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RESEARCH

A catalytically competent sperm PLCzeta variant causes fertilization failure through protein insufficiency

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Abstract

Purpose The PLCζ p.Arg385Gln (R385Q) variant was recently identified in infertile patients with recurrent fertilization failure and markedly reduced sperm PLCζ levels. However, whether this variant causes infertility through loss of catalytic function or protein insufficiency remains unknown.

Methods The structural and functional consequences of the R385Q variant were investigated using structural modelling, protein stability prediction, recombinant PLCζ enzymatic assays, and mouse oocyte microinjection followed by Ca^{2+} oscillation analysis.

Results Structural modelling predicted that the R385Q substitution destabilizes the catalytic domain without disrupting the active site. Consistent with this prediction, recombinant PLCζ^{R385Q} exhibited phosphatidylinositol 4,5-bisphosphate (PIP_2) hydrolytic activity comparable to wild-type PLCζ. Likewise, microinjection of PLCζ^{R385Q} cRNA into mouse oocytes induced Ca^{2+} oscillations with frequencies and patterns indistinguishable from those elicited by wild-type PLCζ, demonstrating preserved catalytic and oocyte activation activity.

Conclusion These findings indicate that the R385Q variant is catalytically competent and does not cause infertility through loss of enzymatic function. Instead, together with our previous demonstration of markedly reduced PLCζ protein levels in patient sperm, the results support a protein insufficiency mechanism in which fertilization failure arises when PLCζ levels fall below the threshold required for physiological oocyte activation. This study expands the spectrum of pathogenic mechanisms underlying PLCζ-associated male infertility.

Keywords Phospholipase C zeta (PLCζ) · Sperm · Oocyte activation deficiency (OAD) · Infertility · Fertilization · Calcium

Introduction

Fertilization in mammals is initiated by a series of intracellular calcium (Ca^{2+}) oscillations that arise shortly after sperm-oocyte fusion. These repetitive Ca^{2+} transients constitute the universal trigger of oocyte activation and regulate key developmental processes, including cortical granule exocytosis, completion of meiosis, pronuclear formation, and the onset of embryonic gene expression [1, 2]. Failure to initiate these Ca^{2+} oscillations results in oocyte activation deficiency (OAD), a significant cause of fertilization failure in assisted reproductive technologies (ART) [3, 4].

A large body of evidence has established that the sperm-specific phospholipase C zeta (PLCζ) is the physiological trigger of these Ca^{2+} oscillations during mammalian fertilization [5–8]. Following sperm-egg fusion, PLCζ is delivered into the egg cytoplasm, where it hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP_2) to generate inositol

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1,4,5-trisphosphate (IP_3). Binding of IP_3 to its receptors on the endoplasmic reticulum induces Ca^{2+} release and initiates the characteristic oscillatory Ca^{2+} signaling pattern observed at fertilization [5, 7]. Importantly, microinjection of PLCζ cRNA or recombinant protein into mammalian oocytes is sufficient to induce fertilization-like Ca^{2+} oscillations and oocyte activation, demonstrating its central role in this process [5, 9].

Structurally, PLCζ belongs to the phospholipase C family but exhibits distinct features that underlie its unique function in fertilization. The protein comprises an N-terminal EF-hand domain, a catalytic core formed by X and Y domains connected by an XY linker and a C-terminal C2 domain [10]. Unlike somatic PLC isoforms, PLCζ lacks a pleckstrin homology domain and is highly sensitive to Ca^{2+}, enabling activity at the low resting Ca^{2+} concentrations present in the oocyte cytoplasm [11, 12]. The catalytic X and Y domains form the enzymatic core responsible for PIP_2 hydrolysis, while the EF-hand and C2 domains contribute to Ca^{2+} sensitivity and membrane targeting [11–15].

Mutations in the *PLCZ1* gene are increasingly recognized as a cause of male infertility associated with fertilization failure or OAD. Numerous pathogenic variants have been identified in infertile patients, many of which impair PLCζ expression, localization, or catalytic activity [16–22]. Functional analyses of these variants have provided important insights into the molecular basis of PLCζ-mediated oocyte activation and support the use of PLCζ as a diagnostic biomarker in cases of unexplained fertilization failure [23, 24].

Recently, a new PLCζ variant (c.1154G > A; p.Arg385Gln, R385Q) was identified in infertile patients presenting with recurrent fertilization failure, in whom sperm exhibited markedly reduced PLCζ levels and altered localization [25]. Structural predictions suggested that the R385Q substitution may disrupt stabilizing intramolecular interactions within the catalytic domain, potentially affecting protein folding and stability [25]. Notably, while this variant has previously been reported in the heterozygous state [26], these findings revealed its occurrence in the homozygous state in a distinct population, indicating broader clinical relevance.

However, while these findings established the clinical phenotype associated with the R385Q variant, they did not determine whether the mutation itself compromises the intrinsic biochemical function of PLCζ or whether fertilization failure results primarily from reduced protein abundance in sperm.

Although our previous study demonstrated markedly reduced PLCζ protein levels and abnormal localization in sperm from affected patients [25], the molecular mechanism underlying the pathogenicity of the R385Q variant remained unresolved. Specifically, it was unclear whether the mutation directly impairs the catalytic activity of PLCζ, disrupts its ability to induce Ca^{2+} oscillations, or whether the mutant protein retains normal catalytic function despite being present at insufficient levels to support physiological oocyte activation. Distinguishing between these possibilities is essential for understanding the expanding spectrum of pathogenic mechanisms underlying PLCζ-associated male infertility.

The present study was therefore designed as a mechanistic follow-up to our previous clinical investigation. We investigated the intrinsic structural and functional properties of the PLCζ R385Q variant using computational modelling, recombinant protein enzymatic assays, and evaluation of its Ca^{2+}-oscillation-inducing activity in mouse oocytes. By directly assessing the catalytic competence of the mutant protein, we sought to determine whether infertility associated with the R385Q variant results from impaired enzymatic function or from protein insufficiency despite preserved catalytic activity. Our findings demonstrate that the R385Q variant retains both catalytic activity and Ca^{2+}-oscillation-inducing capacity, supporting a pathogenic mechanism based on reduced functional PLCζ dosage rather than catalytic dysfunction.

Materials and methods

Modelling of full-length human PLCζ and in silico analysis of the R385Q mutation

The three-dimensional structure of full-length human PLCζ was not available in the Protein Data Bank (PDB). Therefore, homology modelling was performed to generate a structural model of the full-length protein. The amino acid sequence of human PLCζ was submitted to the SWISS-MODEL server [27], and the closest available template was identified as chicken PLCζ in complex with calcium and phosphorylated threonine (PDB ID: 9BCZ), which was subsequently used for model generation.

To assess the structural and thermodynamic impact of the R385Q substitution, protein stability changes ($\Delta\Delta G$) were predicted using three complementary structure-based computational tools: mCSM, SDM, and CUPSAT. mCSM (mutation Cutoff Scanning Matrix) employs a graph-based approach to model the effects of non-synonymous mutations by analyzing atomic distance patterns surrounding the mutated residue [28]. SDM (Site-Directed Mutator) estimates stability changes based on environment-specific substitution frequencies derived from known protein structures and utilizes PDB coordinate data [29]. CUPSAT (Cologne University Protein Stability Analysis Tool) predicts the impact of point mutations on protein stability by combining amino acid-atom potentials with torsion angle distributions, taking into account solvent accessibility and

secondary structure context [30]. For mCSM and SDM, negative $\Delta\Delta G$ values indicate a predicted destabilizing effect, whereas CUPSAT predictions were interpreted according to the tool-specific stability convention.

A structural model of the R385Q mutant PLCζ was generated based on the wild-type model to enable comparative structural analysis. UCSF ChimeraX [31] was used for visualization and examination of local structural changes and intramolecular interactions in both wild-type and mutant proteins.

To further characterize non-covalent interactions, both wild-type and mutant PLCζ models were analyzed using the Arpeggio web server [32]. Arpeggio calculates multiple classes of interatomic interactions (~ 15 types), including hydrogen bonds, hydrophobic contacts, and van der Waals interactions. PDB files of the wild-type and R385Q mutant structures were submitted, and atom typing was performed using OpenBabel via SMARTS queries. Interactions were identified within a 5 Å distance cutoff, and structural interaction fingerprints (SIFts) were generated for each residue pair. These analyses enabled quantitative and comparative assessment of interaction networks affected by the R385Q mutation.

Plasmid construction, recombinant protein expression, and cRNA synthesis

The PLCζ^{R385Q} mutant was generated by site-directed mutagenesis (GenScript Biotech, Piscataway, NJ, USA) and subcloned into the pCR3-LUC expression vector [33]. The mutant construct was amplified by polymerase chain reaction (PCR) using Phusion High-Fidelity DNA polymerase (Thermo Fisher Scientific) with primers incorporating a 5′ BamHI site and a 3′ NotI site. The PCR product was subsequently cloned into a modified pCR3-LUC vector. The primers used were as follows: forward 5′-ACCCGGATCCATGGAAATGAGATGGTTTTTGTC-3′ and reverse 5′-CCAAGCGGCCGCACATCTGACGTACCAAACATAAAC-3′. The PLCζ^{R385Q} construct was further subcloned into the pETMM41 vector to allow recombinant protein expression in *Escherichia coli* [34].

Recombinant MBP-tagged PLCζ (wild-type and R385Q) was expressed in *Escherichia coli* BL21CodonPlus(DE3)-RILP cells transformed with the pETMM41-PLCζ constructs, as previously described [34]. Briefly, cultures were grown at 37 °C to an OD_{600} of ~ 0.6, and protein expression was induced with 0.1 mM IPTG at 16 °C for 18 h. Cells were harvested by centrifugation and lysed by sonication, and soluble PLCζ proteins were purified by amylose affinity chromatography. Eluted proteins were dialyzed in phosphate-buffered saline (PBS) and concentrated using 30 kDa molecular weight cut-off centrifugal concentrators (Sartorius; Stonehouse, Gloucestershire, UK).

For cRNA synthesis, all plasmids were linearized prior to in vitro transcription. cRNA was then synthesized as previously described [33] using the mMESSAGE mMACHINE T7 transcription kit (Invitrogen, Waltham, MA, USA), followed by poly(A) tailing with the Poly(A) Tailing Kit (Invitrogen), according to the manufacturer's instructions.

Phospholipase C activity assay

Recombinant PLCζ activity was assessed using a commercial colorimetric phospholipase C assay kit (ab273343, Abcam, Cambridge, UK) according to the manufacturer's instructions and as previously described [35] with some modifications. Equal amounts of purified wild-type and the mutant PLCζ protein were incubated with the provided chromogenic PLC substrate, and enzymatic activity was quantified by measuring absorbance at 405 nm using a microplate reader (Synergy LX Multi-Mode reader, BioTek, USA). The increase in absorbance at 405 nm, corresponding to substrate hydrolysis, was measured kinetically using a microplate reader, and enzyme activity was calculated from the linear portion of the reaction curve and expressed as relative activity normalized to wild type PLCζ assayed in parallel. All reactions were performed in triplicate, and background signal from control reactions lacking enzyme was subtracted. Relative PLC activity was expressed as normalized absorbance values compared to wild-type PLCζ.

Microinjection of PLCζ cRNA into mouse oocytes and Ca^{2+} imaging

Metaphase II (MII) oocytes were collected from superovulated CD-1 female mice as previously described [33, 36]. All animal experiments complied with ARRIVE guidelines and were carried out in accordance with the U.K. Animals (Scientific Procedures) Act 1986, EU Directive 2010/63/EU, and the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 8023, revised 1978). Oocytes were microinjected with PLCζ cRNA as described previously [33, 37], using KCl-HEPES injection buffer (100 mM KCl, 20 mM HEPES, pH 7.4). The cRNA was mixed 1:1 with 1 mM Oregon Green BAPTA dextran (OGBD) prior to injection to enable Ca^{2+} imaging. Following injection, oocytes were maintained in HKSOM medium supplemented with 1 mM D-luciferin under mineral oil during imaging.

Fluorescence and luminescence imaging were performed using an inverted microscope (Zeiss Axiovert 100) equipped with a Retiga-LUMO CCD camera, as previously described [33]. Ca^{2+} oscillations were monitored via OGBD fluorescence for up to 5 h, followed by measurement of luciferase activity (relative light units, RLU) over 30 min using 10 min integration intervals.

Image analysis was performed using ImageJ (v1.54p, NIH) and SigmaPlot (v12, Systat Software). Background signals were subtracted using regions of interest of equal size to the oocytes. Statistical comparisons between PLCζ^{R385Q} mutant and wild-type PLCζ were performed using unpaired Student's *t*-tests, with significance determined using SigmaPlot.

Results

Structural modeling reveals destabilizing effects of the PLCζ R385Q mutation on the catalytic domain

A structural model of human PLCζ was generated using SWISS-MODEL, with chicken PLCζ (PDB ID: 9BCZ) serving as the structural template. The modeled structure revealed three well-defined domains: an N-terminal EF-hand domain, a central catalytic domain, and a C-terminal C2 domain. The catalytic domain adopts a TIM-barrel-like topology and is divided into two subdomains, the X-box and Y-box, which are connected by the XY linker region (Fig. 1). This catalytic region forms the functional core of the enzyme and is responsible for substrate binding and Ca^{2+}-dependent catalytic activity. The R385Q mutation is located within the Y-box of the catalytic domain. Structural stability predictions indicated a destabilizing effect of the R385Q substitution according to mCSM ($\Delta\Delta G = -0.75$ kcal/mol) and SDM ($\Delta\Delta G = -0.51$ kcal/mol), while CUPSAT yielded a $\Delta\Delta G$ value of 0.15 kcal/mol (Table 1). The predicted ΔΔG values were interpreted according to the respective tool-specific conventions.

Table 1 Predicted effects of the PLCζ R385Q mutation on protein stability using three structure-based prediction tools. Predicted $\Delta\Delta G$ values are expressed in kcal/mol. For mCSM and SDM, negative $\Delta\Delta G$ values indicate a predicted destabilizing effect, whereas CUPSAT predictions were interpreted according to the tool-specific stability convention. The overall stability classification reflects the combined predictions of the three structure-based tools

S.No	Mutations	Predicted $\Delta\Delta G$ (kcal/mol)			Outcome/overall stability
		mCSM	SDM	CUPSAT	
1	WT	0.00	0.00	0.00	-
2	R385Q	−0.75	−0.51	0.15	Destabilizing

Comparative structural analysis between the wild-type and mutant models revealed notable alterations in local intramolecular interactions. In the wild-type structure, Arg385, a positively charged residue, forms multiple stabilizing interactions with neighboring residues. Specifically, Arg385 participates in six hydrogen-bond interactions with Glu381, Ser388, and Glu422, contributing to the stabilization of the catalytic domain. Substitution with glutamine, which lacks a positively charged side chain, reduces the number of hydrogen-bond interactions to five and disrupts favorable electrostatic interactions with nearby negatively charged residues. In particular, the mutant residue is unable to form a side-chain interaction with Glu381, leading to altered local interaction patterns (Fig. 1).

To further characterize the structural consequences of this mutation, we quantified intramolecular non-covalent interactions, including van der Waals, hydrogen bonding, and electrostatic and hydrophobic interactions, using the Arpeggio server. Notably, the R385Q mutant lacked ionic interactions

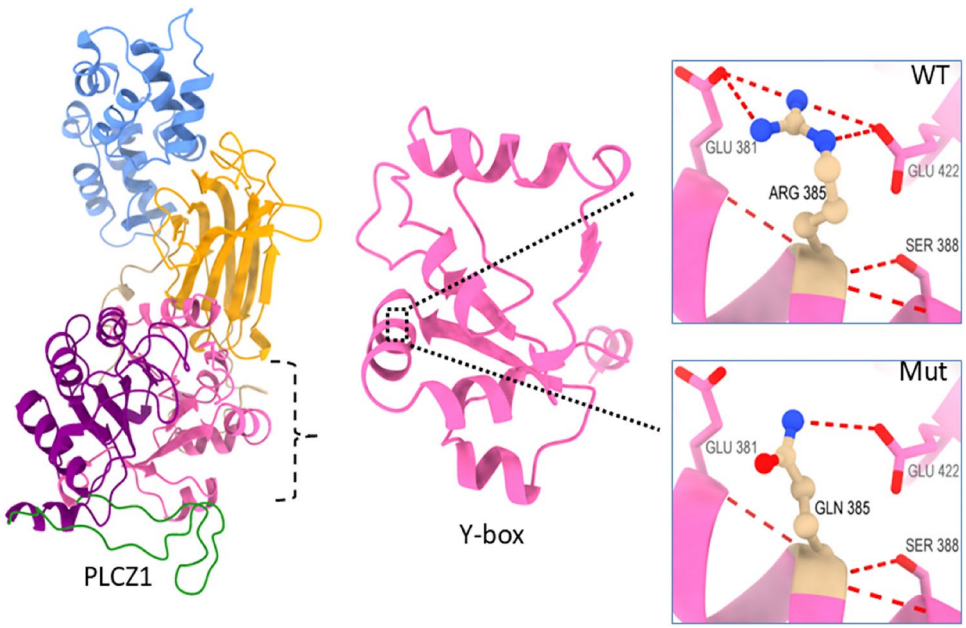

Fig. 1 Structural model of human PLCζ and predicted structural effects of the R385Q mutation. Cartoon representation of the modeled human PLCζ protein showing the domain organization. The Y-box region of the catalytic domain is highlighted in pink. The wild-type residue (R385) and the mutant residue (Q385) are shown in tan. Predicted hydrogen-bond interactions are indicated by red dashed lines

Table 2 Comparative analysis of intramolecular non-covalent interactions in PLCζ wild-type and R385Q mutant derived from the modeled protein structures

Non-covalent interactions	WT	R385Q
Van der Waals interactions	130	128
Hydrogen bonds	6	5
Ionic interactions	7	0
Polar contacts	9	7
Hydrophobic contacts	0	3

entirely, whereas seven ionic interactions were detected in the wild-type structure (Table 2).

Collectively, these analyses indicate that the R385Q mutation disrupts key stabilizing interactions within the catalytic domain of PLCζ, leading to reduced structural stability compared with the wild-type protein.

The PLCζ R385Q mutation does not affect in vitro PIP_2 hydrolysis activity

To evaluate whether the R385Q mutation affects the enzymatic activity of PLCζ, the coding sequences of $PLC\zeta^{WT}$ and $PLC\zeta^{R385Q}$ were subcloned into the pETMM41 expression vector to enable prokaryotic expression as 6xHis-MBP fusion recombinant proteins. We have previously shown that the MBP tag greatly enhances PLCζ expression, purification efficiency, and the long-term protein stability [14]. Following expression in *E. coli*, recombinant proteins were purified using amylose affinity chromatography, and the enzymatic activities of $PLC\zeta^{WT}$ and $PLC\zeta^{R385Q}$ were then directly compared using an in vitro colorimetric PIP_2 hydrolysis assay, as previously described [35]. As shown in Fig. 2, both proteins exhibited comparable enzymatic activities, with no significant difference between the wild-type and the mutant protein. These results indicate that the R385Q mutation does not impair the ability of PLCζ to hydrolyze PIP_2 in vitro.

The PLCζ R385Q mutation does not impair Ca^{2+} oscillation-inducing activity in mouse eggs

To determine the effect of the PLCζ R385Q mutation on Ca^{2+} oscillation activity, cRNA encoding luciferase-tagged human $PLC\zeta^{WT}$ and $PLC\zeta^{R385Q}$ mutant constructs was microinjected into mouse eggs, and Ca^{2+} oscillations were monitored as previously described [33, 37]. To confirm comparable intracellular protein expression levels, relative luminescence units (RLUs) were measured in each egg 5 h after injection. Following optimization experiments, microinjection of $PLC\zeta^{WT}$ induced high-frequency and sustained Ca^{2+} oscillations, with a mean number of 8.00 oscillations per egg (Table 3; Fig. 3A). Similarly, microinjection of the

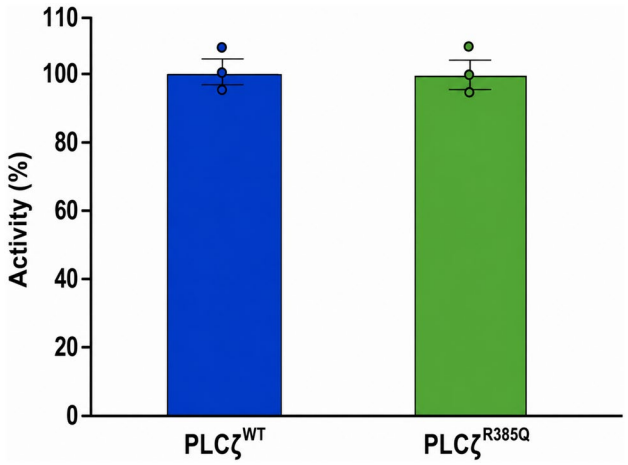

Fig. 2 Comparison of enzymatic activity between wild-type PLCζ and PLCζ R385Q mutant. Phospholipase C activity was quantified using 100 ng of recombinant PLCζ wild-type or R385Q mutant protein using a commercial colorimetric phospholipase C assay kit according to the manufacturer's instructions and as described in the "Materials and methods" section. Results are expressed as a percentage of wild-type activity (set to 100%). Individual data points are shown for each group, with bars representing the mean ± SEM ($n = 3$). No statistically significant difference was observed between PLCζWT and PLCζR385Q (Student's *t*-test, $p > 0.05$)

$PLC\zeta^{R385Q}$ mutant triggered a comparable pattern of high-frequency and persistent Ca^{2+} oscillations, with a mean of 7.65 oscillations per egg, comparable to that observed for the wild-type protein (Table 3; Fig. 3B). These findings indicate that the R385Q mutation does not impair the Ca^{2+} oscillation-inducing activity of PLCζ in mouse eggs.

Discussion

The present study is a mechanistic extension of the recently published clinical report describing the homozygous $PLC\zeta^{R385Q}$ variant [25]. In that study, sperm from affected patients were shown to exhibit markedly reduced PLCζ protein levels together with abnormal localization, establishing a clinical association between the R385Q variant and recurrent fertilization failure. However, whether this variant caused infertility through intrinsic catalytic dysfunction or through reduced protein abundance remained unresolved. The objective of the present work was to determine whether the R385Q substitution intrinsically alters the catalytic competence of PLCζ. By combining structural modelling, recombinant protein enzymatic assays and mouse oocyte Ca^{2+} oscillation analysis, our findings demonstrate that the mutant protein remains catalytically competent despite its clinical association with infertility.

Our study provides new mechanistic insight into the functional consequences of the $PLC\zeta^{R385Q}$ mutant, revealing a

Table 3 Comparison of the Ca^{2+} oscillation-inducing activity of luciferase-tagged $PLC\zeta^{R385Q}$ mutant and wild-type PLCζ ($PLC\zeta^{WT}$)

Luciferase-tagged PLCζ construct	RLU (luciferase)	Number of Ca^{2+} oscillations (mean value)	Number of eggs	Std. Dev. (number of Ca^{2+} oscillations)
$PLC\zeta^{WT}$	91	8.00	27	2.55
$PLC\zeta^{R385Q}$	72	7.65	20	1.90

clear dissociation between predicted structural destabilization and preserved catalytic and physiological activity. While in silico modelling consistently indicates that substitution of Arg385 perturbs local intramolecular interactions within the catalytic Y-domain, our experimental data demonstrate that this mutation does not impair either PIP_2 hydrolysis in vitro or Ca^{2+} oscillation-inducing activity in mouse oocytes. This apparent paradox challenges the conventional interpretation of PLCζ mutants, as primarily catalytic loss-of-function mutations and instead supports a model in which subtle structural perturbations can lead to functional insufficiency in vivo without compromising intrinsic enzymatic competence.

Structurally, Arg385 is positioned within the Y-box of the catalytic TIM-barrel-like domain, which forms the enzymatic core responsible for phosphoinositide hydrolysis. Our modelling suggests that Arg385 contributes to the stabilization of this domain through an extensive network of hydrogen bonds and electrostatic interactions, particularly with negatively charged residues, such as Glu381 and Glu422. Substitution with glutamine abolishes the positive charge at this position, leading to disruption of ionic contacts and a measurable reduction in intramolecular interaction density. Notably, Arpeggio analysis revealed a complete loss of ionic interactions in the mutant structure, despite only a modest predicted destabilization ($\Delta\Delta G \sim -0.5$ to -0.7 kcal/mol). These findings are consistent with previous structural studies of phospholipase C enzymes, which have highlighted the importance of electrostatic networks in maintaining catalytic domain integrity without necessarily determining catalytic activity [38–40].

Importantly, this structural perturbation does not translate into functional impairment under experimental conditions. Both the in vitro recombinant protein activity assays and cRNA microinjection experiments demonstrate that $PLC\zeta^{R385Q}$ retains full catalytic activity and is capable of triggering fertilization-like Ca^{2+} oscillations indistinguishable from those induced by the wild-type PLCζ protein. This observation aligns with earlier studies showing that the

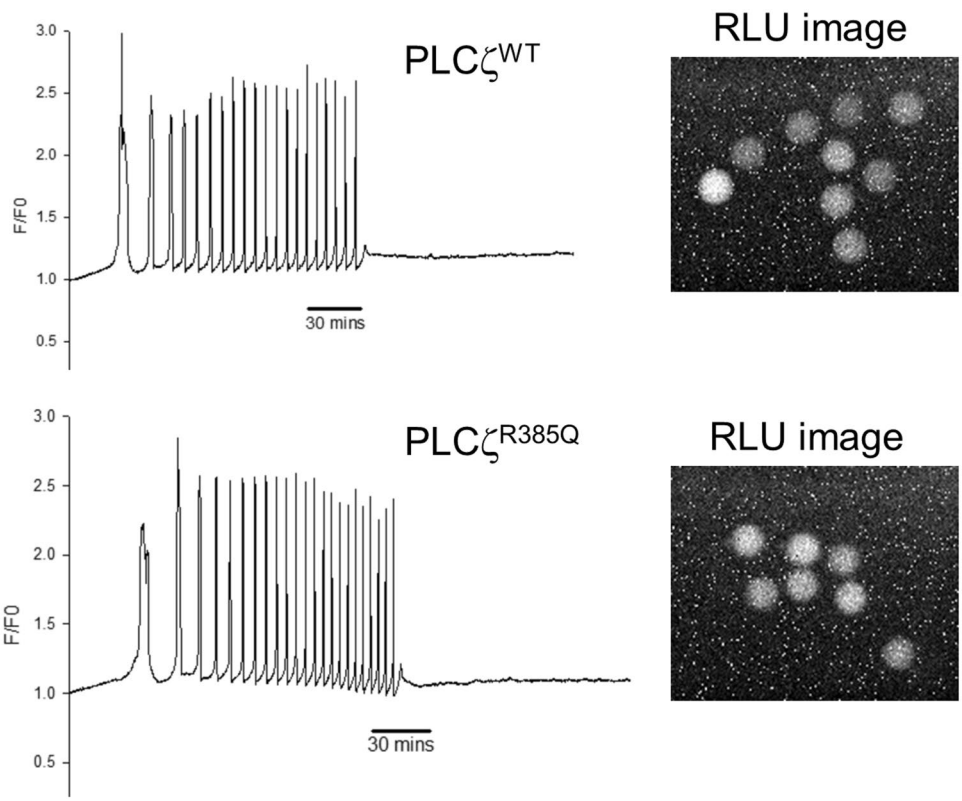

Fig. 3 Ca^{2+} oscillations and luciferase expression in mouse eggs following injection of luciferase-tagged wild-type PLCζ and the PLCζ R385Q mutant. Fluorescence and luminescence measurements from mouse eggs following microinjection of PLCζ mRNA. Each trace shows Ca^{2+} oscillations recorded during the first 5 h after mRNA injection. Fluorescence intensities were normalized to the starting values and are presented as fluorescence (F) divided by the initial fluorescence (F_0). Representative traces are shown for (**A**) wild-type PLCζ ($PLC\zeta^{WT}$) and (**B**) PLCζ carrying the R385Q mutation ($PLC\zeta^{R385Q}$). Images on the right show the luminescence signal from luciferase expression (10-min integration) from the group of eggs from which the single OGBD fluorescence trace displayed on the left was obtained

catalytic machinery of PLCζ is highly robust and that many residues outside the active site can tolerate substitution without abolishing enzymatic function [13, 41]. The preserved oscillatory activity further indicates that key processes such as substrate recognition, catalytic turnover, and IP_3 receptor-mediated Ca^{2+} release remain intact.

The coexistence of structural destabilization and preserved function is consistent with a protein insufficiency model of pathogenicity. In this framework, the R385Q mutation does not impair enzyme activity directly but instead reduces the effective amount of functional PLCζ protein delivered to the oocyte. Clinical and experimental evidence increasingly supports the notion that PLCζ levels in sperm are a critical determinant of fertilization competence. Reduced PLCζ expression and abnormal localization have been consistently associated with oocyte activation deficiency (OAD) and fertilization failure following ICSI [16, 17, 19, 25]. Even modest reductions in protein stability during spermatogenesis could therefore lead to insufficient accumulation of PLCζ, ultimately falling below the threshold required to initiate Ca^{2+} oscillations at fertilization.

An important aspect of the present experimental design is that it specifically isolates the intrinsic functional consequences of the R385Q substitution. By expressing equivalent amounts of recombinant wild-type and mutant PLCζ, we minimized biological variables inherent to patient sperm, including differences in sperm quality, PLCζ abundance, sperm heterogeneity, and other patient-specific factors. Consequently, the preserved catalytic activity observed in our experiments can be attributed directly to the mutant protein itself rather than differences in protein expression. When considered together with our previous demonstration of markedly reduced PLCζ protein levels and abnormal localization in patient sperm [25], the present findings support protein insufficiency, rather than intrinsic catalytic dysfunction, as the most plausible mechanism underlying infertility associated with the R385Q variant. However, this dosage/insufficiency model remains an inference from the combined clinical and experimental evidence and is not directly demonstrated by the present study.

This concept is further reinforced by the well-established dose-dependent nature of PLCζ-induced Ca^{2+} oscillations. Microinjection studies have shown that both the frequency and persistence of Ca^{2+} oscillations depend on the intracellular concentration of PLCζ, with a defined threshold required to reproduce physiological oscillatory patterns [5, 11, 12]. In our experimental system, supraphysiological expression levels achieved via cRNA injection likely compensate for any destabilizing effects of the mutation, thereby masking subtle defects. In contrast, in the physiological context of sperm delivery, where PLCζ is present in limited quantities, even small reductions in protein stability or accumulation may have significant functional consequences. In our experiments, mouse oocytes were used, which are considerably more sensitive to PLCζ than human oocytes [42]. Consequently, under physiological conditions in the human oocyte, PLCζ activity may be closer to the threshold required for successful oocyte activation.

Thus, it is possible that the $PLC\zeta^{R385Q}$ variant represents a threshold-dependent insufficiency variant, rather than a classical catalytic loss-of-function allele.

An additional layer of complexity arises from the potential impact of the R385Q mutation on protein dynamics and regulatory interactions. The catalytic XY region of PLCζ, including the XY-linker, is increasingly recognized as a hub for regulatory modulation, influencing Ca^{2+} sensitivity, membrane association, and protein–protein interactions [13, 38]. Subtle conformational changes induced by the R385Q substitution may alter the accessibility or flexibility of this region, thereby affecting interactions with regulatory partners. This suggests that PLCζ activity is not solely determined by its catalytic domain but is dynamically regulated within a broader molecular network that fine-tunes Ca^{2+} oscillatory patterns during fertilization.

Within this context, it is plausible that the R385Q mutation influences PLCζ function indirectly by modulating its interaction landscape. For example, destabilization of the catalytic domain may increase transient exposure of hydrophobic or flexible regions, enhancing susceptibility to chaperone binding or proteasomal degradation. Alternatively, altered electrostatic properties could affect membrane association or PIP_2 accessibility, particularly under physiological conditions where substrate availability is tightly regulated. These possibilities highlight the importance of considering protein context and regulatory environment, rather than relying solely on in vitro enzymatic assays, when assessing the functional impact of PLCζ variants.

Our findings therefore expand the pathogenic spectrum of PLCζ mutations to include structurally destabilizing, catalytically competent variants. This distinction has important implications for both diagnosis and treatment of male infertility. Traditional approaches have focused on identifying mutations that abolish enzymatic activity; however, our data suggest that variants affecting protein stability or expression may be equally clinically relevant. Accordingly, comprehensive assessment of PLCζ function should include not only enzymatic assays but also evaluation of protein abundance, localization, and stability in sperm. This integrated approach has been advocated in recent diagnostic frameworks for OAD and may improve the identification of patients who could benefit from assisted oocyte activation or PLCζ-based therapeutic strategies [42–44].

The present study has several limitations that should be acknowledged. First, the functional experiments were performed using recombinant protein and cRNA microinjection,

which assess the intrinsic activity of the mutant protein under controlled experimental conditions rather than reproducing physiological sperm-mediated PLCζ delivery. Consequently, supraphysiological PLCζ expression following cRNA injection may compensate for subtle effects of the R385Q mutation that become biologically relevant when only physiological quantities of PLCζ are delivered by sperm. Furthermore, mouse oocytes are known to be more sensitive to PLCζ than human oocytes [42], which may further reduce the ability to detect mild functional defects.

We noted that all mouse oocytes that showed Ca^{2+} oscillations formed 2nd polar bodies during the recordings; hence, they underwent early signs of activation. Although assessment of 2nd polar body formation or pronuclear formation following recombinant protein injection would provide an additional downstream measure of oocyte activation, the number or frequency of Ca^{2+} oscillations remains the most quantitative and sensitive functional readout of PLCζ activity because they represent the direct physiological consequence of PLCζ function, whereas activation events are all-or-none and less sensitive as an assay of PLCζ activity.

Finally, recombinant PLCζ protein titration studies across a range of physiological concentrations, together with assessment of patient sperm-induced Ca^{2+} oscillations in mouse oocytes, would further test the threshold model proposed in the present study. Such experiments represent important future directions for determining the quantitative PLCζ requirements necessary for successful human oocyte activation.

In conclusion, we demonstrate that the PLCζ ^{R385Q} mutation induces local structural destabilization while preserving intrinsic enzymatic activity and Ca^{2+}-oscillation-inducing capacity under the experimental conditions tested. Taken together with our previous demonstration of markedly reduced PLCζ protein abundance and abnormal localization in patient sperm, these findings support a reduced functional PLCζ dosage/protein insufficiency model as the most plausible mechanism underlying the fertilization failure associated with the R385Q variant, rather than intrinsic catalytic inactivation. However, this mechanism remains an inference from the combined clinical and experimental evidence and is not directly established by the present study. Further studies examining PLCζ^{R385Q} across physiological protein concentrations and in the context of sperm-mediated delivery will be required to directly test this model. More broadly, these findings demonstrate that preservation of intrinsic catalytic competence does not exclude pathogenicity and emphasize the importance of integrating protein abundance, localization, and functional analysis when evaluating PLCζ variants associated with human infertility.

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Author contribution Conceptualization: K.S., M.N.; Methodology: Z.I., K.S., M.N.; Formal Analysis: A.S., Z.H., Z.I., K.S., M.N.; Validation: Z.I., K.S., M.N.; Investigation: A.S., Z.H., Z.I., K.S., M.N.; Resources: M.N.; Data Curation: A.S., Z.H., Z.I., K.S., M.N.; Supervision: K.S., M.N.; Writing—Original Draft Preparation: M.N.; Writing—Review & Editing: A.S., Z.H., Z.I., K.S., M.N.; All authors have read and agreed to the published version of the manuscript. ***please note for Alaaeldin Saleh1#, Zizhen Huang2# –- #These authors contributed equally to this work.

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Data availability All data generated or analyzed during this study are included within the paper.

Declarations

Ethics approval and consent to participate Procedures on mice were reviewed and approved by the Animal Welfare and Review Body at Cardiff University and carried out in the Biological Services Unit in the School of Biosciences at Cardiff University. They were in compliance with, and under the authority, of a UK Home Office Licence held by K.S. at Cardiff University.Procedures on mice were reviewed and approved by the Animal Welfare and Review Body at Cardiff University and carried out in the Biological Services Unit in the School of Biosciences at Cardiff University. They were in compliance with, and under the authority, of a UK Home Office Licence held by K.S. at Cardiff University.

Consent for publication Not applicable.

Competing interests The authors declare no competing interests.

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