

# SH2 domain-mediated activation of an SRC family kinase is not required to initiate $\text{Ca}^{2+}$ release at fertilization in mouse eggs

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## Abstract

SRC family kinases (SFKs) function in initiating  $\text{Ca}^{2+}$  release at fertilization in several species in the vertebrate evolutionary line, but whether they play a similar role in mammalian fertilization has been uncertain. We investigated this question by first determining which SFK proteins are expressed in mouse eggs, and then measuring  $\text{Ca}^{2+}$  release at fertilization in the presence of dominant negative inhibitors. FYN and YES proteins were found in mouse eggs, but other SFKs were not detected; based on this, we injected mouse eggs with a mixture of FYN and YES Src homology 2 (SH2) domains. These SH2 domains were effective inhibitors of  $\text{Ca}^{2+}$  release at fertilization in starfish eggs, but did not inhibit  $\text{Ca}^{2+}$  release at fertilization in mouse eggs. Thus the mechanism by which sperm initiate  $\text{Ca}^{2+}$  release in mouse eggs does not depend on SH2 domain-mediated activation of an SFK. We also tested the small molecule SFK inhibitor SU6656, and found that it became compartmentalized in the egg cytoplasm, thus suggesting caution in the use of this inhibitor. Our findings indicate that although the initiation of  $\text{Ca}^{2+}$  release at fertilization of mammalian eggs occurs by a pathway that has many similarities to that in evolutionarily earlier animal groups, the requirement for SH2 domain-mediated activation of an SFK is not conserved.

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## Introduction

At fertilization,  $\text{Ca}^{2+}$  elevation in the egg cytosol establishes a block to polyspermy, and also causes the resumption of the cell cycle, leading to embryonic development (Ducibella *et al.* 2002, Runft *et al.* 2002, Hyslop *et al.* 2004, Markoulaki *et al.* 2004). In mammals (Miyazaki *et al.* 1992, 1993), as well as other species in the vertebrate evolutionary line (see Runft *et al.* 2002), the  $\text{Ca}^{2+}$  is released from the egg's endoplasmic reticulum by inositol trisphosphate ( $\text{IP}_3$ ), but the mechanisms leading to  $\text{IP}_3$  production are incompletely understood. SRC family kinases (SFKs) function in this signalling pathway in echinoderms (Jaffe *et al.* 2001, O'Neill *et al.* 2004), ascidians (Runft & Jaffe 2000), fish (Wu & Kinsey 2002, Kinsey *et al.* 2003), and frogs (Sato *et al.* 1996, 2000), but whether the same is true in mammals is uncertain.

For echinoderms, the evidence for the function of SFKs in fertilization signalling includes immune complex kinase assays (Kinsey 1996, Abassi *et al.* 2000, O'Neill *et al.* 2004), fertilization-dependent association of SFKs with phospholipase C ( $\text{PLC}\gamma$ ) (Giusti *et al.* 1999a, Kinsey & Shen 2000, Runft *et al.* 2004), inhibition of  $\text{Ca}^{2+}$  release

by dominant negative SFK constructs (Giusti *et al.* 1999b, 2003, Abassi *et al.* 2000, Kinsey & Shen 2000, O'Neill *et al.* 2004), and stimulation of  $\text{PLC}\gamma$ -dependent  $\text{Ca}^{2+}$  release by injection of SRC protein or SFK activating antibodies (Giusti *et al.* 2000, 2003). These findings support a model, for echinoderm species, in which sperm–egg interaction activates an SFK, which directly or indirectly activates  $\text{PLC}\gamma$ , leading to  $\text{IP}_3$  production and  $\text{Ca}^{2+}$  release; some but not all aspects of this pathway have been established for ascidians, fish, and frogs.

For mammals, tyrosine phosphorylation of several proteins has been found to increase within 1–3 h after fertilization of rat eggs; however, it is not known if this is due to activation of SFKs or other tyrosine kinases (Ben-Yosef *et al.* 1998). Tyrosine kinase inhibitors have been applied during mouse fertilization, and this delayed the initiation of  $\text{Ca}^{2+}$  release, but the inhibitors used were not specific for SFKs (Dupont *et al.* 1996). Also, it is not known whether the delay was due to direct effects on the initiation of  $\text{Ca}^{2+}$  release, or to indirect effects on the sperm prior to its binding and fusion with the egg. In a recent study, such effects on sperm physiology were avoided by injecting sperm or sperm extracts into eggs,

rather than applying sperm to the outside of eggs as occurs normally (Kurokawa *et al.* 2004). PP2 (4-amino-5(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine), which inhibits SFKs, did not inhibit  $\text{Ca}^{2+}$  release in response to injection of sperm or sperm extracts into the mouse egg cytoplasm (Kurokawa *et al.* 2004). Although this experiment argued against a role for SFKs in initiation of  $\text{Ca}^{2+}$  release, critical signalling events related to sperm–egg membrane binding and fusion could have been bypassed, because the experiment tested the effect of PP2 on the response to sperm injection vs the response to the multistep process of fertilization.

In the work to be described here, we determined which SFKs are present in mouse eggs, and then microinjected the eggs with membrane impermeant inhibitors of these SFKs. By use of microinjection, we were able to isolate effects on the egg from effects on the sperm, thus allowing us to examine if inhibition of SFKs in the egg inhibited  $\text{Ca}^{2+}$  release during normal fertilization.

Mammalian genomes encode eight different SFKs: SRC, FYN, YES, FGR, LYN, LCK, HCK, and BLK (Bolen *et al.* 1991), and several different SFKs can function in activation of PLC $\gamma$ , leading to  $\text{Ca}^{2+}$  release (Quek *et al.* 2000, Rhee 2001, Ozdener *et al.* 2002). We used immunoblotting to test which of the SFK proteins are present in mouse eggs, and then used the Src homology 2 (SH2) domains of these SFKs as dominant negative inhibitors, to investigate the function of SFKs in mediating  $\text{Ca}^{2+}$  release at fertilization. Although many signalling proteins contain SH2 domains, sequence differences within these ~100 amino acid regions confer specificity on their binding to phosphotyrosine-containing sequences in other proteins (Songyang & Cantley 1995). Because excess SFK SH2 domains can interfere with the interaction of SFKs with their binding partners, intracellular injection of SH2 domains has been an effective and specific way of inhibiting SFK activation in fibroblasts (Roche *et al.* 1995) as well as eggs (Giusti *et al.* 1999b, Abassi *et al.* 2000, Kinsey & Shen 2000, Runft & Jaffe 2000, Sette *et al.* 2002, Kinsey *et al.* 2003, O'Neill *et al.* 2004). Single cell kinase assays of zebrafish eggs injected with FYN SH2 domains have shown that the SH2 domains prevent the increase in SFK activity at fertilization; they prevent the stimulation of SFK activity, rather than directly inhibiting the kinase activity, since addition of the SH2 domains to an *in vitro* kinase assay had very little effect on kinase activity (Kinsey *et al.* 2003).

## Materials and Methods

### Preparation of gametes

Fully grown, immature mouse oocytes were collected from NSA (CF-1) mice (Harlan, Indianapolis, IN, USA) after killing the mice using  $\text{CO}_2$ . Oocytes were collected in minimal essential medium (MEM) supplemented with 250  $\mu\text{M}$  dbcAMP (Mehlmann & Kline 1994). Oocytes were washed into MEM without dbcAMP to induce oocyte maturation; such *in vitro* matured eggs exhibit a normal

pattern of  $\text{Ca}^{2+}$  oscillations following fertilization (Mehlmann *et al.* 2001). Sperm were collected from the caudal epididymides and vas deferens in Whittingham's medium (Hogan *et al.* 1994) containing 3% (w/v) bovine serum albumin (BSA) (Mehlmann & Kline 1994), and were allowed to capacitate for  $\geq 90$  min before use. For fertilization, the zona was removed from eggs using acid Tyrode's solution (Mehlmann *et al.* 1996). Eggs in Whittingham's medium containing no BSA were adhered to a glass coverslip coated with Cell-Tak (Collaborative Research, Bedford, MA, USA) that formed the bottom of a chamber fitting into a heated microscope stage perfused with 5%  $\text{CO}_2$  (Medical Systems Corp., Greenvale, NY, USA). Sperm were applied at a final concentration of  $\sim 2\text{--}5 \times 10^5$  sperm/ml. Eggs of starfish (*Asterina miniata*) were obtained and fertilized as previously described (Carroll *et al.* 1997).

### Immunoblotting

Immunoblotting was performed as described previously (Mehlmann *et al.* 1998). Aliquots of mouse eggs were frozen in liquid  $\text{N}_2$  after removal of excess medium, and stored at  $-80^\circ\text{C}$  until use. Cell lysates used as positive controls were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Primary antibodies are listed in Table 1.

### SH2 domain experiments

Plasmids encoding the glutathione *S*-transferase fusion proteins GST-FYN SH2 (chicken, amino acids 148–251) and GST-YES SH2 (mouse, amino acids 142–265) were obtained from K Vuori (Cancer Center, The Burnham Institute, La Jolla, CA, USA) and D Flynn (MBR Cancer Center, West Virginia University, Morgantown, WV, USA) respectively. The amino acid sequence of the SH2 domain of chicken FYN is 99% identical to that of mouse FYN (SH2

**Table 1** Primary antibodies used for immunoblotting.

SFK	Antibody (source) <sup>a</sup>	Epitope <sup>b</sup>
SRC	mAb327 (EMD OP07)	SH3 domain of avian SRC
FYN	SC-16	N-terminus
YES	SK6 (A L Burkhardt)	Amino acids 5–71 of human YES
YES	BD 610375	Amino acids 10–193 of human YES
FGR	SC-17	C-terminus
LYN	SC-7274	N-terminus
LCK	SC-13	C-terminus
HCK	SC-1428	N-terminus
BLK	SC-329	N-terminus

<sup>a</sup> Antibodies designated SC were obtained from Santa Cruz Biotechnology. EMD refers to EMD Calbiochem (La Jolla, CA, USA). BD refers to BD Transduction Laboratories (San Diego, CA, USA). The SK6 antibody from A L Burkhardt (Millennium Pharmaceuticals, Inc., Cambridge, MA, USA) is a rabbit polyclonal (Li *et al.* 1992).

<sup>b</sup> Antibodies from Santa Cruz Biotechnology were generated against peptide antigens based on human SFK sequences, which are identical or very similar to the corresponding mouse sequences. Amino acid numbers for the SC antigens are not available. All of the antibodies listed here have been shown to cross-react with mouse proteins.

domain region as defined in Xu *et al.* 1997). SH2 domain proteins were produced in bacteria, and purified, dialyzed, and concentrated as described previously (Carroll *et al.* 1997). Zona-intact eggs were quantitatively microinjected using pipettes backfilled with mercury (Mehlmann & Kline 1994, Jaffe & Terasaki 2004). Concentrations of injected substances were calculated based on cytoplasmic volumes of 200 pl and 3000 pl for mouse and starfish eggs respectively. Ca<sup>2+</sup> changes were measured using calcium green 10 kDa dextran (Molecular Probes, Eugene, OR, USA), at a final concentration of 16 μM (mouse) or 10 μM (starfish) (Carroll *et al.* 1997, Mehlmann *et al.* 1998). For injection of FYN and YES SH2 domains into mouse eggs, the stock solution contained 10 mg/ml FYN SH2, 10 mg/ml YES SH2, and 330 μM calcium green dextran in phosphate-buffered saline. Injections of the SH2 domain fusion proteins into the mouse eggs were made 2–7 h before insemination. The delay between injection and insemination was a consequence of performing the injections using zona-intact eggs (the eggs were held in place by use of a suction pipette that attaches to the zona). We then removed the zonae with low pH medium, and afterwards allowed the eggs to equilibrate in medium of normal pH for at least 2 h prior to insemination (fertilization is inefficient if the zona is present). Although degradation of the SH2 domains during the 2–7 h period between injection and insemination is a possible concern, we consider this to be unlikely, since SH2 domains of PLCγ are stable in the egg cytoplasm for at least 6.5 h (Mehlmann *et al.* 1998).

### Experiments with SU6656

SU6656 (2-oxo-3-(4,5,6,7-tetrahydro-1H-indol-2-ylmethylene)-2,3-dihydro-1H-indole-5-sulfonic acid dimethylamide; Calbiochem) was prepared as a 10 mM stock in dimethyl sulfoxide, and then diluted to 10 μM in MEM. Oocytes that had been incubated with SU6656 were observed using a BioRad MRC600 confocal microscope, with a 40×/1.2 numerical aperture water immersion objective (Carl Zeiss, Inc., Thornwood, NY, USA). Fluorescence was excited using the 488 nm line of a krypton/argon laser, and detected using a 515 nm long pass filter.

## Results

### FYN and YES proteins are present in mouse eggs, but other SFKs are not detectable

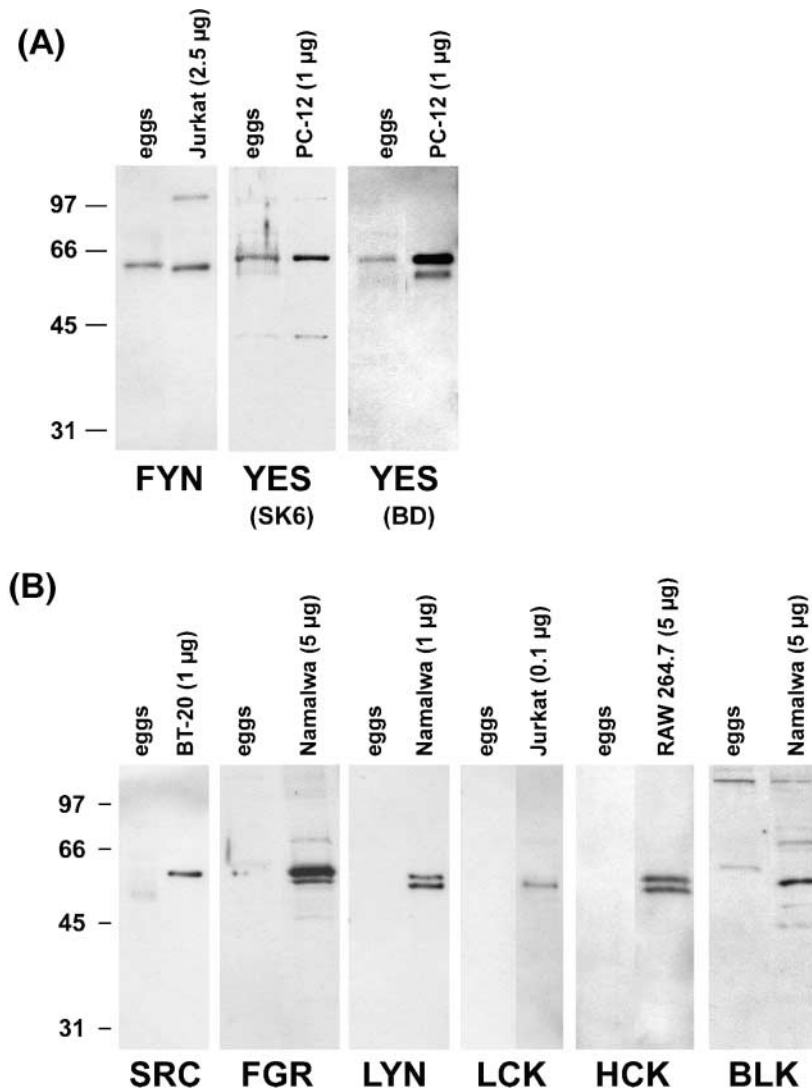
A previous study has shown that FYN protein is present in mouse eggs (Sette *et al.* 2002), but it has been uncertain whether other SFK proteins are present as well. To determine which of the eight SFKs are expressed in mouse eggs, immunoblotting was used to compare eggs with cell lines known to express each of these proteins. In addition to FYN, YES protein was detected in mouse eggs (Fig. 1A), using two different antibodies. FYN and YES proteins have also been found in rat eggs (Talmor-Cohen *et al.* 2004a).

Other SFK proteins were not detectable in mouse eggs, under conditions where these SFKs were detectable in comparable amounts of control cell protein (Fig. 1B). The absence of a detectable amount of SRC protein in mouse eggs was surprising, since SRC is present in rat eggs (Talmor-Cohen *et al.* 2004a). However, based on three independent experiments in which we observed a strong SRC signal from 1 μg of a control cell lysate, and no SRC signal from 2.5–5 μg of mouse egg lysate, we concluded that mouse eggs contain little if any SRC protein. LCK was also of particular interest since it was previously reported to be present based on immunofluorescence and an immune complex kinase assay (Mori *et al.* 1991), and RT-PCR (Mori *et al.* 1992). However, these studies did not test for the presence of LCK protein by immunoblotting, so a possible explanation for the difference in our findings could be antibody non-specificity in the previous work. We concluded from our immunoblots that the major SFK proteins present in mouse eggs are FYN and YES.

### Ca<sup>2+</sup> release at fertilization of mouse eggs does not require SH2 domain-mediated SFK activation

To investigate if FYN and YES function in the signalling pathway leading to Ca<sup>2+</sup> release at fertilization in mouse eggs, we injected eggs with a mixture of FYN and YES SH2 domain proteins (Fig. 2A). The FYN SH2 domain is an effective inhibitor of mouse egg activation in response to injection of a truncated form of a receptor tyrosine kinase (tr-KIT) (Sette *et al.* 2002), and in sea urchin (Abassi *et al.* 2000, Kinsey & Shen 2000), starfish (Giusti *et al.* 1999b), ascidian (Runft & Jaffe 2000), and fish (Kinsey *et al.* 2003) eggs, 2.5–25 μM FYN SH2 partially or completely inhibits Ca<sup>2+</sup> release at fertilization. We confirmed the effectiveness of the FYN SH2 domain by showing that, as previously described (Giusti *et al.* 1999b), it inhibited Ca<sup>2+</sup> release at fertilization in starfish eggs (Fig. 2B and C). We also tested the YES SH2 domain in starfish eggs; seven eggs injected with 2.5–25 μM YES SH2 all showed either no Ca<sup>2+</sup> release or a delayed and reduced Ca<sup>2+</sup> release when fertilized, compared with controls injected with the Ca<sup>2+</sup> indicator only (Fig. 2D). SH2 domains of non-SRC family tyrosine kinases (ABL, SYK, ZAP-70) are not inhibitory (Giusti *et al.* 1999b, Abassi *et al.* 2000, Kinsey & Shen 2000, Runft & Jaffe 2000, Kinsey *et al.* 2003, O'Neill *et al.* 2004).

Injection of mouse eggs with a mixture of FYN and YES SH2 domains (12 μM each) did not inhibit Ca<sup>2+</sup> release at fertilization (Fig. 2E and F). Of seven eggs tested, six showed Ca<sup>2+</sup> release, and the time between sperm addition and the Ca<sup>2+</sup> rise was 14 ± 5 min (means ± s.d.). Four of five control eggs injected with the Ca<sup>2+</sup> indicator only showed Ca<sup>2+</sup> release, beginning at 14 ± 2 min after sperm addition. The amplitude of the Ca<sup>2+</sup> release and the pattern of oscillations were also the same for FYN SH2 + YES SH2 domain-injected eggs vs controls.



**Figure 1** Immunoblots of SFK proteins in mouse eggs and control lysates of cell lines. All egg lanes contained 2.5 µg protein (100 eggs). See Table 1 for antibodies used. (A) Eggs contain FYN and YES proteins; YES was detected with two different antibodies. BD; BD 610375. (B) No other SFK proteins were detected in eggs. Jurkat, PC-12, BT-20, Namalwa and RAW 264.7; positive control cell lines.

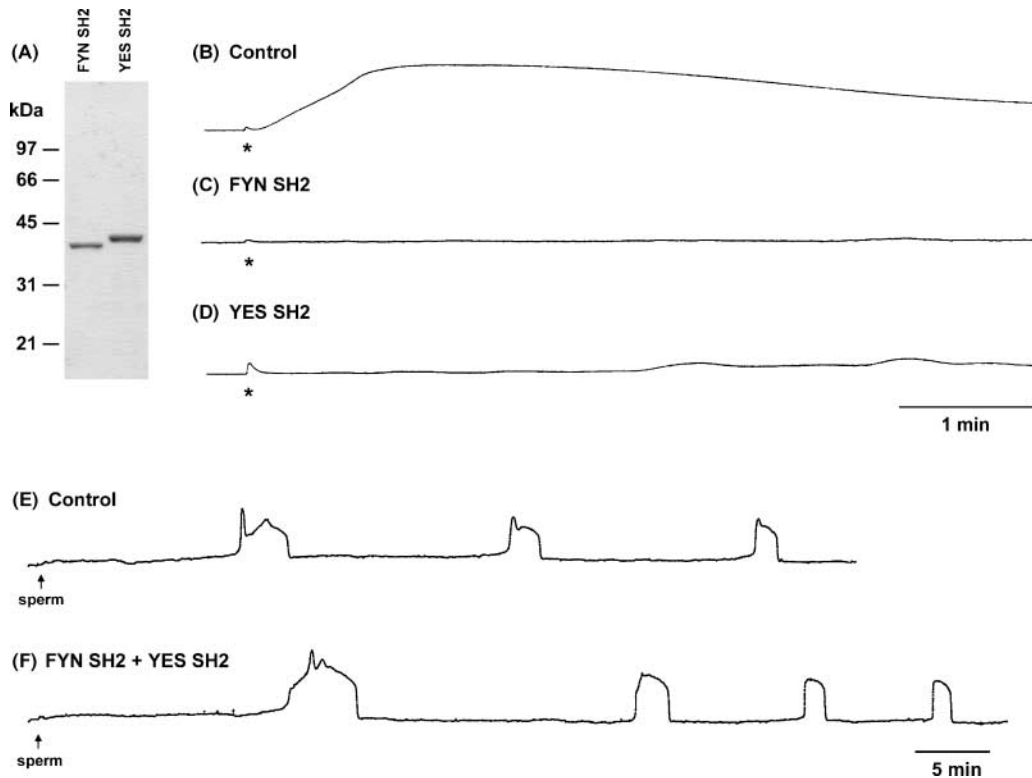
In another series of experiments,  $\text{Ca}^{2+}$  release at fertilization was unaffected by injection of 25 µM FYN SH2 domain protein (six eggs). As discussed above, our control experiments showed that both FYN and YES SH2 domains were effective SFK inhibitors in starfish eggs, and previously published work has also indicated that, at a comparable concentration, FYN SH2 domains are effective inhibitors in mouse eggs stimulated with tr-KIT (Sette *et al.* 2002). Thus, we concluded that in mouse fertilization,  $\text{Ca}^{2+}$  release in the egg does not depend on SH2 domain-mediated activation of an SRC.

#### **Intracellular compartmentalization of the SFK inhibitor SU6656**

To examine the SFK dependence of  $\text{Ca}^{2+}$  release at fertilization of mouse eggs using a different method, we attempted to use a small molecule inhibitor of SFK

activity, SU6656 (Blake *et al.* 2000). We chose not to use another commonly used SFK inhibitor, PP2, because preliminary experiments showed that PP2 inhibited mouse sperm motility.

To determine if SU6656 was an effective inhibitor in an egg known to require SFK activity for  $\text{Ca}^{2+}$  release at fertilization, we initially applied it to starfish eggs; surprisingly, SU6656 (10 µM) had no inhibitory effect on fertilization-induced  $\text{Ca}^{2+}$  release ( $n = 4$  eggs). SU6656 is fluorescent, so it was possible to check that it entered the oocyte cytoplasm by fluorescence microscopy. These observations showed that SU6656 did enter the cytoplasm but, within the cytoplasm, it was compartmentalized within vesicles near the oocyte surface (Fig. 3A). This sequestration may explain why it was not an effective SFK inhibitor. Likewise, in mouse oocytes, SU6656 was not distributed homogeneously within the cytoplasm,



**Figure 2** FYN and YES SH2 domains inhibit Ca<sup>2+</sup> release at fertilization in starfish but not mouse eggs. (A) GST-SH2 domain fusion proteins used for microinjection. 10% SDS-PAGE gel stained with Coomassie blue; 1 µg protein per lane. (B) Ca<sup>2+</sup> release at fertilization in a starfish egg. (C) Inhibition of Ca<sup>2+</sup> release at fertilization in a starfish egg by injection of FYN SH2, at a cytoplasmic concentration of 1.0 mg/ml = 25 µM. (D) Inhibition of Ca<sup>2+</sup> release at fertilization in a starfish egg by injection of YES SH2, at a cytoplasmic concentration of 0.5 mg/ml = 12 µM. (E) Ca<sup>2+</sup> release at fertilization in a mouse egg. (F) No inhibition of Ca<sup>2+</sup> release at fertilization in a mouse egg by injection of a mixture of FYN and YES SH2 domains, each at a cytoplasmic concentration of 0.5 mg/ml = 12 µM. Traces in (B–F) show calcium green fluorescence as a function of time. Asterisks indicate the action potential, which marks the time of sperm–egg fusion in starfish eggs (McCulloh & Chambers 1992).

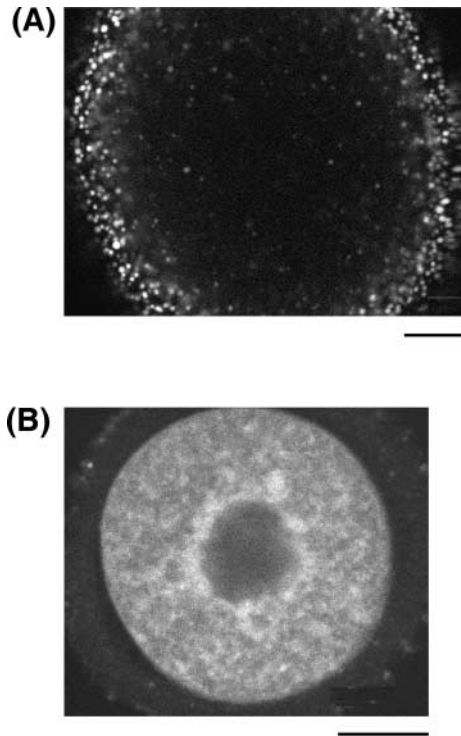
indicating compartmentalization within organelles (Fig. 3B). For this reason, we did not use this inhibitor to test the SFK dependence of Ca<sup>2+</sup> release at fertilization in mouse eggs. These findings suggest caution in the use of SU6656 as an inhibitor of SFKs. Since SU6656 compartmentalizes within the cytoplasm, it may not be accessible to the inner surface of the plasma membrane, where SFKs function in transducing extracellular signals.

## Discussion

At fertilization in species of the vertebrate evolutionary line, Ca<sup>2+</sup> is released from the egg's endoplasmic reticulum by a process requiring IP<sub>3</sub>, and in non-mammalian vertebrates, an SFK functions in this pathway (Runft *et al.* 2002, see Introduction). The present results show that Ca<sup>2+</sup> release at fertilization in mouse eggs is not inhibited by excess SH2 domains of the two SFK proteins found in the eggs, FYN and YES. These findings indicate that the signalling events leading to IP<sub>3</sub>-dependent Ca<sup>2+</sup> release are fundamentally different in eggs of mice, compared with those of echinoderms (Giusti *et al.* 1999b, 2003, Abassi *et al.* 2000, Kinsey & Shen 2000, O'Neill *et al.* 2004), ascidians (Runft & Jaffe 2000), and fish (Kinsey

*et al.* 2003), where SFK SH2 domains do inhibit Ca<sup>2+</sup> release at fertilization. Other differences between echinoderms and mammals are that in echinoderms (Carroll *et al.* 1997, 1999, Shearer *et al.* 1999), but not mice (Mehlmann *et al.* 1998), PLCγ SH2 domains inhibit Ca<sup>2+</sup> release at fertilization, and the time between sperm fusion and Ca<sup>2+</sup> release is greater in mouse eggs (~1–5 min) compared with echinoderm eggs (~4–12 s) (see Runft *et al.* 2002).

Although our work argues against a role for SH2 domain-dependent activation of an SFK in initiating Ca<sup>2+</sup> release at fertilization in mouse eggs, other studies indicate that SFKs may function downstream of Ca<sup>2+</sup> to cause the resumption of meiosis that occurs at fertilization. Injection of constitutively active FYN protein causes metaphase II-arrested mouse and rat eggs to resume meiosis (Sette *et al.* 2002, Talmor-Cohen *et al.* 2004b), and the SFK kinase inhibitors PP2 and SU6656 inhibit the resumption of meiosis in rat eggs in response to the Ca<sup>2+</sup> ionophore ionomycin (Talmor-Cohen *et al.* 2004a). FYN kinase is localized to the meiotic spindle of rat eggs (Talmor *et al.* 1998, Talmor-Cohen *et al.* 2004a,b), and SU6656 causes disintegration of the meiotic spindle (Talmor-Cohen *et al.* 2004b); in addition, tubulin coimmunoprecipitates with FYN in ionomycin-activated rat eggs (Talmor-Cohen *et al.* 2004b).



**Figure 3** SU6656 is compartmentalized in starfish and mouse oocytes. (A) A starfish oocyte that had been incubated for 80 min in  $10\ \mu\text{M}$  SU6656 in sea water before imaging. The confocal section shows that SU6656 is localized in vesicles near the oocyte surface. (B) A mouse oocyte that had been incubated for 120 min in  $10\ \mu\text{M}$  SU6656 in MEM containing  $250\ \mu\text{M}$  dbcAMP before imaging. The confocal section shows that SU6656 is localized in compartments throughout the cytoplasm and concentrated around the prophase nucleus (germinal vesicle). Scale bars =  $20\ \mu\text{m}$ .

All of these findings support a role for FYN in the  $\text{Ca}^{2+}$ -dependent activation of meiosis at fertilization of mammalian eggs, but leave open the question of how  $\text{Ca}^{2+}$  release is initiated.

Much of the recent work on egg activation at fertilization in mammalian eggs has concerned the hypothesis that a substance from the sperm cytoplasm that enters the egg cytoplasm as a consequence of sperm–egg fusion could be the activator of the  $\text{Ca}^{2+}$  release pathway (see Runft *et al.* 2002). Current candidates include tr-KIT (Sette *et al.* 2002) and PLC $\zeta$  (Cox *et al.* 2002, Saunders *et al.* 2002, Fujimoto *et al.* 2004, Kouchi *et al.* 2004, Yoda *et al.* 2004, Knott *et al.* 2005). Our findings argue against tr-KIT, since FYN SH2 domains inhibit  $\text{Ca}^{2+}$  release in response to tr-KIT injection (Sette *et al.* 2002), but not in response to fertilization.

PLC $\zeta$  has several properties consistent with an egg-activating function. (1) The protein is present in sperm (Cox *et al.* 2002, Saunders *et al.* 2002) and is localized to the part of the sperm head that first enters the egg at fertilization (Fujimoto *et al.* 2004). (2) Sperm extract fractions containing PLC $\zeta$  correlate with fractions that can activate eggs (Fujimoto *et al.* 2004). (3) PLC $\zeta$  exhibits high PLC activity

at low free  $\text{Ca}^{2+}$  levels as found in unfertilized eggs (Kouchi *et al.* 2004). (4) Microinjection of PLC $\zeta$  RNA or protein causes  $\text{Ca}^{2+}$  oscillations in eggs, identical to those at fertilization (Cox *et al.* 2002, Saunders *et al.* 2002, Kouchi *et al.* 2004, Yoda *et al.* 2004). (5) Immunodepletion of sperm extracts with a PLC $\zeta$  antibody removes the ability of the extract to cause  $\text{Ca}^{2+}$  release (Saunders *et al.* 2002). (6) Fertilization of eggs with sperm from transgenic mice expressing short hairpin RNAs that target PLC $\zeta$  results in a decreased duration of the  $\text{Ca}^{2+}$  transients (Knott *et al.* 2005). However, it is not known whether other proteins might have been removed from the sperm extract by the PLC $\zeta$  antibody that was used for immunodepletion, and the short hairpin RNAs had only a slight effect on the initiation of the  $\text{Ca}^{2+}$  transients. In addition, some uncertainty remains as to whether a single sperm contains sufficient PLC $\zeta$  protein (Saunders *et al.* 2002, Fujimoto *et al.* 2004, Kouchi *et al.* 2004) or PLC activity (Mehlmann *et al.* 2001, Kouchi *et al.* 2004) to initiate  $\text{Ca}^{2+}$  release. Issues contributing to this uncertainty include the use of partially purified recombinant protein and incompletely characterized antibodies for the quantification of PLC $\zeta$  in sperm.

Nevertheless, PLC $\zeta$  is at present the best supported candidate for a sperm factor causing mammalian egg activation. Regardless of what the sperm factors that activate eggs turn out to be, our results have indicated that these molecules are likely to differ among species that do or do not require SH2 domain-mediated SFK activation in the signalling pathway.

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