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## Male infertility-linked point mutation disrupts the $\text{Ca}^{2+}$ oscillation-inducing and $\text{PIP}_2$ hydrolysis activity of sperm $\text{PLC}\zeta$

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**Short title:** Mutation-specific inactivation of sperm  $\text{PLC}\zeta$  as a cause of male infertility

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**Abbreviations:** phospholipase C-zeta,  $\text{PLC}\zeta$ ; phosphatidylinositol 4,5-bisphosphate,  $\text{PIP}_2$ ; inositol 1,4,5-trisphosphate,  $\text{IP}_3$ ; human chorionic gonadotrophin, hCG;

### SYNOPSIS

A male infertility-linked human  $\text{PLC}\zeta$  mutation introduced into mouse  $\text{PLC}\zeta$  completely abolishes both *in vitro*  $\text{PIP}_2$  hydrolysis activity and the ability to trigger *in vivo*  $\text{Ca}^{2+}$  oscillations in mouse eggs. Wild-type  $\text{PLC}\zeta$  initiated a normal pattern of  $\text{Ca}^{2+}$  oscillations in eggs in the presence of 10-fold higher mutant  $\text{PLC}\zeta$  suggesting that infertility is not mediated by a dominant-negative mechanism.

**Keywords:** phospholipase C,  $\text{PLC}\zeta$ , calcium oscillations, infertility, egg activation, fertilization

### INTRODUCTION

During mammalian fertilization, the fertilizing spermatozoon initiates a series of biochemical and morphological events in the egg known as ‘egg activation’. In all species examined, the earliest egg activation event is an increase in the cytosolic free  $\text{Ca}^{2+}$  concentration [1, 2]. In mammals, this  $\text{Ca}^{2+}$  signal is delivered in the form of long-lasting  $\text{Ca}^{2+}$  oscillations that commence after gamete fusion and persist beyond the completion of meiosis [3]. This  $\text{Ca}^{2+}$  signaling phenomenon is necessary and sufficient for the completion of all the events of egg activation [4, 5]. Much controversy existed over how the sperm induces this fundamental developmental event, but growing evidence supports the notion that during mammalian fertilization, egg activation is triggered by a sperm-specific phospholipase C isoform, PLC-zeta ( $\text{PLC}\zeta$ ) [6-9].  $\text{PLC}\zeta$  introduced into the ooplasm is able to hydrolyse phosphatidylinositol 4,5-bisphosphate ( $\text{PIP}_2$ ) to yield inositol 1,4,5-trisphosphate ( $\text{IP}_3$ ), thus triggering  $\text{Ca}^{2+}$  oscillations within the egg via the  $\text{IP}_3$  receptor-mediated  $\text{Ca}^{2+}$  signaling pathway [10].  $\text{PLC}\zeta$  has the smallest molecular mass and most elementary domain organization among mammalian PLC isoforms [10, 11].  $\text{PLC}\zeta$  consists of a tandem pair of EF hand domains at the N-terminus, followed by catalytic X and Y domains and a C-terminal C2 domain [6, 10].

Further support for the importance of  $\text{PLC}\zeta$  in fertilization has arisen from two recent clinical reports demonstrating either a reduced protein level or mutated forms of  $\text{PLC}\zeta$  in cases of human male infertility [12, 13]. One infertility case identified following failed *in vitro* fertilization treatment was associated with a point mutation in the  $\text{PLC}\zeta$  catalytic Y domain [13] where substitution of histidine by a proline residue (H398P) correlated with the absence of  $\text{Ca}^{2+}$  oscillation-inducing activity of human  $\text{PLC}\zeta$  [13]. His-398 is conserved in  $\text{PLC}\zeta$  from various mammalian species as well as in  $\text{PLC}\delta 1$ , the most closely related isoform to  $\text{PLC}\zeta$  [14]. In this study, we have introduced the

infertility-linked human PLC $\zeta$  H398P mutation into the equivalent His-435 residue of mouse PLC $\zeta$  to give PLC $\zeta^{\text{H435P}}$  (Fig.1A) and analysed the effect of this mutation on *in vivo* Ca $^{2+}$  oscillation-inducing and *in vitro* PIP $_2$  hydrolysis activity. For comparative analysis, we also substituted His-435 for a neutral, non helix-destabilizing residue, alanine, to produce PLC $\zeta^{\text{H435A}}$ . An additional charge-reversal mutant, PLC $\zeta^{\text{D210R}}$ , which produces an inactive enzyme [6] served as a negative-control. We also examined the effect on PIP $_2$  hydrolysis activity of substituting in PLC $\delta$ 1 the equivalent conserved His-542 to yield PLC $\delta$ 1 $^{\text{H542P}}$ . Further, we investigated potential dominant-negative inhibitory effects of PLC $\zeta^{\text{H435P}}$  on the Ca $^{2+}$  oscillation-inducing activity of wild-type mouse PLC $\zeta$  (PLC $\zeta^{\text{WT}}$ ) and mouse sperm.

## MATERIALS AND METHODS

### Plasmid construction and cRNA synthesis

Mouse PLC $\zeta$ -luciferase in pCR3 [15] was subjected to site-directed mutagenesis (QuikChange II, Stratagene) to generate the PLC $\zeta^{\text{H435P}}$ , PLC $\zeta^{\text{H435A}}$  and PLC $\zeta^{\text{D210R}}$  mutants. PLC $\zeta^{\text{WT}}$  and mutants were amplified by PCR from the corresponding pCR3 plasmid using *Phusion* polymerase (Finnzymes) to incorporate a 5'-EcoRI site and a 3'-SalI site and cloned into pGEX-6P1 (GE Healthcare). The primers used for amplification of wild-type and mutant PLC $\zeta$  were: 5'-ACATGAATTCATGGAAAGCCAACTTCATGA-3' (forward) and 5'-TAACGTCGACTCACTCTCTGAAGTACCAAAC-3' (reverse). Similarly, rat PLC $\delta$ 1 in pGEX-5X2 [15] was subjected to site-directed mutagenesis to generate PLC $\delta$ 1 $^{\text{H542P}}$ . Following linearization of wild-type and mutant PLC $\zeta$ s, cRNA was synthesized using mMessage Machine T7 kit (Ambion) and poly(A) tailing kit (Ambion), as per the manufacturer's instructions.

### Preparation and handling of gametes

Experiments were carried out with mouse eggs in Hepes-buffered saline (H-KSOM) as described previously [15]. Female mice were superovulated by injection of human chorionic gonadotrophin (hCG; Intervet). Eggs were collected 13.5-14.5 h later and maintained in 100  $\mu$ l H-KSOM under mineral oil at 37 °C. Egg microinjection was carried out 14.5-15.5 h after hCG administration [16]. *In vitro* fertilization (IVF) experiments were carried out as described previously [6].

### Microinjection and measurement of intracellular Ca $^{2+}$ and luciferase expression

Mouse eggs were microinjected with cRNA encoding the particular PLC(s) mixed with an equal volume of 1 mM Oregon Green BAPTA-dextran (Molecular Probes) in injection buffer (120 mM KCl, 20 mM Hepes, pH 7.4). All injections were 3-5 % of the egg volume. Eggs were then maintained in H-KSOM containing 100  $\mu$ M luciferin and imaged on a Nikon TE2000 microscope equipped with a cooled intensified-CCD camera (Photek Ltd, UK). Ca $^{2+}$  was monitored for 4 h after injection by measuring Oregon Green BAPTA-dextran fluorescence with low-level excitation light from a halogen lamp. After Ca $^{2+}$  measurements, eggs were monitored for luminescence by integrating light emission (in the absence of fluorescence excitation) for 20 min using the same intensified-CCD camera. Fluorescence signals were 10-100 times that for luminescence. Ca $^{2+}$  measurements were considered valid only if the egg was also luminescent. To estimate expressed protein levels from luminescence values, luminescent eggs collected in a tube containing PBS with 1 mM MgATP + 100  $\mu$ M luciferin were lysed with 0.5% Triton X-100 and the steady-state light emitted (counts per second; cps) was calibrated with recombinant firefly luciferase (Sigma) to give the mean protein expression of each PLC $\zeta$ -luciferase construct [15]. Lower levels of protein at the start of experiments were estimated by linear extrapolation of the luminescence calibration curve.

### Protein expression and purification

For GST-fusion protein expression, *E.coli* (Rosetta (DE3), Novagen) transformed with the appropriate pGEX plasmid, was cultured at 37 °C until  $A_{600}$  of 0.6 then protein expression induced for 18 h, 16°C with 0.1 mM isopropyl  $\beta$ -d-thiogalactopyranoside (Promega). Cells were centrifuged at 6000  $\times$  g for 10 min, resuspended in phosphate-buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na $_2$ HPO $_4$ ·7H $_2$ O, 1.4 mM KH $_2$ PO $_4$ , pH 7.4) containing 2 mM dithiothreitol and protease inhibitor mixture (Roche) then sonicated 4  $\times$  15 s on ice. After centrifugation at 15,000  $\times$  g, 15 min,

4 °C, soluble GST-fusion proteins were purified by affinity chromatography using glutathione-Sepharose™ 4B following standard procedures (GE Healthcare). Eluted proteins were dialyzed overnight (Pierce; SnakeSkin 10,000 MWCO) at 4 °C in 4 litres of PBS, and concentrated with centrifugal concentrators (Sartorius, 10,000 MWCO).

### PLC activity assay, PAGE and Western blotting

PIP<sub>2</sub> hydrolytic activity of PLC $\zeta$ <sup>WT</sup>, PLC $\delta$ 1<sup>WT</sup> and mutant constructs was assayed as described previously [15]. The assay mixture final volume was 50  $\mu$ l containing 100 mM NaCl, 0.4% sodium cholate (w/v), 2 mM CaCl<sub>2</sub>, 4 mM EGTA, 20  $\mu$ g of bovine serum albumin, 5 mM 2-mercaptoethanol, and 20 mM Tris-HCl buffer, pH 6.8. The PIP<sub>2</sub> concentration in the reaction mixture was 220  $\mu$ M, containing 0.05  $\mu$ Ci of [<sup>3</sup>H]PIP<sub>2</sub>. Assay conditions were optimized for linearity, requiring a 10 min, 25 °C incubation with 20 pmol protein. Recombinant proteins were separated by SDS-PAGE and immunoblot analysis performed as described previously [15]. Proteins were probed with anti-GST polyclonal antibody (1:10,000 dilution).

## RESULTS

To assess the Ca<sup>2+</sup> oscillation-inducing activity of the PLC $\zeta$ <sup>WT</sup>, PLC $\zeta$ <sup>H435P</sup>, PLC $\zeta$ <sup>H435A</sup> and PLC $\zeta$ <sup>D210R</sup> mutants and to verify these constructs were faithfully expressed as functional proteins in cRNA-microinjected mouse eggs, we prepared PLC $\zeta$ -luciferase fusion constructs to enable quantitation of relative protein expression, as previously described [15]. PLC $\zeta$ <sup>WT</sup>-injected eggs displayed prominent Ca<sup>2+</sup> oscillations, with the first Ca<sup>2+</sup> spike occurring at a luminescence of 0.35 cps which we estimate is equivalent to a protein expression level of 35 fg/egg, similar to that previously reported [6]. In contrast, microinjecting cRNA encoding either PLC $\zeta$ <sup>H435P</sup> or PLC $\zeta$ <sup>D210R</sup> failed to cause any Ca<sup>2+</sup> oscillations (Fig.1B), even after relatively high levels of protein expression (Table 1). However, microinjecting PLC $\zeta$ <sup>H435A</sup> mutant caused Ca<sup>2+</sup> oscillations in all injected eggs (Fig.1B), exhibiting a broadly similar potency to PLC $\zeta$ <sup>WT</sup>, with the first Ca<sup>2+</sup> spike detected after protein expression of 0.73 cps (Table 1). These results show that the mouse PLC $\zeta$  H435P mutation is inactive in mouse eggs, whereas the H435A mutation retains Ca<sup>2+</sup> oscillation-inducing activity.

PLC $\zeta$ <sup>H435P</sup> and PLC $\zeta$ <sup>D210R</sup> were subcloned into pGEX-6P1 and purified as GST-fusion proteins. We also prepared the corresponding substitution, H452P in PLC $\delta$ 1 and expressed PLC $\delta$ 1<sup>WT</sup> and PLC $\delta$ 1<sup>H452P</sup> to enable comparative isoform analysis of *in vitro* PIP<sub>2</sub> hydrolysis activity. Optimal protein yield for PLC $\zeta$ <sup>WT</sup>, PLC $\delta$ 1<sup>WT</sup> and their mutants required maintaining 37 °C cultures until OD<sub>600</sub> of 0.5 followed by induction of expression with 0.1 mM IPTG for 18 hours at 16 °C. Figure 2 shows glutathione affinity-purified recombinant proteins analyzed by SDS-PAGE (left panels) and anti-GST immunoblot analysis (right panels). The predicted molecular mass for GST-PLC $\zeta$  and its corresponding mutants was 100 kDa (Fig.2A), while for GST-PLC $\delta$ 1<sup>WT</sup> and the H452P mutant it was 111 kDa (Fig.2B). The corresponding proteins with appropriate molecular mass were observed as the highest band in both the gel and immunoblot. The band at 26 kDa is consistent with cleaved GST, which together with several other intermediate molecular mass bands detected by GST antibody, are the likely result of some degradation occurring through the protein expression and purification.

The enzyme activity of GST-fusion proteins for PLC $\zeta$ <sup>WT</sup>, PLC $\delta$ 1<sup>WT</sup> and corresponding mutants were determined using the [<sup>3</sup>H]PIP<sub>2</sub> hydrolysis assay at 1  $\mu$ M and 1 mM Ca<sup>2+</sup>, as previously described [15]. PLC $\zeta$ <sup>H435P</sup> and PLC $\zeta$ <sup>D210R</sup> were enzymatically inactive at both low and high Ca<sup>2+</sup>, in contrast with PLC $\zeta$ <sup>WT</sup> which showed a specific activity of 449 $\pm$ 36 nmol/min/mg at 1  $\mu$ M Ca<sup>2+</sup> and 382 $\pm$ 47 nmol/min/mg at 1 mM Ca<sup>2+</sup> (Table 2). PLC $\delta$ 1<sup>WT</sup> displayed high activity at 1 mM Ca<sup>2+</sup> with PIP<sub>2</sub> hydrolysis of 1385 $\pm$ 42 nmol/min/mg *versus* 395 $\pm$ 27 nmol/min/mg at 1  $\mu$ M Ca<sup>2+</sup>. In contrast, PLC $\delta$ 1<sup>H452P</sup> was completely inactive at 1  $\mu$ M Ca<sup>2+</sup> and retained only 16% of the PLC $\delta$ 1<sup>WT</sup> activity at 1 mM Ca<sup>2+</sup> (Table 2). These results show that the above His-Pro mutation completely inactivates the PIP<sub>2</sub> hydrolysis activity of both PLC $\zeta$  and PLC $\delta$ 1, whereas the His-Ala substitution in PLC $\zeta$  retains *in vivo* function.

To investigate whether PLC $\zeta$ <sup>H435P</sup> can alter the Ca<sup>2+</sup> oscillation-inducing activity of PLC $\zeta$ <sup>WT</sup>, we co-microinjected into mouse eggs an equal mixture of cRNA encoding luciferase-tagged

PLC $\zeta^{\text{H435P}}$  and PLC $\zeta^{\text{WT}}$  (Fig.3, top panel). The co-injected cRNA produced Ca $^{2+}$  oscillations with comparable properties to those observed with PLC $\zeta^{\text{WT}}$  alone (Fig.1B; Table 1), showing a time to 1 $^{\text{st}}$  peak of ~30 minutes (Table 3). This suggests that expression of PLC $\zeta^{\text{H435P}}$  at similar levels to PLC $\zeta^{\text{WT}}$  does not interfere with Ca $^{2+}$  oscillations. To determine whether an excess of PLC $\zeta^{\text{H435P}}$  was required to block PLC $\zeta^{\text{WT}}$ -induced Ca $^{2+}$  oscillations, we performed sequential cRNA microinjections; PLC $\zeta^{\text{H435P}}$  was introduced first, followed after a period of 1 or 2 hours by a second injection of PLC $\zeta^{\text{WT}}$  (Fig.3, middle and lower panels). This protocol employing prior expression of PLC $\zeta^{\text{H435P}}$  for 1/2 hours followed by PLC $\zeta^{\text{WT}}$  expression did not interfere with the induction of normal Ca $^{2+}$  oscillations ~30 mins after PLC $\zeta^{\text{WT}}$  cRNA injection (Table 3). Table 3 shows that in the double cRNA injection experiments, the cumulative luminescence for both PLC $\zeta^{\text{H435P}}$  and PLC $\zeta^{\text{WT}}$  at the time of 1 $^{\text{st}}$  spike (7.06 and 18.68 cps for 1 and 2 h, respectively) was much higher than for control PLC $\zeta^{\text{WT}}$  alone (0.35 cps; Table 1). This suggests that an excess of PLC $\zeta^{\text{H435P}}$  protein does not interfere with PLC $\zeta^{\text{WT}}$ -induced Ca $^{2+}$  oscillations. The estimated PLC $\zeta^{\text{H435P}}$  protein level of 400 fg in the egg after 2.5 hour (Fig.3; Table 3), when the 1 $^{\text{st}}$  spike is observed ~30 mins after injection of PLC $\zeta^{\text{WT}}$ , is well above the ~35 fg estimated to be required for Ca $^{2+}$  oscillations with PLC $\zeta^{\text{WT}}$  alone (Fig.1; Table 1) [6, 9]. This data therefore suggests that expressing the PLC $\zeta^{\text{H435P}}$  protein even in 10-fold excess remains unable to block Ca $^{2+}$  oscillation-inducing activity of PLC $\zeta^{\text{WT}}$ . To examine whether normal sperm-induced Ca $^{2+}$  oscillations are affected by the presence of PLC $\zeta^{\text{H435P}}$ , IVF experiments with mouse sperm were performed. Even after high protein expression levels of PLC $\zeta^{\text{H435P}}$  were achieved in the mouse eggs (>40 cps) there was no discernable effect on sperm-induced Ca $^{2+}$  oscillations (Fig.4).

## DISCUSSION

Accumulating evidence suggests that sperm-specific PLC $\zeta$  is the likely agent that stimulates Ca $^{2+}$  oscillations and consequent egg activation after sperm-egg membrane fusion. Identification of PLC $\zeta$  in several different mammalian species indicates that PLC $\zeta$  should play a pivotal role at fertilization in all mammalian kingdoms. The importance of PLC $\zeta$  in mammalian fertilization has been supported by two recent clinical studies which linked PLC $\zeta$  with some cases of human male infertility [12, 13]. Intracytoplasmic sperm injection (ICSI) is a powerful *in vitro* fertilization technique that has been extensively employed to overcome many male infertility conditions such as severe oligospermia, asthenospermia and teratospermia [12]. A recent study identified a number of patients with repeatedly unsuccessful ICSI due to egg activation failure. Sperm from these patients were unable to initiate Ca $^{2+}$  oscillations and this deficiency was associated with reduced or absent PLC $\zeta$  expression in their sperm [12]. A second clinical study reported identification of a point mutation in the PLC $\zeta$  gene of a patient with failed ICSI [13]. In this mutant PLC $\zeta$ , His-398 in the catalytic Y domain was substituted to a proline residue. Injection of infertile sperm or the mutant PLC $\zeta$  from this patient failed to induce the typical pattern of Ca $^{2+}$  oscillations in unfertilized mouse eggs [13]. The His-398 residue is conserved not only in PLC $\zeta$  of all mammalian species, but also in PLC $\delta$ 1, the most closely related PLC isoform to PLC $\zeta$ . Interestingly, His-398 does not correlate with one of the five critical active site residues within the catalytic domain of PLC $\delta$ 1 (His $^{311}$ , Glu $^{341}$ , Asp $^{343}$ , His $^{356}$  and Glu $^{390}$ ) that were previously identified by structural studies [17] and which are conserved in PLC $\zeta$ .

In this study, we introduced the infertility-linked human PLC $\zeta$  H398P mutation into the equivalent His-435 residue of mouse PLC $\zeta$  (H435P; Fig.1A) and assessed the effects of PLC $\zeta^{\text{H435P}}$  upon *in vivo* Ca $^{2+}$  oscillation-inducing and *in vitro* PIP $_2$  hydrolysis activity. Our data indicates that the H435P mutation totally abolishes the ability of PLC $\zeta$  to trigger Ca $^{2+}$  oscillations in mouse eggs (Fig.1B, Table 1) and also fully abrogates enzyme activity in a PIP $_2$  hydrolysis assay (Table 2). To examine whether the His-435 mutation to a proline, which in protein structure prediction is known to have helix-destabilising ability, specifically causes sufficient perturbation of the catalytic Y domain to annul PLC $\zeta$  enzymatic activity, we employed an additional PLC $\zeta$  mutant in which His-435 was substituted by a neutral, non helix-destabilizing amino acid, alanine. Interestingly, in contrast to the inactive PLC $\zeta^{\text{H435P}}$ , robust Ca $^{2+}$  oscillations were generated by PLC $\zeta^{\text{H435A}}$  cRNA injection with a similar potency to PLC $\zeta^{\text{WT}}$  (Fig.1B, Table 1). This suggests that the H435P substitution may cause

inactivation of PLC $\zeta$  due to the introduction of major protein structural changes, consistent with that proposed previously [13]. We further examined the effect of the PLC $\zeta^{\text{H435P}}$  equivalent mutation upon the *in vitro* enzymatic properties of a closely related PLC isoform, PLC $\delta$ 1. We observed very similar results with the equivalent PLC $\delta$ 1<sup>H542P</sup> mutant, which retained only 16% of the PIP<sub>2</sub> hydrolysis activity of the PLC $\delta$ 1<sup>WT</sup> at high Ca<sup>2+</sup> levels, a condition where PLC $\delta$ 1<sup>WT</sup> is known to be fully enzymatically active [11, 15].

Interestingly, Heytens *et al.*, reported that the H398P mutation identified to correlate with male infertility appeared to be heterozygous, suggesting that in humans PLC $\zeta^{\text{H398P}}$  may exert a dominant-negative effect on PLC $\zeta^{\text{WT}}$  [13]. To investigate whether mouse PLC $\zeta^{\text{H435P}}$  acts in a dominant-negative fashion, we performed both cRNA co-microinjection and sequential injection experiments using PLC $\zeta^{\text{H435P}}$  and PLC $\zeta^{\text{WT}}$ . The aim of these experiments was to determine whether an equivalent or excess level of PLC $\zeta^{\text{H435P}}$  protein expressed in mouse eggs can block the Ca<sup>2+</sup> oscillation-inducing activity of PLC $\zeta^{\text{WT}}$ . However, when PLC $\zeta^{\text{H435P}}$  was expressed at up to 10-fold higher levels relative to PLC $\zeta^{\text{WT}}$ , this did not block the ability of PLC $\zeta^{\text{WT}}$  to trigger Ca<sup>2+</sup> oscillations, suggesting that in the mouse, PLC $\zeta^{\text{H435P}}$  does not display dominant-negative behaviour. This observation is intriguing as it leaves unexplained why the sperm of this heterozygous infertile male is unable to cause Ca<sup>2+</sup> oscillations after ICSI. Further analysis is required to confirm if there are any other defects, such as PLC $\zeta$  mislocalization in the sperm of this infertile patient. Transgenic, heterozygous animal models, carrying this mutant allele would also help to enable further investigation of the reason for failure of egg activation in this clinical case.

The present study extends the previous work of Heytens *et al.* by revealing that (i) a mouse PLC $\zeta$  H435P mutation, equivalent to the infertile human H398P, is functionally inactive both *in vivo* and *in vitro*, as is the equivalent PLC $\delta$ 1 mutation H542P, (ii) injection of luciferase-tagged mutant PLCs enables quantitative analysis of their *in vivo* expression in eggs and the demonstration that a dominant-negative inhibition mechanism by the mouse H435P mutant PLC $\zeta$  does not appear to operate. Further advances in understanding the precise role and importance of PLC $\zeta$  in mammalian fertilization may provide a major step in overcoming some cases of male infertility. It has been reported that in some cases of egg activation failure after ICSI, treatment with Ca<sup>2+</sup> ionophore has been successful in producing assisted egg activation [18]. However, it remains to be determined whether routine ionophore treatment to assist with activation carries the risk of abnormal embryo development, or whether it is the most effective means of overcoming activation failure [19]. The application of recombinant PLC $\zeta$  might represent a potential alternative, physiological therapeutic agent that can overcome certain cases of failed fertilization after ICSI.

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## FIGURE LEGENDS

### Figure 1.

#### $\text{Ca}^{2+}$ oscillation-inducing activity of PLC $\zeta$ -luciferase and mutants in mouse eggs.

A, Schematic representation of mouse PLC $\zeta$  domain structure identifying the location of the H435P mutation within the catalytic Y domain, as well as the D210R control mutation in the X domain. B, Panels on the left show representative fluorescence (a.u.; arbitrary units) and luminescence (cps; counts per second) recordings reporting the  $\text{Ca}^{2+}$  concentration changes (black traces;  $\text{Ca}^{2+}$ ) and luciferase expression (red traces; Lum), respectively, in a mouse egg following microinjection of the indicated PLC $\zeta$ -luciferase cRNA (encoding either PLC $\zeta^{\text{WT}}$ , PLC $\zeta^{\text{H435P}}$ , PLC $\zeta^{\text{H435A}}$  or PLC $\zeta^{\text{D210R}}$ ). Panels on the right show integrated image of luciferase luminescence from eggs microinjected with the corresponding PLC $\zeta$ -luciferase cRNA. The peak luminescence (Lum) recorded is shown in counts per second.

### Figure 2.

#### Expression and purification of GST-fusion proteins for PLC $\zeta$ , PLC $\delta$ 1 and mutants.

A, Affinity-purified GST-fusion proteins for PLC $\zeta^{\text{WT}}$ , PLC $\zeta^{\text{H435P}}$  and PLC $\zeta^{\text{D210R}}$  (2 $\mu\text{g}$ ) analyzed by 8% SDS-PAGE (left panel) or immunoblot analysis using anti-GST polyclonal antibody (right panel). B, Affinity-purified GST-fusion proteins for PLC $\delta 1^{\text{WT}}$  and PLC $\delta 1^{\text{H542P}}$  (2  $\mu\text{g}$ ) analyzed by 8% SDS-PAGE (left panel) or immunoblot analysis using anti-GST polyclonal antibody (right panel).

### Figure 3.

#### Co-expression of PLC $\zeta^{\text{H435P}}$ and PLC $\zeta^{\text{WT}}$ in mouse eggs.

Panels on the left show representative fluorescence and luminescence recordings reporting Ca $^{2+}$  concentration changes (black traces; Ca $^{2+}$ ) and luciferase expression (red traces; Lum), respectively, in a mouse egg. The egg was co-microinjected with equal amounts of PLC $\zeta$ -luciferase cRNA encoding PLC $\zeta^{\text{WT}}$  and PLC $\zeta^{\text{H435P}}$  (top panel), or was initially microinjected with cRNA for PLC $\zeta^{\text{H435P}}$  followed, after a period of 1 hour (middle panel) or 2 hours (bottom panel), by the microinjection of cRNA for PLC $\zeta^{\text{WT}}$ . Panels on the right show integrated image of luciferase luminescence from eggs microinjected with PLC $\zeta^{\text{H435P}}$  and PLC $\zeta^{\text{WT}}$  cRNA. The peak luminescence (Lum) recorded is shown in counts per second.

### Figure 4.

#### Effect of PLC $\zeta^{\text{H435P}}$ on sperm-induced Ca $^{2+}$ oscillations.

Mouse eggs were either untreated (IVF control) or injected with PLC $\zeta^{\text{H435P}}$  cRNA (IVF + PLC $\zeta^{\text{H435P}}$ ) 3 hours prior to the start of recording. PLC $\zeta^{\text{H435P}}$  expression produced luminescence of 41.7  $\pm$  1.8 cps (mean  $\pm$  SEM, n=11). Fluorescence recordings (arbitrary units) reporting Ca $^{2+}$  concentration changes were monitored after addition of capacitated mouse sperm. Following IVF both control and PLC $\zeta^{\text{H435P}}$  cRNA-injected eggs exhibited robust Ca $^{2+}$  oscillations and formed pronuclei. The number of Ca $^{2+}$  spikes in control eggs was 33.4  $\pm$  3.8 (mean  $\pm$  sem, n=7), and the number of Ca $^{2+}$  spikes in PLC $\zeta^{\text{H435P}}$  cRNA-injected eggs was 32.9  $\pm$  4.5 (mean, sem, n=11).

## TABLE LEGENDS

### Table 1.

#### Properties of PLC $\zeta$ -luciferase and mutants expressed in mouse eggs.

Ca $^{2+}$  oscillation-inducing activity (Ca $^{2+}$  spike number in 2 hours; Time to 1 $^{\text{st}}$  Ca $^{2+}$  spike) and luciferase luminescence levels (Peak luminescence; Luminescence at 1 $^{\text{st}}$  spike) are summarized for mouse eggs microinjected with each PLC-luciferase construct (see Fig.1B). Each egg was microinjected with a pipette cRNA concentration of 1.6 g/l.

### Table 2.

#### [ $^3\text{H}$ ]PIP $_2$ hydrolysis activity of purified GST-PLC fusion proteins (n=3).

Specific enzyme activity values obtained for the various GST-PLC fusion proteins showing that PLC $\zeta^{\text{H435P}}$  and PLC $\zeta^{\text{D210R}}$  were both unable to hydrolyze [ $^3\text{H}$ ]PIP $_2$  at either 1  $\mu\text{M}$  or 1 mM Ca $^{2+}$ . Similarly, PLC $\delta 1^{\text{H542P}}$  was enzymatically inactive at 1  $\mu\text{M}$  Ca $^{2+}$  but retained  $\sim$ 16% of the activity of PLC $\delta 1^{\text{WT}}$  at 1 mM Ca $^{2+}$ .

### Table 3.

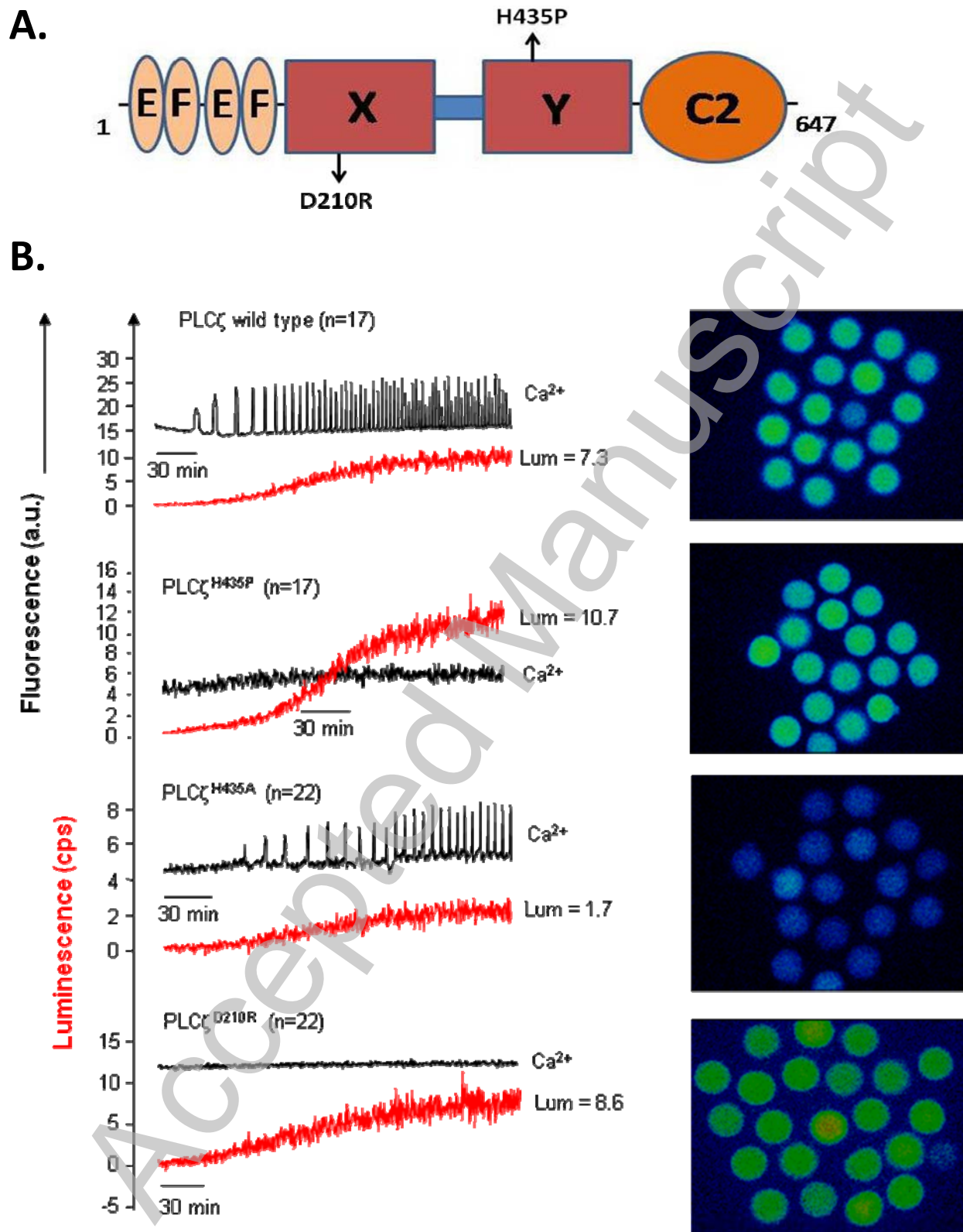
#### Properties of PLC $\zeta^{\text{WT}}$ and PLC $\zeta^{\text{H435P}}$ co-expressed in mouse eggs.

Ca $^{2+}$  oscillation-inducing activity (Ca $^{2+}$  spike number in 2 hours; Time to 1 $^{\text{st}}$  Ca $^{2+}$  spike) and luciferase luminescence levels (Peak luminescence; Luminescence at 1 $^{\text{st}}$  spike) are summarized for mouse eggs microinjected with each PLC-luciferase construct (see Fig.3). Each egg was microinjected with a pipette cRNA concentration of 1.6 g/l. Asterisk denotes that this value is the time taken after the second injection of cRNA for PLC $\zeta^{\text{WT}}$ .



FIGURES

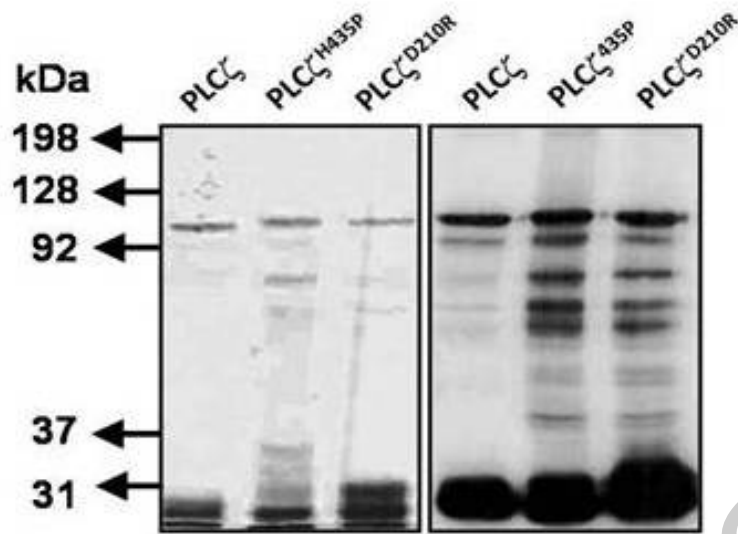
Fig. 1



THIS IS NOT THE VERSION OF RECORD - see doi:10.1042/BJ20101772

Fig. 2

A.



B.

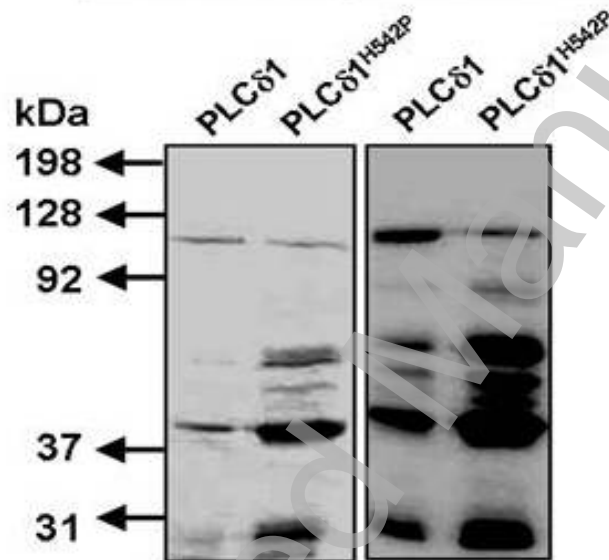


Fig. 3

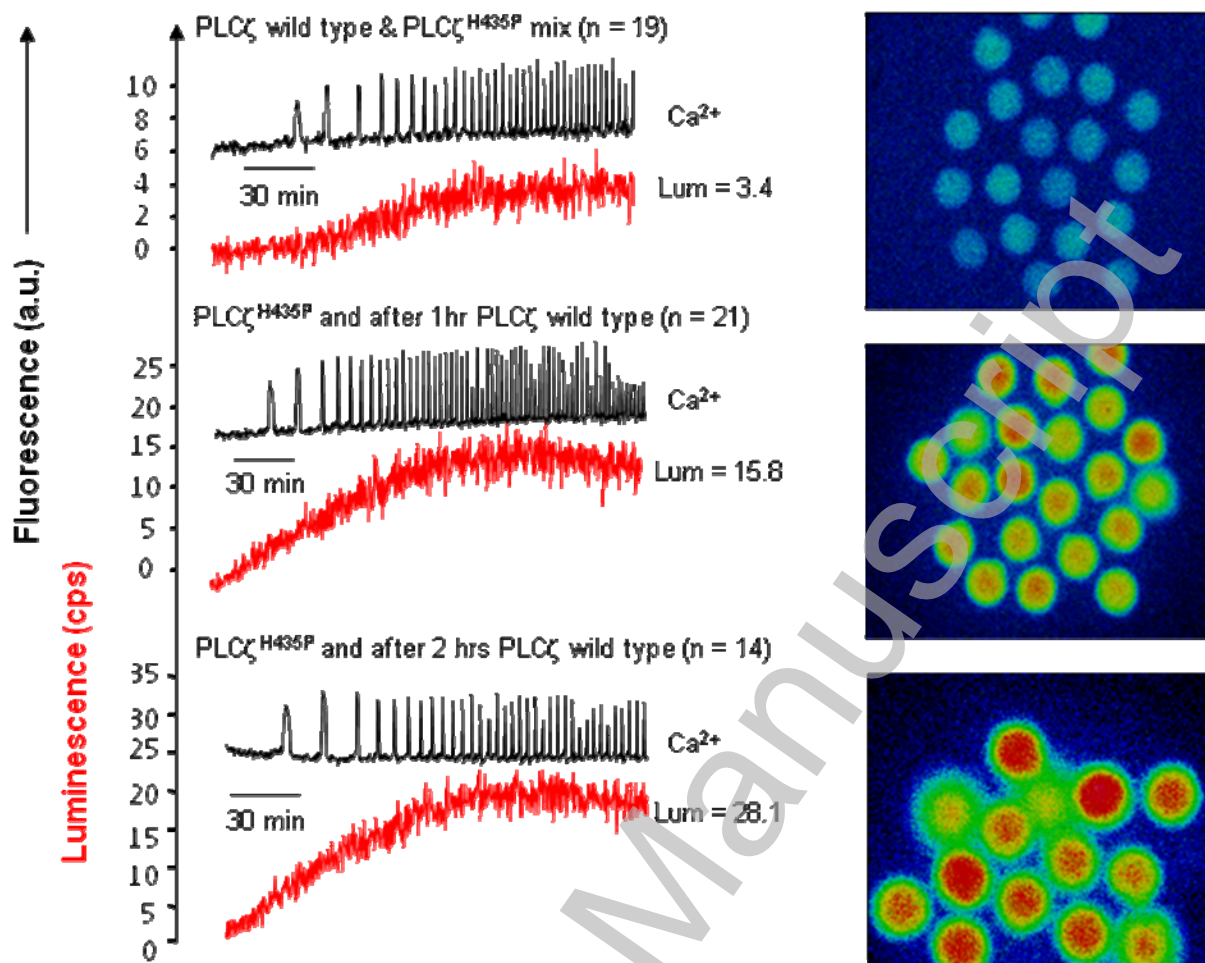
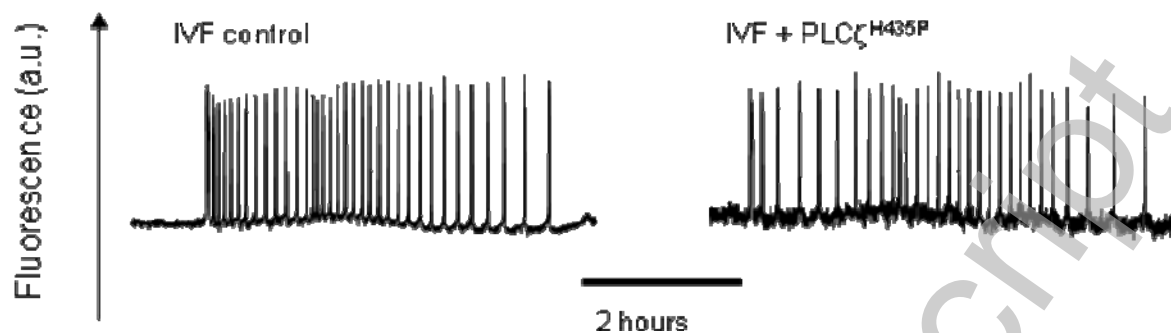


Fig. 4



## TABLES

Table 1

PLC $\zeta$ -luciferase	Ca <sup>2+</sup> spikes in the first 2 hours	Peak luminescence (cps)	Time of 1 <sup>st</sup> spike (min)	Luminescence at 1 <sup>st</sup> spike (cps)	Number of eggs
PLC $\zeta$ <sup>WT</sup>	19±0.14	7.33±0.38	~30	0.35 ±0.037	17
PLC $\zeta$ <sup>H435P</sup>	0	10.7±0.62	-	-	17
PLC $\zeta$ <sup>H435A</sup>	10±0.65	1.74±0.14	~55	0.73 ±0.038	22
PLC $\zeta$ <sup>D210R</sup>	0	8.6±0.55	-	-	22

Table 2

GST-fusion protein	[ <sup>3</sup> H]PIP <sub>2</sub> hydrolysis activity (nmol/min/mg) at 1 $\mu$ M Ca <sup>2+</sup>	[ <sup>3</sup> H]PIP <sub>2</sub> hydrolysis activity (nmol/min/mg) at 1mM Ca <sup>2+</sup>
PLC $\zeta$ <sup>WT</sup>	449±36	382±47
PLC $\zeta$ <sup>H435P</sup>	0	0
PLC $\zeta$ <sup>D210R</sup>	0	0
PLC $\delta$ 1 <sup>WT</sup>	395±27	1385±42
PLC $\delta$ 1 <sup>H542P</sup>	0	222±36

Table 3

PLC $\zeta$ -luciferase injected	Ca <sup>2+</sup> spikes in the first 2 hrs	Peak luminescence (cps)	Time of 1 <sup>st</sup> spike (min)	Luminescence at 1 <sup>st</sup> spike (cps)	Number of eggs
PLC $\zeta$ <sup>H435P</sup> & PLC $\zeta$ <sup>WT</sup> mix	21±1.7	3.4±0.14	~30	0.68±0.045	19
PLC $\zeta$ <sup>H435P</sup> +1hr, then PLC $\zeta$ <sup>WT</sup>	17.2±1.0	15.8± 0.47	~30*	7.06 ±0.72	21
PLC $\zeta$ <sup>H435P</sup> +2hr, then PLC $\zeta$ <sup>WT</sup>	15.5±1.1	28.1±1.6	~30*	18.68 ±1.2	13