATATGTAAGTGCCCTTGTGAAAGGATGC  588

Query  481    CGTTGTTTGGAGATTGACTGCTGGGATGGAGCACAAAATGAACCTGTTGTATATCATGGC  540
          |||
Sbjct  589     CGTTGTTTGGAGATTGACTGCTGGGATGGAGCACAAAATGAACCTGTTGTATATCATGGC  648

```

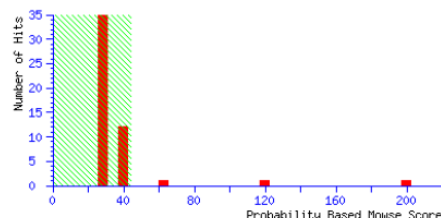
Supplementary Appendix 8: Representative NCBI BLAST alignment of the PLC ζ ORF (Sbjct) and sequencing results from pcDNA3.1-PLC ζ^{WT} (Query). The title at the top indicates a summary of the alignment result. Score: length of sequence input into software; Expect: parameter that describes the number of hits one can "expect" to see by chance when searching a database of a particular size; Identities: homology of the match, i.e. 100% indicates the full sequence is a match; Gaps: indicates any gaps within the input sequences; Strand: Direction of the input strand. Bases which exhibit a match are indicated by a line between the two sequences. Bases which do not show a match exhibit a blank space (arrow) between the sequences. A: Adenine; C: Cytosine; T: Thymine; G: Guanine. In this case, this sequencing result represents a colony which had an unwanted point mutation, and cannot therefore be used for downstream applications.

Mascot Search Results

User : bmk
 Email :
 Search title : junaaid upper.mgf
 MS data file : D:\Data\February 2009\junaaid upper.mgf
 Database : UniProt_SwissProt_sprot (410518 sequences; 148080998 residues)
 Taxonomy : Mammalia (mammals) (64201 sequences)
 Timestamp : 17 Feb 2009 at 09:27:46 GMT
 Protein hits : [P02754](#) RecName: Full=Beta-lactoglobulin; Short=Beta-LG; AltName: Allergen=Bos d 5; Flags: Precursor;
 [P67975](#) RecName: Full=Beta-lactoglobulin; Short=Beta-LG;
 [Q95JS1](#) RecName: Full=1-phosphatidylinositol-4,5-bisphosphate phosphodiesterase zeta-1; EC=3.1.4.11; AltName: Full=Phosphol
 [Q96K76](#) RecName: Full=Ubiquitin carboxyl-terminal hydrolase 47; EC=3.1.2.15; AltName: Full=Ubiquitin thioesterase 47; AltNa
 [Q2M1P5](#) RecName: Full=Kinesin-like protein KIF7;

Probability Based Mowse Score

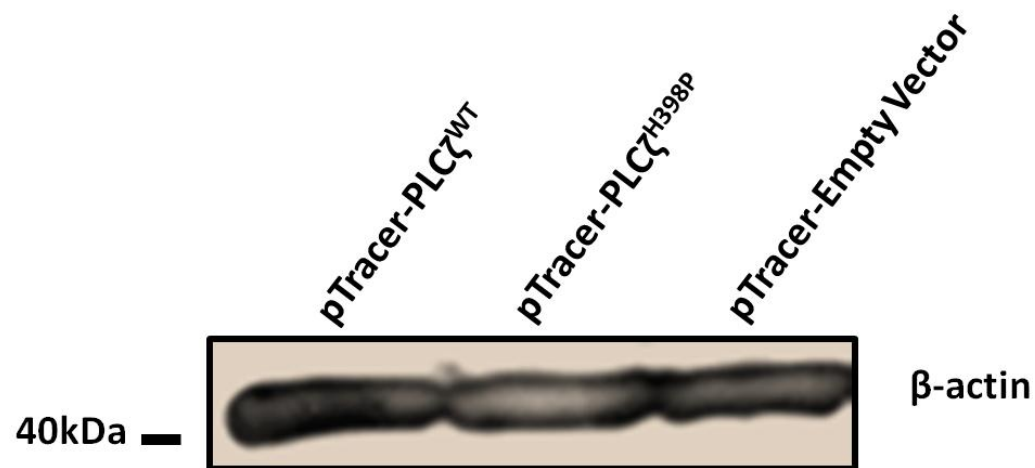
Ions score is $-10 \cdot \log(P)$, where P is the probability that the observed match is a random event.
 Individual ions scores > 44 indicate identity or extensive homology ($p < 0.05$).
 Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.



3. [Q95JS1](#) Mass: 74504 Score: 63 Queries matched: 5
RecName: Full=1-phosphatidylinositol-4,5-bisphosphate phosphodiesterase zeta-1; EC=3.1.4.11; AltName: Full=Phospholipase C-zeta-1; Shc
☐ Check to include this hit in error tolerant search or archive report

	Query	Observed	Mr (expt)	Mr (calc)	Delta	Miss	Score	Expect	Rank	Peptide
☒	54	397.5860	793.1574	792.3766	0.7808	0	(24)	4.2	1	K.IQDDFR.G
☒	55	397.6740	793.3334	792.3766	0.9568	0	31	0.87	1	K.IQDDFR.G
☒	102	518.3220	1034.6294	1033.5080	1.1215	0	28		2	K.YEPIEEVR.K
	329	1119.1750	2236.3354	2237.0699	-0.7345	1	10	1.6e+02	5	K.ESEIFESVLGENQDKETGVK.K
	330	1119.1790	2236.3434	2237.0699	-0.7265	1	(9)	1.7e+02	9	K.ESEIFESVLGENQDKETGVK.K

Supplementary Appendix 9: Representative screen shot illustrating match of protein band submitted for LC/MS with PLC ζ (red arrow). The screenshot also illustrates the peptides which were matched, and the score representing the confidence of the match (red highlight). Data provided courtesy of Dr. Mariola Edelman and Dr. Benedikt Kessler (University of Oxford, UK)



Supplementary Appendix 10: Representative immunoblot utilising an anti- β -actin monoclonal antibody to demonstrate equal loading of lysates obtained from HEK293T cells transfected with pTracer-PLC ζ^{WT} , -PLC ζ^{H398P} , and -Empty vector.



List of publications

International peer-reviewed articles

- 1) **Kashir J**, Heindryckx B, Jones C, De Sutter P, Parrington J, Coward K. Oocyte activation, phospholipase C zeta, and human infertility. *Human Reproduction Update* 2010;**16**:690-703.
- 2) **Kashir J**, Heynen A, Jones C, Durrans C, Craig J, Gadea J, Turner K, Parrington J, Coward K. Effects of cryopreservation and density-gradient washing on phospholipase C zeta concentrations in human spermatozoa. *Reproductive Biomedicine Online* 2011;**23**:263-267.
- 3) Coward K, Ponting CP, Zhang N, Young C, Huang CJ, Chou CM, **Kashir J**, Fissore RA, Parrington J. Identification and functional analysis of an ovarian form of the egg activation factor phospholipase C Zeta (PLC ζ) in pufferfish. *Molecular Reproduction and Development* 2011;**78**:48-56.
- 4) **Kashir J**, Jones C, Lee HC, Rietdorf K, Nikiforaki D, Durrans C, Ruas M, Tee ST, Heindryckx B, Galione A, Sutter PD, Fissore RA, Parrington J, Coward K. Loss of activity mutations in phospholipase c zeta (PLC ζ) abolishes calcium oscillatory ability of human recombinant protein in mouse oocytes. *Human Reproduction* 2011 [In Press].
- 5) **Kashir J**, Konstantinidis M, Jones C, Lemmon B, Lee HC, Hamer R, Heindryckx B, Deane CM, Sutter PD, Fissore RA, Parrington J, Wells D, Coward K. A maternally-inherited autosomal point mutation in the oocyte activation factor phospholipase C zeta (PLC ζ) leads to male infertility. *Human Reproduction* [Submitted for review, 2011].

Presentations at international and university conferences (presented by JK)

- 1) **Kashir J**, Jones C, Young C, Heytens E, Grasa P, De Sutter P, Parrington J, Coward K. Abnormal localization patterns and a mutant isoform of the oocyte activation factor, phospholipase C zeta, in spermatozoa from infertile men. *2nd Congress of the International Network for Young Researchers in Male Fertility*. 2009. Munster, Germany. [Oral presentation; **Prize for best oral presentation of session**].
- 2) **Kashir J**, Young C, Heytens E, Grasa P, Jones C, Ruas M, Yoon SY, Fissore RA, Deane CM, Nikiforaki D, Tee ST, Sutter PD, Parrington J, Coward K. (2009). Abnormal localization patterns and mutant isoforms of the oocyte activation factor,

phospholipase c zeta, in spermatozoa from infertile men. *5th Medical Sciences DPhil Day*, University of Oxford [Poster presentation].

- 3) **Kashir J**, Jones C, Young C, Ruas M, Grasa P, Rietdorf K, Heytens E, Yoon SY, Fissore RA, Deane CM, Nikiforaki D, Tee ST, De Sutter P, Parrington J, Coward K. Identification and functional characterization of a point mutation in the gene encoding the sperm factor phospholipase C zeta in an infertile male. *26th Annual meeting of the European Society for Human Reproduction and Embryology (ESHRE)*. 2010. Rome, Italy. [Poster presentation].
- 4) **Kashir J**, Jones C, Lemmon B, Konstantinidis M, Heindryckx B, Fissore RA, Deane CM, Sutter PD, Wells D, Parrington J, Coward K. The identification and functional characterisation of mutant isoforms of the oocyte activation factor phospholipase C zeta (PLC ζ), and their genetic modes of inheritance. *Fertility 2011*, Dublin, Ireland. [Oral presentation; **Prize for best scientific oral communication**].

International and university conference abstracts (presented by authors other than JK)

- 1) Coward K, Young C, **Kashir J**, Heytens E, Yoon SY, Grasa P, Jones C, Ruas M, Deane CM, Hamer R, Moser JM, Jensen NM, Schmitt-John T, Fissore RA, Sutter PD, Parrington J. Abnormal expression, localisation, and mutant isoforms of the oocyte activation factor, phospholipase c zeta, in infertile mice and men. *Gordon Research Conference on Egg Activation and Fertilization*. 2009. New Jersey, USA. [Poster presentation; Parrington J].
- 2) **Kashir J**, Jones C, Lemmon B, Konstantinidis M, Heindryckx B, Lee HC, Fissore RA, Hamer R, Deane CM, Sutter PD, Wells D, Parrington J, Coward K. Egg activation as a model for understanding fertilization and the molecular basis of intracellular calcium signalling. *Egg Activation Symposium*. 2010. Seattle, USA. [Oral presentation; Parrington J].
- 3) Parrington J, **Kashir J**, Jones C, Lemmon B, Konstantinidis M, Heindryckx B, Lee HC, Fissore RA, Hamer R, Deane CM, Sutter PD, Wells D, Coward K. Identification and functional characterisation of mutant isoforms of the oocyte activation factor phospholipase C zeta (PLC ζ) and their genetic modes of inheritance. *Gordon Research Conference on Fertilization and Activation of Development*. 2011. New Jersey, USA. July. [Oral presentation; Parrington J].

- 4) Ramadan WM, Jones C, Thurston E, **Kashir J**, Parrington J, Coward K. The effect of age and epididymal maturation upon levels of the oocyte activation factor phospholipase C zeta (PLC ζ) in mouse sperm. *33rd Annual Meeting of the British Andrology Society*. 2011. Birmingham, United Kingdom. [Poster presentation; Ramadan WM].
- 5) **Kashir J**, Jones C, Lemmon B, Konstantinidis M, Heindryckx B, Lee HC, Fissore RA, Hamer R, Deane CM, Sutter PD, Wells D, Parrington P, Coward K.. Identification and functional characterisation of mutant isoforms of the oocyte activation factor phospholipase C zeta (PLC ζ) and their genetic modes of inheritance. *14th World Congress on Human Reproduction*. Nov/Dec 2011. Melbourne, Australia. [Oral presentation; Parrington J].

Popular science articles

- 1) Guner J, Jones C, **Kashir J**, Turner K, & Coward K.. Fertile prospects for human embryology training. *Biologist (London)* 2010;**57**:69-76.
- 2) Shivare SB, Ladas I, **Kashir J**, Jones C, Griffiths T, Turner K, Coward K. Light microscopy in clinical embryology: Lighting the way for infertility treatment. *Microscopy and Analysis* 2011;**March**:9-13.
- 3) Thurston E, Foote L, Jones C, **Kashir J**, Coward K. Life from the laboratory bench: The everlasting legacy of Sir Robert Edwards. *Biologist (London)* [Submitted, 2011].

Book Chapters

- 1) **Kashir J**, Jones C, Coward K. Calcium Oscillations, Oocyte Activation, and Phospholipase C zeta. In: *Calcium Signaling: Advances in Experimental Medicine and Biology Series* (Dr. Islam MS Editor). Springer Science + Business media. Springer Dordrecht Heidelberg London New York. [In Press].
- 2) **Kashir J**, Jones C, Parrington J, Coward K. Chapter 11: Fertilization and Egg Activation. In: *Textbook of Clinical Embryology* (Coward K & Wells D; Editors). Cambridge University Press. Cambridge, UK. [In Press].

- 3) Jones C, **Kashir J**, Coward K. Chapter 23: Fundamental Skills in Clinical Embryology. In: *Textbook of Clinical Embryology* (Coward K & Wells D; Editors). Cambridge University Press, Cambridge, UK. [In Press].
- 4) Gadea J, Parrington J, **Kashir J**, Coward K. Chapter 3: The Male Reproductive Tract and Spermatogenesis. In: *Textbook of Clinical Embryology* (Coward K & Wells D; Editors). Cambridge University Press, Cambridge, UK. [In Press].

Miscellaneous

- 1) **Letter to the Editor:** A sperm's tail: the need to consider temporal aspects of specific physiological mechanisms during and following gamete fusion. **Reply to:** Ramalho-Santos J. A sperm's tail: the importance of getting it right. *Hum Reprod* 2011;**26**:2590-2591 [Submitted, 2011].

Prizes and awards

- 1) **Best Oral Presentation of Session:** 2nd Congress of the International Network for Young Researchers in Male Fertility. 2009. Munster, Germany.
- 2) **Best Scientific Oral Communication:** Fertility 2011, Dublin, Ireland.