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STIM1 is required for Ca^{2+} signaling during mammalian fertilization

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ABSTRACT

During fertilization in mammals, a series of oscillations in the oocyte's intracellular free Ca^{2+} concentration is responsible for oocyte activation and stimulation of embryonic development. The oscillations are associated with influx of Ca^{2+} across the plasma membrane that is probably triggered by the depletion of the intracellular stores, a mechanism known as store-operated Ca^{2+} entry. Recently, STIM1 has been identified in oocytes as a key component of the machinery that generates the Ca^{2+} influx after store depletion. In this study, the involvement of STIM1 in the sperm-induced Ca^{2+} oscillations and its significance in supporting subsequent embryo development were investigated. Downregulation of STIM1 levels in pig oocytes by siRNA completely inhibited the repetitive Ca^{2+} signal triggered by the fertilizing sperm. In addition, a significantly lower percentage of oocytes cleaved or formed blastocysts when STIM1 was downregulated prior to fertilization compared to the control groups. Restoring STIM1 levels after fertilization in such oocytes by means of mRNA injection could not rescue embryonic development that in most cases was arrested at the 2-cell stage. On the other hand, STIM1 overexpression prior to fertilization did not alter the pattern of sperm-induced Ca^{2+} oscillations and development of these fertilized oocytes up to the blastocyst stage was also similar to that registered in the control group. Finally, downregulation of STIM1 had no effect on oocyte activation when activation was stimulated artificially by inducing a single large elevation in the oocyte's intracellular free Ca^{2+} concentration. These findings suggest that STIM1 is essential for normal fertilization as it is involved in the maintenance of the long-lasting repetitive Ca^{2+} signal.

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Introduction

During fertilization the sperm delivers half of the chromosomes of the future embryo and at the same time it also activates the developmental program of the oocyte. In all species studied so far, embryonic development is stimulated by an increase in the oocyte's intracellular free calcium ion (Ca^{2+}) concentration (Stricker, 1999). In mammalian oocytes the activating signal takes the form of low-frequency Ca^{2+} oscillations that last for several hours (Kline and Kline, 1992a; Swann and Ozil, 1994). The repetitive increases in the cytoplasmic Ca^{2+} level are responsible for the induction of a series of additional events such as cortical granule exocytosis, recruitment of maternal mRNAs, resumption of meiosis and pronuclear formation (Kline and Kline, 1992a; Runft et al., 2002; Markoulaki et al., 2004). It is generally accepted that the oscillations are initiated when phospholipase C zeta (PLCζ) diffuses from the sperm head into the oocyte cytoplasm (Saunders et al., 2002). The enzyme catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) into inositol 1,4,5-triphosphate (IP_3) and diacylglycerol. IP_3 then binds to its receptor on the surface of the endoplasmic reticulum and triggers the release of Ca^{2+} from the intracellular stores (reviewed by Berridge, 1993). The initial Ca^{2+} release is then followed by additional transients resulting from the repetitive release and re-uptake of Ca^{2+} by the stores and it is believed that the amplitude, duration, and frequency of the oscillations encode information critical for stimulating proper embryo development (Vitullo and Ozil, 1992). In fact, the pattern of the oscillatory signal influences not only events that take place immediately after fertilization such as pronuclear formation (Ozil and Swann, 1995) but also has long-term effects, such as determining gene expression in the developing embryo (Ozil et al., 2006). All these findings strongly indicate the importance of proper Ca^{2+} signaling during oocyte activation.

Although the long-lasting oscillatory Ca^{2+} signal is a prerequisite for normal fertilization in mammals, little is known about its underlying regulatory mechanisms. It was demonstrated long time ago that sustaining the repetitive Ca^{2+} transients requires an influx of Ca^{2+} through the oocyte plasma membrane; the oscillations stop prematurely in the absence of extracellular Ca^{2+} (Igusa and Miyazaki, 1983) or in the presence of Ca^{2+} influx channel antagonists (Shiina et al., 1993). In non-excitable cells, a key mechanism to support Ca^{2+} signals of extended duration is

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store-operated Ca^{2+} entry (Putney, 1986, 1990). It is believed that in such cells a Ca^{2+} signal is generated when Ca^{2+} is released from the endoplasmic reticulum; depletion of the intracellular stores then stimulates an influx of extracellular Ca^{2+} through Ca^{2+} channels in the plasma membrane (reviewed by Parekh and Putney, 2005). The Ca^{2+} influx induced by store-depletion is believed to be important in sustaining the long-lasting Ca^{2+} oscillations; the mechanism was identified in a great number of somatic cells and its existence was also demonstrated in oocytes (Kline and Kline, 1992b; McGuinness et al., 1996; Machaty et al., 2002). However, the molecular components of the pathway that is responsible for maintaining the fertilization Ca^{2+} signal has not been completely identified.

A protein central in the regulation of store-operated Ca^{2+} entry is the stromal interaction molecule 1 (STIM1). The gene encoding STIM1 was first described as a putative tumor growth suppressor gene mapping to human chromosome region 11p15.5 (Parker et al., 1996). Later, the STIM1 protein was shown to function as a sensor of luminal Ca^{2+} in human T lymphocytes and Drosophila S2 cells conveying the empty signal from the endoplasmic reticulum to the plasma membrane Ca^{2+} entry channels (Liou et al., 2005; Roos et al., 2005). In these studies, suppression of STIM1 blocked store-operated Ca^{2+} entry and its co-overexpression with the Orai1 Ca^{2+} influx channel increased store-operated Ca^{2+} entry activity. Furthermore, STIM1 was found to be involved in maintaining the Ca^{2+} oscillations induced by agonist stimulation in human embryonic kidney cells suggesting that STIM1 is closely associated with replenishing the stores during oscillations (Wedel et al., 2007). A previous report from our laboratory has indicated that STIM1 is also expressed in porcine oocytes and it is essential for Ca^{2+} entry after artificial depletion of the intracellular stores (Koh et al., 2009). Because Ca^{2+} entry across the plasma membrane is critical for the maintenance of Ca^{2+} oscillations at fertilization, we hypothesized that STIM1 may be responsible for mediating Ca^{2+} entry and thus maintaining the sperm-induced Ca^{2+} oscillations in oocytes. In this study, the role of STIM1 during fertilization and its significance in the ensuing embryo development was investigated using the pig as a model.

Materials and methods

Chemicals

All chemicals were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO) unless otherwise indicated.

Oocyte maturation

Ovaries from prepubertal gilts were obtained from a local abattoir. Immature oocytes were aspirated from medium size (3 to 6 mm) follicles using a 20-gauge hypodermic needle attached to a 10-ml syringe. Oocytes with intact surrounding cumulus cells and evenly dark cytoplasm were selected. The oocyte-cumulus complexes were collected in Hepes-buffered Tyrode's Lactate (TL-Hepes) medium (Hagen et al., 1991), rinsed three times in Tissue Culture Medium 199 (TCM-199) and incubated in the same medium supplemented with 0.1 mg/ml cysteine, 10 ng/ml epidermal growth factor (EGF), 10 IU/ml luteinizing hormone (LH) and 10 IU/ml follicle stimulating hormone (FSH) at 39 °C in 5% CO_2 in air for 42–44 h (Abeydeera et al., 1998b). At the end of the maturation period the surrounding cumulus cells were removed from the oocytes by vigorous vortexing for 6 min in the presence of 2 mg/ml hyaluronidase. Oocytes with intact plasma membrane and evenly dark cytoplasm were then selected and used for the experiments.

Immunocytochemistry

Mature oocytes were fixed in 4% paraformaldehyde for 30 min at room temperature. After fixation, the oocytes were washed and permeabilized in PBS containing 0.5% Triton X-100 for 1 h. Non-specific binding sites were blocked by incubation in PBS containing 0.1% Tween−20, 0.01% Triton X-100 and 1% bovine serum albumin (BSA); this was followed by an incubation with an antibody raised against protein disulfide isomerase (DPI), a protein associated with the endoplasmic reticulum (mouse IgG2b monoclonal, from Invitrogen Corporation; Carlsbad, CA, dilution 1:200), overnight at 4 °C. The oocytes were then washed in washing solution (0.1% Tween−20 and 0.01% Triton X-100 in PBS) and incubated overnight in the presence of a rabbit polyclonal antibody raised against STIM1 (dilution 1:200; Millipore, Billerica, MA) at 4 °C. The next day, the oocyes were washed in washing solution and stained with Alexa Fluor 488-conjugated goat anti-mouse IgG (to label the endoplasmic reticulum; Invitrogen, dilution 1:500) and rhodamine-conjugated goat anti-rabbit antibody (to label STIM1; Millipore, dilution 1:500) for 1 h at room temperature. After washing, the chromosomes were stained with Hoechst 33342 for 10 min. Oocytes undergoing the same procedure but not treated with the primary antibody were used as control.

In vitro transcription

The plasmid encoding the yellow fluorescent protein conjugated to the N-terminus of human STIM1 (YFP-STIM1) was a generous gift from Drs. Jen Liou and Tobias Meyer of Stanford University. In order to express the fusion protein in the transcriptionally inactive oocytes, mRNA encoding YFP-STIM1 was generated by in vitro transcription. First, a DNA fragment encoding YFP-STIM1 was amplified from the plasmid using a forward primer that contained the T7 promoter at its 5′ end. The sequences of the primers used were forward primer 5′–AGAATGGATGTATGCGTCCGTCTTGC-3′ and reverse primer 5′–CGCCTACTTCTTAAGAGGCTTCTTAAAGATTTTGAGAGG-3′. The PCR reactions were conducted using Platinum® Taq DNA Polymerase High Fidelity (Invitrogen). The conditions for the PCR were 95 °C for 2 min, followed by 94 °C for 30 s, 55 °C for 30 s, and 68 °C for 3 min for 25 cycles. The expected size of the PCR product was 2900 bp. The mRNA encoding YFP was produced in a similar manner to serve as control. The PCR products were separated on a 0.8% agarose gel and purified using the QIAquick PCR Purification Kit (Qiagen; Valencia, CA). The mMESSAGE mMACHINE Kit (Ambion; Austin, TX) was used to produce mRNA from the amplified PCR products in vitro. For this purpose the reaction mixture was incubated at 37 °C for 1 h, the template DNA was then degraded by a 15-min incubation in the presence of TURBO DNase (Ambion) at 37 °C. Synthesized RNA was purified by lithium-chloride precipitation and the pellet re-suspended in nuclease-free water to a final concentration of 600 ng/μl. The sample was distributed into 4 μl aliquots and stored at −80 °C.

RNA interference

A set of siRNA that previously showed effective STIM1 knockdown in pig oocytes in our previous study (Koh et al., 2009) was used. This set of siRNA could downregulate STIM1 at both the transcript and protein levels when injected into oocytes prior to maturation. The siRNAs were designed to target the SAM domain-encoding region of the STIM1 gene. Targeting this region with

siRNA demonstrated successful STIM1 knockdown in HeLa cells (Jousset et al., 2007). The designed siRNAs were compared with all available porcine sequences in GenBank to determine specificity. The following siRNAs were used in the experiment: sense, 5′–UCACGUACGUGGAGCUGCCUCAG-UA-3′; and antisense, 5′–UACUGAGGCAGCUCCACGUACGUGA-3′. The siRNA was diluted to a final concentration of 1 μM in DEPC-treated nuclease-free water (Invitrogen) according to the manufacturer's recommendations, aliquoted and stored at −80 °C until use. Oocytes injected with a scrambled siRNA duplex (sense, 5′–UCAAUGCGUAGCGG-UCCCUAGCGUA-3′; antisense, 5′–UACGCUAGGGACCGCUACGCAUUGA-3′; from Invitrogen) were used as negative control. These siRNAs were then used for microinjection into oocytes.

RNA microinjection

For STIM1 downregulation, siRNA designed against STIM1 was injected into the cytoplasm of immature oocytes using a FemtoJet microinjector (Eppendorf; Hamburg, Germany). For this purpose, oocyte-cumulus complexes were selected after collection and the siRNA against STIM1 was injected together with 1% fluorescein-labeled dextran to visualize microinjection. Microinjection was performed in Ca^{2+}-free TL-Hepes on the heated stage of a Nikon TE2000-U inverted microscope (Nikon Corporation; Tokyo, Japan). Successful microinjection into the cumulus-enclosed oocytes was verified immediately after injection; only oocytes showing green fluorescence under UV light due to the presence of the fluorescent dextran in the ooplasm were collected. Controls included oocytes injected with scrambled siRNA or those injected with the fluorescent dextran only. Following injection the cumulus-oocyte complexes were transferred into maturation medium and matured for 44 h. (In preliminary experiments the siRNA was introduced into the oocytes towards the end of maturation. For this purpose the oocytes were denuded 35 h after the beginning of the maturation period, and either STIM1 siRNA or scrambled siRNA was injected into the ooplasm.) For STIM1 overexpression, mRNA encoding YFP-STIM1 was injected into cumulus-free oocytes 35 h after the beginning of maturation. Injected oocytes were rinsed with TCM-199 three times and further incubated in maturation medium but this time without hormonal supplementation. After 10 h of incubation (this time was sufficient to allow for translation of the injected mRNA based on previous experiments) the oocytes with first polar bodies and intact plasma membranes were selected and used for further analyses.

SDS-PAGE and western blot analysis

Five hundred mature oocytes were pooled and lysed in SDS sample buffer; the lysates were boiled for 3 min and centrifuged for 4 min at 12,000 x g. The proteins were separated by SDS-PAGE on ice using a 5% stacking gel and a 12% separating gel for 30 min at 90 V and 2.5 h at 120 V, respectively. They were then transferred electrophoretically onto nitrocellulose membranes using the semi-dry transfer method at 180 mA for 1 h. After blocking for 1 h in TBST buffer (0.1% Tween-20 in Tris-buffered saline) containing 5% non-fat milk, the membrane was incubated with rabbit polyclonal antibody against STIM1 (dilution 1:500; Millipore, Billerica, MA) overnight at 4 °C. The membrane was then washed in TBST and incubated for 1 h at room temperature with horseradish peroxidase (HRP)-conjugated anti-rabbit immunoglobulin G (1:1500; Promega; Madison, WI). Following incubation the membrane was washed in TBST, processed using the enhanced chemiluminescence (ECL) detection system (Pierce Thermo Scientific; Rockford, IL) and exposed to an x-ray film. The analysis was replicated 3 times.

In vitro fertilization

Mature oocytes were rinsed in fertilization medium (a modified Tris-buffered medium) consisting of 113.1 mM NaCl, 3 mM KCl, 7.5 mM $CaCl_2 \times 2H_2O$, 20 mM Tris (crystallized free base), 11 mM glucose, 5 mM sodium pyruvate, 0.1% BSA, and 1 mM caffeine (Abeydeera et al., 1998a). Groups of 30 to 35 oocytes were placed into 50 μl droplets of the medium covered with mineral oil. Fresh semen previously collected from a Large White boar and diluted in Modena extender was washed three times by centrifugation at 900 g for 4 min in Dulbecco's PBS (without Ca^{2+} and Mg^{2+}) containing 0.1% BSA, 75 μg/ml potassium penicillin G, and 50 μg/ml streptomycin sulfate (pH 7.2, 39 °C). The final sperm pellet was diluted with the fertilization medium and the sperm suspension at a final concentration of 5×10^5 cells/ml was added to each 50 μl droplet containing the oocytes. The gametes were co-incubated for 5 h at 39 °C in an atmosphere of 5% CO_2 in air; potential zygotes were then transferred to PZM-3 medium for culture. On day 7, the nuclei of the cultured embryos were stained with Hoechst 33342 and the developmental stages of the embryos were determined using a Nikon Eclipse 50i microscope fitted for epifluorescence.

Cytosolic Ca^{2+} measurements

The zonae pellucidae were removed after maturation and zona-free oocytes were loaded with the Ca^{2+} indicator dye fura-2. For this purpose, they were incubated in the presence of 2 μM of the acetoxymethyl ester form of the dye and 0.02% pluronic F-127 for 40–50 min (both from Invitrogen). The oocytes were pre-incubated with porcine spermatozoa for 1 h in an incubator at 39 °C and transferred into a chamber with a coverglass bottom; the chamber was then placed on the heated stage of an inverted microscope and changes in the intracellular free Ca^{2+} concentration were recorded using InCyt Im2, a dual-wavelength fluorescence imaging system (Intracellular Imaging, Inc.; Cincinnati, OH). During measurements the emitted fluorescence was detected at 510 nm after exciting the dye alternately at 340 and 380 nm. The ratio of the two emitted fluorescence intensities was calculated and the data are presented as fluorescence ratio values; ratios of 1.0 and 5.0 correspond to about 100 and 1200 nM Ca^{2+}, respectively. The average duration of Ca^{2+} measurements was 4 h and in each treatment group the measurements were repeated at least 10 times using different oocytes.

Thimerosal/dithiothreitol activation

We also investigated the effect of STIM1 downregulation on oocyte activation when the activation was induced artificially by a single Ca^{2+} transient. For this purpose mature oocytes (STIM1 siRNA-injected oocytes together with various controls) were incubated in TL-Hepes containing 200 μM thimerosal for 10 min followed by 30 min incubation in the presence of 8 mM dithiothreitol. Potential zygotes were then transferred to PZM-3 medium for culture. On day 7 the percentage of embryos that reached the blastocyst stage was evaluated after staining with Hoechst 33342.

Statistical analysis

The data were subjected to analysis of variance (ANOVA) using the PROC MIX procedure of the Statistical Analysis System (SAS Institute; Cary, NC). Percentage data were transformed by arc sine transformation before the ANOVA analysis. Differences among treatment means were analyzed using the Tukey test. Differences with $p < 0.05$ were considered significant in all comparisons.

Results

STIM1 is essential for maintaining Ca^{2+} oscillations at fertilization

To better understand the involvement of STIM1 in signal transduction during fertilization, we used indirect immunocytochemistry to determine the intracellular localization of STIM1 relative to the intracellular Ca^{2+} store. As shown in the confocal images of Fig. 1, STIM1 co-localized with the endoplasmic reticulum in the porcine ooplasm. The endoplasmic reticulum is distributed uniformly as a reticular network in the cytoplasm of metaphase II oocytes and STIM1 follows a similar pattern. This is consistent with STIM1 serving as a sensor of luminal Ca^{2+} content in cells.

Next, to investigate the role of STIM1 in the sperm-induced Ca^{2+} oscillations, STIM1 levels were suppressed in oocytes prior to fertilization. STIM1 siRNA was successfully injected into cumulus-enclosed immature oocytes and the injected oocytes were able to reach the metaphase II stage as indicated by the presence of their first polar bodies by the end of the maturation period. Fig. 2 shows the effect of siRNA injection on the expression of STIM1 in the mature oocytes. The injection of 1 μM STIM1 siRNA effectively knocked down STIM1 protein levels; this suggested that the designed siRNA could successfully suppress the expression of STIM1 in oocytes.

We then measured a series of Ca^{2+} transients when control non-treated oocytes were co-incubated with spermatozoa (Fig. 3A) and a similar, long-lasting signal was detected in oocytes injected with scrambled siRNA as well (Fig. 3B). In these cases the first Ca^{2+} rise was measured approximately 1.5 h after the addition of sperm and it was followed by a number of additional spikes. The average interval between the spikes was ~20 min and the oscillations lasted for several hours. On the other hand, the overwhelming majority of oocytes with downregulated STIM1 levels were unable to generate repetitive Ca^{2+} signals. Most of these oocytes (8 out of 10 measured) showed only one Ca^{2+} transient after fertilization; additional Ca^{2+} rises were not detected in these gametes (Fig. 3C). Two oocytes displayed repetitive Ca^{2+} spikes probably due to ineffective STIM1 downregulation. When the siRNA against STIM1 was microinjected 35 h after the beginning of maturation, the ablation of the repetitive signal was also successful. In most cases (16/20) the Ca^{2+} signal ceased after a single transient (data not shown). Interestingly, in the remaining 4 oocytes we observed Ca^{2+} oscillations with low amplitude and very high frequency (Fig. 3D). The average amplitude of the Ca^{2+} transients in these oocytes was $R=2.4\pm0.1$ with an average frequency of 6.9 ± 1.2 transients per hour whereas these parameters in the control scrambled siRNA-injected group were $R=6.1\pm0.6$ and 2.2 ± 0.2, respectively ($p<0.05$). Finally, in order to demonstrate that the termination of the oscillations was caused by the lack of store refilling due to the absence of STIM1, ionomycin (final conc. 10 μM) was added to the oocytes after the cessation of the sperm-induced Ca^{2+} transients. The addition of the ionophore in Ca^{2+}-free medium caused only a small increase in the cytoplasmic Ca^{2+} levels indicative of the release of a small amount of Ca^{2+} from the stores in oocytes with downregulated STIM1 (Fig. 4), while in control oocytes a large amount of Ca^{2+} was mobilized (in these control cells the ionophore was added after Ca^{2+} levels reached baseline values following a Ca^{2+} transient during the course of the repetitive Ca^{2+} signal). This implies that the stores were not properly refilled in the absence of STIM1. Overall, these data indicate that STIM1 downregulation had a profound effect on the characteristics of the fertilization Ca^{2+} signal.

To further investigate the role of STIM1 in Ca^{2+} signaling during fertilization, the STIM1 protein was overexpressed in porcine oocytes by microinjection of YFP-STIM1 mRNA. Using western blot analysis we have previously demonstrated that the mRNA is effectively expressed in pig oocytes, the YFP-STIM1 fusion protein was detected at high levels in the oocytes following microinjection (Koh et al., 2009). Oocytes with overexpressed STIM1 displayed Ca^{2+} oscillations with characteristics very similar to those detected in control cells (data not shown).

STIM1 downregulation impairs embryo development

Next, we studied the importance of STIM1-mediated signaling in supporting embryo development. The suppression of STIM1 expression at fertilization led to a decrease in the in vitro

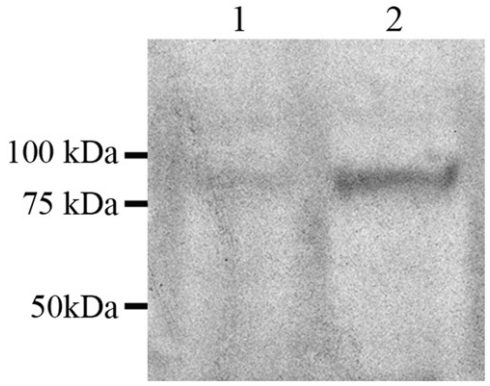

Fig. 2. Western blot showing the downregulation of STIM1 by RNA interference. The siRNAs were injected into immature oocytes and the protein levels were assessed in the cells after maturation. Total proteins from 500 oocytes were loaded into each well of the gel. Lane 1: STIM1 siRNA-injected oocytes; Lane 2: scrambled siRNA-injected oocytes. The analysis was replicated three times.

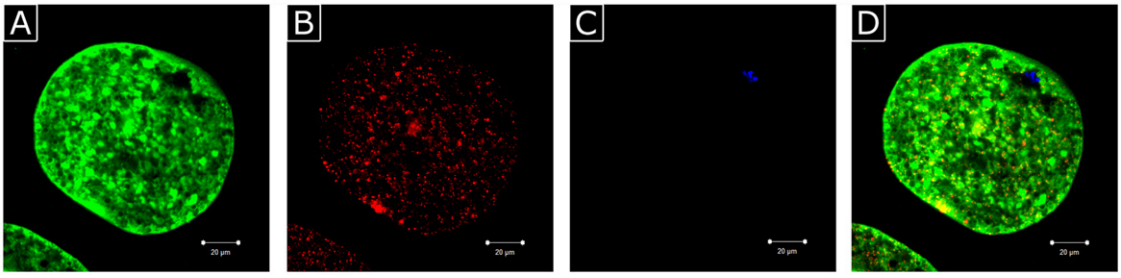

Fig. 1. Confocal images showing the immunocytochemical localization of STIM1 and the endoplasmic reticulum in porcine oocytes. (A) green fluorescence denoting the endoplasmic reticulum; (B) red fluorescence indicating STIM1; (C) metaphase chromosomes stained with Hoechst 33342; (D) composite image showing the endoplasmic reticulum network, STIM1 and the chromosomes. In control oocytes no specific staining was detected in the absence of the primary antibodies (not shown). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

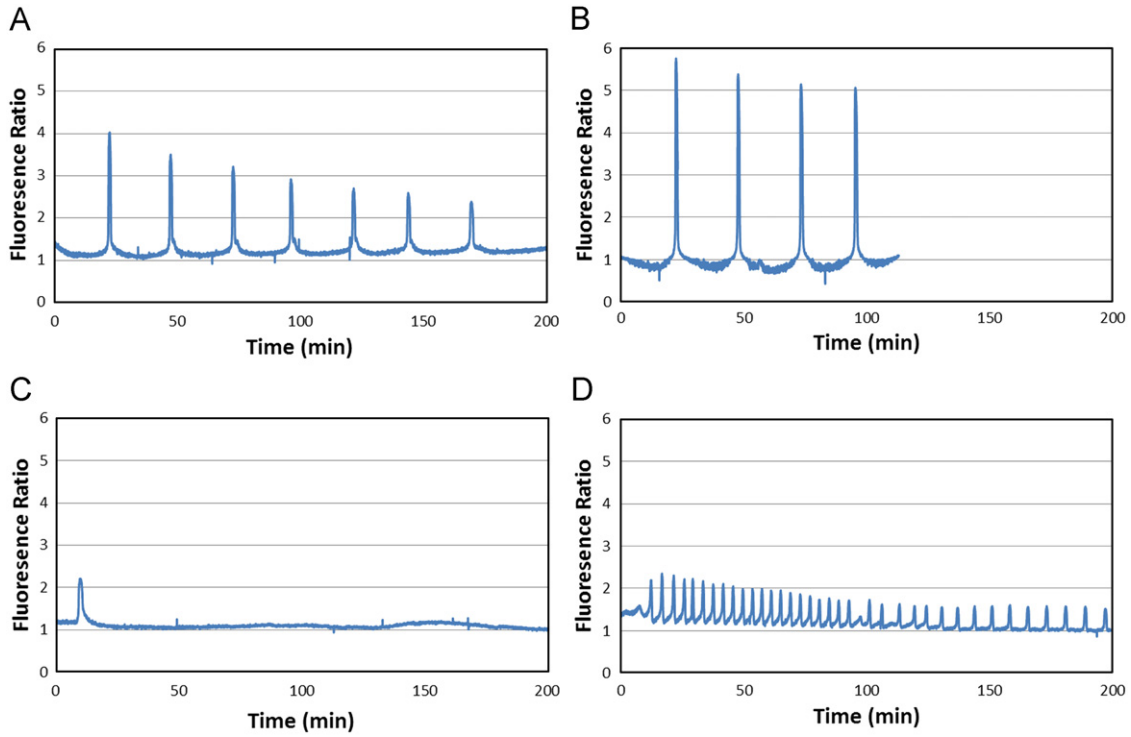

Fig. 3. Effect of STIM1 downregulation on sperm-induced Ca^{2+} oscillations. (A) Ca^{2+} oscillations in a non-treated control oocyte after insemination. (B) Fertilization Ca^{2+} signal in an oocyte injected with scrambled siRNA. (C) Sperm-induced Ca^{2+} signal in an oocyte injected with STIM1 siRNA at the beginning of maturation. Note the lack of oscillations in these oocytes. (D) In 4 out of 20 oocytes injected 35 h after the beginning of maturation with STIM1 siRNA, Ca^{2+} oscillations with low amplitude and high frequency were monitored.

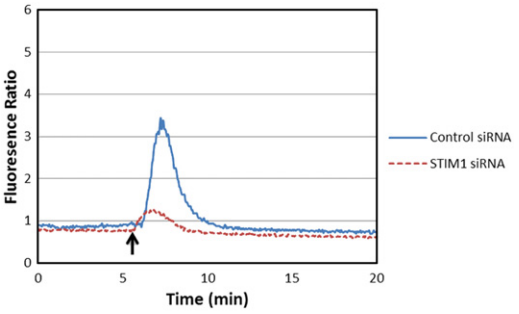

Fig. 4. Ionomycin-induced Ca^{2+} release from the endoplasmic reticulum in fertilized oocytes. GV-stage oocytes were injected with siRNA against STIM1 or with scrambled siRNA (control), matured and fertilized. When the Ca^{2+} oscillations stopped in the STIM1 siRNA-injected oocytes, ionomycin (10 μM) was added to the cells in Ca^{2+}-free medium; in the control group ionomycin was added during the series of oscillations, approximately 5 min after a Ca^{2+} transient. The amount of Ca^{2+} mobilized by the ionophore was notably smaller in oocytes with downregulated STIM1. Each trace is a representative response from a single oocyte; a total of 12 oocytes were examined.

Table 1
Effect of STIM1 downregulation on embryo development after fertilization.

Treatment	Total number of oocytes	No. of replications	2-cell embryos (% ± S.E.M.)	Blastocysts (% ± S.E.M.)
Non-treated control	87	4	67.8 ± 8.5[a]	14.9 ± 3.1[a]
Control siRNA injection	120	4	72.5 ± 3.5[a]	14.2 ± 4.4[a]
STIM1 siRNA injection	125	4	28.8 ± 3.4[a]	0.8 ± 0.8[a]

[a] Different superscript letters in the same column denote significant differences ($p < 0.05$).

developmental potential of the resulting embryos (Table 1). We found that as a result of STIM1 downregulation there was a significant drop in the percentage of 2-cell embryos following fertilization (28.8% vs. 67.8%; $p < 0.05$). At the same time, cleavage frequency of oocytes injected with scrambled siRNA (72.5%) was not different from that found in the non-injected control group. The detrimental effect of lacking STIM1 at fertilization was more dramatic by the end of the culture period: the percentage of embryos that formed blastocysts in the non-injected and control siRNA-injected groups were 14.9% and 14.2%, respectively; while only 0.8% of the embryos that received STIM1 siRNA reached the blastocyst stage ($p < 0.05$).

To test whether the absence of STIM1 at the time of fertilization was responsible for the perturbed embryo development, STIM1 levels were restored after fertilization in oocytes lacking STIM1. In these experiments STIM1 expression was first suppressed by injecting siRNA into GV-stage oocytes. At the end of the maturation period the cumulus cells were removed, sperm was added to the oocytes and 6 h later mRNA encoding YFP-STIM1 was introduced into the ooplasm. Oocytes injected with non-coding siRNA before fertilization and receiving water-injection after fertilization served as control. We found that restoring STIM1 levels after fertilization did not rescue embryo development; both cleavage frequency (25.6% vs. 73.0%) and blastocyst formation (0% vs. 13.5%) were significantly lower in oocytes that were injected with STIM1 siRNA prior to fertilization in spite of subsequent expression of the protein, compared to the control group (Table 2; $p < 0.05$).

As described above, STIM1 overexpression in oocytes prior to fertilization did not alter Ca^{2+} oscillation patterns induced by the fertilizing sperm. To test the effect of STIM1 overexpression on embryo development, mRNA coding for YFP-STIM1 was injected into mature oocytes 35 h after the beginning of maturation. Ten hours later the oocytes were fertilized and cultured in vitro to

Table 2
Results of the rescue experiment. STIM1 levels were downregulated in immature oocytes and after fertilization they were restored by injecting STIM1 mRNA into the cytoplasm.

Treatment	Total no. of oocytes	No. of replications	2-cell embryos (% ± S.E.M.)	Blastocysts (% ± S.E.M.)
Scrambled siRNA + water injection	74	3	73.0 ± 5.6[a]	13.5 ± 1.8[a]
STIM1 siRNA + STIM1 mRNA injection	78	3	25.6 ± 4.1[a]	0[a]

[a] Different superscript letters in the same column indicate significant differences ($p < 0.05$).

Table 3
Effect of STIM1 overexpression on embryo development.

Treatment	Total no. of oocytes	No. of replications	2-cell embryos (% ± S.E.M.)	Blastocysts (% ± S.E.M.)
YFP mRNA injection	110	3	70.0 ± 5.8[a]	15.4 ± 2.6[a]
STIM1 mRNA injection	97	3	72.2 ± 4.1[a]	14.4 ± 2.1[a]

[a] Different superscripts in the same column indicate statistically significant differences ($p < 0.05$).

monitor development. Overexpression of STIM1 had no effect on oocyte activation (as determined by the percentage of oocytes cleaved) or blastocyst formation by the end of the 7-day incubation period (Table 3).

STIM1 downregulation has no effect on oocyte activation induced by a single Ca^{2+} rise

The aforementioned experiments have shown that STIM1 is required for proper signaling during fertilization and that disrupting STIM1-mediated signaling prior to sperm-oocyte fusion has a negative impact on subsequent embryo development. Under physiological conditions, the oocyte's developmental program is stimulated by a series of Ca^{2+} transients that take place in the ooplasm. On the other hand, most methods that are used to induce parthenogenetic activation of oocytes trigger only a single large Ca^{2+} elevation. Therefore, to better understand the function of STIM1 in signaling we investigated the effect of STIM1 downregulation on oocyte activation when development is triggered by a single Ca^{2+} transient. Thimerosal is known to mobilize Ca^{2+} from intracellular stores and if the Ca^{2+} release is followed by a treatment with dithiothreitol, oocyte activation will follow (Machaty et al., 1999); the combined thimerosal/dithiothreitol activation method has successfully induced oocyte activation and supported term development after nuclear transfer in the pig (Rogers et al., 2008). STIM1 levels were downregulated by injecting STIM1 siRNA into cumulus-enclosed oocytes prior to maturation; the oocytes were then matured, denuded, and treated with thimerosal. When oocyte activation was to be evaluated, the thimerosal treatment was followed by incubation in the presence of dithiothreitol. We found that the thimerosal-induced Ca^{2+} signal was similar in all oocytes tested; STIM1 downregulation had no effect on the Ca^{2+} response generated by thimerosal (Fig. 5). In addition, there was no difference in the percentage of activated oocytes between the non-injected control, control siRNA-injected, and STIM1 siRNA-injected groups; approximately 75–80% of the oocytes cleaved as a result of the activation treatment (Table 4).

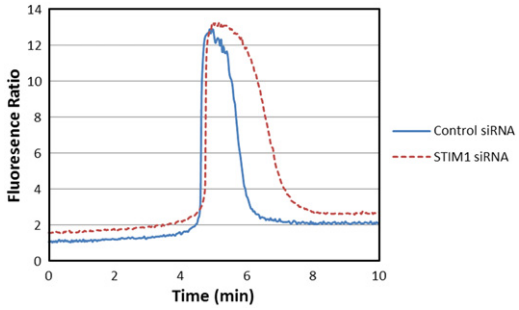

Fig. 5. Thimerosal-induced Ca^{2+} transients in oocytes with downregulated STIM1 levels and in scrambled siRNA-injected (control) oocytes. Decreased STIM1 levels did not affect the amount of Ca^{2+} released as a result of the thimerosal (conc. 100 μM) treatment. Each profile is representative of 10 oocytes.

Table 4
Effect of STIM1 downregulation on oocyte activation after a single Ca^{2+} transient induced by the thimerosal/dithiothreitol treatment.

Treatment	Total no. of oocytes	No. of replications	Oocytes cleaved (% ± S.E.M.)
Non-injected control	118	4	78.0 ± 7.3[a]
Control siRNA injection	129	4	75.2 ± 7.5[a]
STIM1 siRNA injection	126	4	79.4 ± 6.0[a]

[a] Different superscript letters in a column indicate significant differences ($p < 0.05$).

Discussion

In various cell types, the STIM1 protein has been described to localize at the endoplasmic reticulum with its EF hand sensing the Ca^{2+} content of the intracellular store. It is also believed to serve as a signal transducer that upon the release of Ca^{2+} from the store moves to the plasma membrane and opens Ca^{2+} channels to generate store-operated Ca^{2+} entry. The Ca^{2+} entering the cell after store depletion is important for replenishing the intracellular stores and also, in cells such as T lymphocytes, for maintaining elevated intracellular Ca^{2+} levels for an extended period of time (reviewed by Hogan et al., 2010). In addition, in human embryonic kidney cells store-operated Ca^{2+} entry mediated by STIM1 has been shown to play a role in supporting Ca^{2+} oscillations induced by the muscarinic agonist methacoline (Bird and Putney, 2005; Wedel et al., 2007). Although the oscillatory Ca^{2+} signal in fertilized mammalian oocytes is critical for the stimulation of normal embryo development, the mechanism that sustains the long-lasting oscillations has not been characterized. Our laboratory provided the first evidence that STIM1 was responsible to mediate Ca^{2+} entry in oocytes after the passive depletion of the intracellular stores using thapsigargin, an inhibitor of the sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase (Koh et al., 2009). Later, store-operated Ca^{2+} entry generated by an interplay between STIM1 and the plasma membrane Ca^{2+} channel Orai1 was demonstrated in immature Xenopus oocytes (Yu et al., 2009) and the potential involvement of STIM1 in signaling during fertilization was also shown in the mouse (Gómez-Fernández et al., 2009).

In the present study we unequivocally demonstrated the role of STIM1 in Ca^{2+} signaling during fertilization. STIM1 suppression in oocytes inhibited the sperm-induced Ca^{2+} oscillations indicating that STIM1 is essential for the maintenance of the long-lasting Ca^{2+} signal. In most oocytes injected with STIM1 siRNA we detected only one Ca^{2+} transient that was probably the result

of Ca^{2+} released from the endoplasmic reticulum. Additional Ca^{2+} increases did not occur because in the absence of STIM1 no mechanism was available to signal store depletion and stimulate Ca^{2+} influx to replenish the stores. In some cases during the preliminary experiments, Ca^{2+} oscillations with low amplitude and very high frequency were observed. In those experiments STIM1 siRNA was microinjected into the oocytes towards the end of the maturation period and because of their limited life span the injected oocytes were incubated for only 15 h prior to insemination. This relatively short incubation time may have led to incomplete downregulation of the endogenous STIM1 protein that resulted in altered oscillation patterns but without complete elimination of the repetitive signal. It is possible that following store depletion, the reduced number of STIM1 available in the endoplasmic reticulum stimulated the entry of only a limited amount of Ca^{2+} across the plasma membrane that could not contribute significantly to the Ca^{2+} increase in the cytoplasm leading to the generation of Ca^{2+} transients with lower amplitude. It is not clear why the frequency of the Ca^{2+} oscillations was higher under such conditions. It is possible that the limited amount of STIM1 sensed store fullness earlier and this somehow led to premature release of Ca^{2+} and hence, shorter intervals between spikes, but this needs further verification. To eliminate the possibility of incomplete downregulation, during additional experiments siRNA against STIM1 was delivered into immature, GV-stage oocytes. This timing was based on literature data indicating that STIM1 expression is effectively inhibited by 48 h after siRNA injection (Liou et al., 2005; Roos et al., 2005). Because immature pig oocytes require complete cumulus cell investment for maturation, the siRNA was microinjected into cumulus-enclosed oocytes. This facilitated successful maturation of the injected oocytes and allowed for effective downregulation of endogenous STIM1 levels during the subsequent culture period of $\sim$44 h. When such oocytes were fertilized we detected a complete termination of Ca^{2+} oscillations after just one Ca^{2+} rise; no additional transients of any size were measured in these cells. Overall, these results indicate that STIM1 is required for maintaining the long lasting Ca^{2+} oscillations at fertilization.

The absence of STIM1 at the time of gamete fusion also had a negative impact on subsequent embryo development: when STIM1 was downregulated prior to fertilization, a significantly lower percentage of embryos cleaved and reached the blastocyst stage by the end of the culture period. This is probably due to the abnormal Ca^{2+} signal caused by the lack of store-operated Ca^{2+} entry to refill the stores. It was suggested that the duration, amplitude and frequency of the Ca^{2+} oscillations encode information that affect not only the initiation of activation but has consequences much later in time, during peri- and post-implantation (Bos-Mikich et al., 1997; Ozil et al., 2006). The results indicate that the suppression of STIM1 before fertilization prevented the onset of repetitive Ca^{2+} oscillations in the fertilized oocytes that eventually resulted in poor embryonic development. This may seem to be in contradiction with the fact that term development can be achieved following nuclear transfer where development of the reconstructed oocyte is most often induced by a physical or chemical stimulus that triggers only a single Ca^{2+} transient in the ooplasm. However, one must keep in mind that the Ca^{2+} elevations induced by the artificial stimuli are, in most cases non-physiological, large Ca^{2+} rises similar to what we detected after using thimerosal in the present study. In mouse it was demonstrated that development up to the blastocyst stage does not rely on a specific pattern of Ca^{2+} signaling if the total Ca^{2+} signal input 'experienced' by the egg reaches a minimal threshold (Tóth et al., 2006). Therefore, a single Ca^{2+} transient of sufficient duration and amplitude may successfully activate the oocyte's developmental program after nuclear transfer whereas the relatively smaller Ca^{2+} elevations that did take place in fertilized oocytes with downregulated STIM1 levels were inefficient.

It is possible that the lack of STIM1 also influenced Ca^{2+} signals potentially associated with cell cycle progression or mitotic cell division(s) during embryo development. STIM1 was reported to be essential for cell cycle progression in human hepatoma and endothelial cells (Abdullaev et al., 2008; Aubart et al., 2009) although this was not the case in HEK293 cells (El Boustany et al., 2010). Whether STIM1 is required for signaling during mitosis in preimplantation embryos remains to be elucidated. Store-operated Ca^{2+} entry is suppressed during mitosis in somatic cells (Preston et al., 1991; Tani et al., 2007); in the fertilized mouse oocyte the Ca^{2+} oscillations stop during interphase but resumes as the embryo enters first mitosis (Kono et al., 1996; Marangos et al., 2003). This indicates that at least in mice the sperm-derived enzyme PLCζ is still active and triggers Ca^{2+} oscillations during the first mitotic division; it also implies that store-operated Ca^{2+} entry, and thus STIM1 may be involved in the process. However, in our experiments, when the level of STIM1 was restored in zygotes whose STIM1 expression was knocked down prior to fertilization, embryonic development could not be rescued: the frequencies of cleavage and blastocyst formation were still significantly lower than those in the control group. This suggests that the abnormal fertilization Ca^{2+} signal, as a result of STIM1 suppression, was responsible primarily for the poor embryo development. It seems that restoring STIM1 levels after fertilization did not ameliorate embryonic development because the developmental program in the embryos had not been activated by the appropriate Ca^{2+} signal. This further strengthens the notion that store-operated Ca^{2+} entry mediated by STIM1 is essential for proper oocyte activation and normal embryonic development.

Overexpression of STIM1 before fertilization did not alter the Ca^{2+} oscillations triggered by sperm. This seems to indicate that extra STIM1 alone is not sufficient to further stimulate Ca^{2+} influx during the oscillatory signal. STIM1 overexpression had various effects on store-operated Ca^{2+} entry in different cell types. In mouse pulmonary arterial smooth muscle cells it enhanced the rise in the intracellular free Ca^{2+} levels after pharmacologically-induced depletion of the cytoplasmic Ca^{2+} stores (Ng et al., 2009). In HEK293 cells, Jurkat T cells, and Xenopus oocytes on the other hand, STIM1 overexpression alone had only a small-to-modest effect on Ca^{2+} influx, and overexpression together with Orai1 was necessary to significantly increase Ca^{2+} influx (Mercer et al., 2006; Peinelt et al., 2006; Yu et al., 2009). In a similar study, overexpression of STIM1 in HEK293 cells did not change the pattern of Ca^{2+} oscillations induced by the PLC-linked agonist methacholine (Bird et al., 2009). These authors hypothesized that the endogenous STIM1 was probably sufficient to completely activate plasma membrane-resident Orai1 and additional STIM1was not able to stimulate further Ca^{2+} influx. This observation was also reported in Human Liver (HL)-7702 cells indicating that the phenomenon is not limited to HEK293 cells (Zhang et al., 2010). We have also found that although STIM1 overexpression causes a moderate elevation in Ca^{2+} influx after thapsigargin-induced store depletion (Koh et al., 2009), this increase is markedly higher when STIM1 is overexpressed together with Orai1 (Wang et al., 2012). These findings probably indicate a fine stoichiometry between STIM1 and Orai1. In addition, because STIM1 overexpression did not alter the pattern of the sperm-induced Ca^{2+} oscillations, we did not expect changes in the development of embryos arising from these oocytes. Consistent with our assumption, cleavage frequency and blastocyst formation were not affected by elevated STIM1 levels in the oocytes.

When STIM1 expression was suppressed in oocytes prior to thimerosal/dithiothreitol-induced parthenogenetic activation, oocyte activation as assessed by cleavage of the activated oocytes was not altered. The combined thimerosal/dithiothreitol treatment activates oocytes by triggering a single Ca^{2+} rise in the ooplasm. We found no difference in the shape of the Ca^{2+} transients between control and STIM1-suppressed oocytes; the absence of STIM1 did not change the pattern of Ca^{2+} signals induced. Thimerosal stimulates Ca^{2+} release from the intracellular stores through a mechanism different from that utilized by the fertilizing sperm. Although the process is not fully understood it probably involves the oxidization of critical sulfhydryl groups on the InP_3- (and where applicable, the ryanodine) Ca^{2+} release channels (Galione et al., 1993). The observation that oocyte activation was unaffected by STIM1 downregulation strengthens our hypothesis that STIM1 is relevant in signaling because it helps to maintain the sperm-induced Ca^{2+} oscillations through store-operated Ca^{2+} entry; its absence has no effect on downstream events when development is stimulated by a single large elevation in the cytosolic Ca^{2+} level.

The low-frequency Ca^{2+} oscillations during fertilization are key for the stimulation of proper development because they are responsible for triggering a great number of activation events in the oocyte (reviewed by Runft et al., 2002). Store-operated Ca^{2+} entry was proposed to be the main mechanism responsible for sustaining Ca^{2+} oscillations in non-excitable cells (Berridge, 1990; Thomas et al., 1996). Experimental data later indicated that this may depend on the cell type: store-operated Ca^{2+} entry was necessary for maintaining Ca^{2+} oscillations in HEK293 cells (Bird and Putney, 2005; Wedel et al., 2007) while it was not essential for the repetitive Ca^{2+} signal in *C. elegans* (Yan et al., 2006). In addition, a recent study indicated that Ca^{2+} influx through Ca^{2+} release-activated Ca^{2+} channels, a major channel for store-operated Ca^{2+} entry, stimulated gene expression (Chang et al., 2008). This was confirmed in another study where a spatially localized Ca^{2+} increase through Ca^{2+} release-activated Ca^{2+} channels, but not a general Ca^{2+} rise was sufficient to activate gene expression (Di Capite et al., 2009) indicating that the Ca^{2+} influx stimulated by store depletion may regulate various cellular functions. Previously we have shown that store-operated Ca^{2+} entry in pig oocytes was mediated by STIM1 and the data presented here indicate that STIM1, probably by mediating store-operated Ca^{2+} entry, is also essential during fertilization to sustain the repetitive Ca^{2+} signal and to induce normal embryo development. However, although store-operated Ca^{2+} entry is the primary mechanism to replenish the intracellular stores during Ca^{2+} signaling, we cannot exclude the possibility that there might be additional mechanisms serving this purpose. Arachidonic acid-regulated Ca^{2+}-selective channels are independent of store depletion; instead, they are activated by arachidonic acid generated via agonist-induced receptor activation (Mignen and Shuttleworth, 2000). The channel has been identified in various cell types (Mignen et al., 2003) and recently the involvement of STIM1 in the regulation of arachidonic acid-regulated Ca^{2+}-channel activity has also been reported (Mignen et al., 2007). Suppression of STIM1 by siRNA blocked arachidonic acid-regulated Ca^{2+} channel activity, independent of Ca^{2+} release-activated Ca^{2+} channels or store depletion. Arachidonic acid-regulated Ca^{2+} channels have not yet been identified in oocytes but their potential presence and involvement in Ca^{2+} signaling at fertilization would be an interesting question to address. Non-selective cation channels such as transient receptor potential (TRP) channels may also be involved in Ca^{2+} influx during oscillations. STIM1 is reported to interact and activate TRP channels in HEK293 cells (Huang et al., 2006). Co-localization of STIM1 with TRP channels was demonstrated in such cells and co-immunoprecipitation showed physical interaction between the two molecules. The expression of TRP channels has previously been shown in porcine oocytes (Machaty et al., 2002). Although the role of TRP channels as genuine store-operated Ca^{2+} channels is under debate, it is possible that certain store-operated Ca^{2+} channels may be built of TRP proteins (Smyth et al., 2006). Finally, recent findings indicate the involvement of STIM1 in gating voltage-gated Ca^{2+} channels (Park et al., 2010; Wang et al., 2010). Such channels represent yet another type of membrane channels that may be controlled by STIM1. Thus determining the identity of Ca^{2+} entry channels that interact with STIM1 to sustain the repetitive Ca^{2+} signal is essential to fully understand the signaling mechanism that operates during fertilization. This is all the more important in light of a recent study which demonstrated that Ca^{2+} influx at fertilization not only sustains the repetitive Ca^{2+} signal but may also activate specific signaling pathways required for the extrusion of the second polar body (Miao et al., 2012).

In summary, the results of this study clearly indicate that STIM1 is essential for generating a Ca^{2+} influx after the mobilization of luminal Ca^{2+} at fertilization to refill the intracellular stores. Its function guarantees the maintenance of the repetitive Ca^{2+} signal that is required to induce proper embryo development. Further studies are under way to identify additional components of this signal transduction pathway and elucidate the regulatory mechanisms that operate during fertilization.

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Appendix A. Supporting information

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