Biochimica et Biophysica Acta 1843 (2014) 299–308

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamcr

Calcium signaling and the MAPK cascade are required for sperm activation in *Caenorhabditis elegans*

Zhiyu Liu [a,b,1], Bin Wang [a,2], Ruijun He [a], Yanmei Zhao [a], Long Miao [a,*]

[a] Laboratory of Noncoding RNA, Institute of Biophysics, Chinese Academy of Sciences, Beijing 100101, China
[b] University of Chinese Academy of Sciences, Beijing 100049, China

ARTICLE INFO

Article history:
Received 16 May 2013
Received in revised form 28 October 2013
Accepted 5 November 2013
Available online 13 November 2013

Keywords:
Sperm activation
Calcium
MAPK
Major Sperm Protein
C. elegans

ABSTRACT

In nematode, sperm activation (or spermiogenesis), a process in which the symmetric and non-motile spermatids transform into polarized and crawling spermatozoa, is critical for sperm cells to acquire fertilizing competence. SPE-8 dependent and SPE-8 independent pathways function redundantly during sperm activation in both males and hermaphrodites of *Caenorhabditis elegans*. However, the downstream signaling for both pathways remains unclear. Here we show that calcium signaling and the MAPK cascade are required for both SPE-8 dependent and SPE-8 independent sperm activation, implying that both pathways share common downstream signaling components during sperm activation. We demonstrate that activation of the MAPK cascade is sufficient to activate spermatids derived from either wild-type or *spe-8* group mutant males and that activation of the MAPK cascade bypasses the requirement of calcium signal to induce sperm activation, indicating that the MAPK cascade functions downstream of or parallel with the calcium signaling during sperm activation. Interestingly, the persistent activation of MAPK in activated spermatozoa inhibits Major Sperm Protein (MSP)-based cytoskeleton dynamics. We demonstrate that MAPK plays dual roles in promoting pseudopod extension during sperm activation but also blocking the MSP-based, amoeboid motility of the spermatozoa. Thus, though nematode sperm are crawling cells, morphologically distinct from flagellated sperm, and the molecular machinery for motility of amoeboid and flagellated sperm is different, both types of sperm might utilize conserved signaling pathways to modulate sperm maturation.

1. Introduction

In the nematode *Caenorhabditis elegans*, spermatids are generated in both males and self-fertile hermaphrodites [1]. Within hermaphrodites, the gonad initially generates sperm before switching over to the exclusive production of oocytes during larval to adult transition. The first ovulated oocyte pushes the stored spermatids into the spermatheca [2], where spermatids are exposed to the hermaphrodite-derived sperm activator and activated into spermatozoa rapidly [3]. The SPE-8 group proteins (including three transmembrane proteins SPE-12 [4], SPE-19 [5], and SPE-29 [6] and two cytoplasmic proteins SPE-8 and SPE-27 [7]) play a central role during hermaphrodite self-sperm activation. Mutations in *spe-8* group genes cause hermaphrodite sperm activation defect and therefore self-sterility. However, spermatids of *spe-8* group mutant hermaphrodites could be activated by male-derived TRY-5 (trypsin-like serine protease), a seminal fluid component secreted by male vas deferens, indicating that TRY-5-induced sperm activation is a SPE-8 pathway independent process [8]. The phenomenon that male-derived sperm activators activate *spe-8* group mutant hermaphrodite self-sperm is known as sperm trans-activation [9]. The time and place of male sperm activation are distinguished from those of hermaphrodite sperm activation [3]. Male sperm activation occurs after inseminated into hermaphrodite uterus and is mediated by TRY-5 in a SPE-8 independent manner [8]. During a typical cross, male spermatids are preferentially used to fertilize oocytes promoting outcross progeny. Spermatids can also be activated in vitro by various factors, including an ionophore (monensin) [10], proteases (Pronase and trypsin), a weak base (triethanolamine/TEA) [11] and Calmodulin inhibitors (TFP, CPZ and W7) [9]. However, the physiological relevance of these in vitro sperm activators remains uncertain.

Our previous studies have demonstrated that the labile zinc, enriched in both male and hermaphrodite reproductive tracts, has the capacity to induce sperm activation dependent on SPE-8 pathway [12]. The exogenous zinc induces the mobilization of intracellular zinc and the release of vesicular zinc into the extracellular space during sperm activation. The exogenously applied zinc or the exocytosed zinc from activating sperm, like the zinc released synaptically from the glutamatergic neurons of mammalian cerebral cortex [13], might enter the same or other sperm cells via voltage-gated zinc or calcium channels. This would

* Corresponding author at: Laboratory of Noncoding RNA, Institute of Biophysics, Chinese Academy of Sciences, Beijing 100101, China. Tel.: +86 10 64888425; fax: +86 10 64888478.
E-mail address: lmiao@moon.ibp.ac.cn (L. Miao).
[1] Current address: Department of Molecular Biology and Genetics, Cornell University, Ithaca, NY 14853, USA.
[2] Current address: Centre for Gene Regulation and Expression College of Life Sciences, University of Dundee, Dundee DD1 5EH, UK.

0167-4889/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.bbamcr.2013.11.001

then function as an autocrine or paracrine signal to trigger a chain of molecular events required for sperm activation. In the glutamatergic neurons, the elevation of zinc levels around the neuron terminals activates phospholipase C (PLC), and then the activated PLC cleaves the phosphatidyl inositol bisphosphate (PIP_2) generating the diacyl glycerol (DAG) and inositol 1,4,5 phosphate (IP_3) [13]. IP_3 binds to inositol 1,4,5-trisphosphate receptor (IP_3R) and triggers the release of calcium from endoplasmic reticulum (ER). The hydrophobic DAG is tethered to the inner leaflet of the plasma membrane and recruits protein kinase C (PKC). Recruited PKC is then activated by the increased calcium ions in the cytoplasm [14,15]. This results in a series of cellular responses by stimulation of various calcium-sensitive proteins for signal transduction [16]. The intracellular calcium signaling induced by the changes of extracellular zinc triggers the activation of MAPK and MEK/ERK-dependent activation of Na^+/H^+ exchanger, leading to cell proliferation [17–19]. ERK1/2 and p38 MAPK are identified as the regulators of human sperm motility and acrosome reaction [20,21]. Intracellular calcium signaling is also critical for nematode sperm activation [22] and the inhibition of Calmodulin, a calcium-binding messenger protein, directly triggers *C. elegans* sperm activation [9]. However, whether calcium signaling and the MAPK cascade are involved during zinc-induced sperm activation in nematode remains to be elucidated.

Here, we show that the activity of PLC and IP_3R regulating the intracellular calcium signaling is essential for *C. elegans* sperm activation. Furthermore, the activity of MEK/ERK is also required during sperm activation and the activation of JNK/p38 is sufficient to trigger sperm activation, bypassing the requirement of upstream signaling. Thus, though nematode sperm are morphologically distinct from flagellated sperm, both types of sperm might utilize evolutionarily conserved signaling cascades (such as the MAPK cascade and calcium signaling) during sperm motility acquisition. These observations indicate that some signaling pathways regulating sperm maturation appear to be broadly conserved though sperm cells from different species are under strong selection pressure during evolution.

2. Material and methods

2.1. Chemicals and reagents

The following reagents were used in this study: Pronase (Calbiochem); AEBSF, aprotinin, bestatin, E-64, and pepstatin A (Pierce Biotechnology); trypsin, U73343, U73122, 2-APB (2-aminoethoxydiphenyl borate), PD169316, FR180204, and Anisomycin (Sigma-Aldrich); U0126 (Invitrogen); and FM 1-43 (Molecular Probes). Stock solutions of 10 mM U73122 or U73343, 100 mM 2-APB, 50 mM U0126, 5 mM PD169316, 4 M AEBSF or PMSF, 1 M aprotinin, 1 M bestatin, 1 M E-64, 1 M pepstatin A, 200 mM Anisomycin and 50 mM FR180204 were stored at −80 °C. All stock solutions were prepared in dimethylsulfoxide (DMSO). Prior to treatment, aliquots of each drug or peptide were thawed at room temperature and diluted with sperm medium (SM) buffer (50 mM Hepes, 1 mM $MgSO_4$, 25 mM KCl, 45 mM NaCl, 5 mM $CaCl_2$, 10 mg/mL PVP, pH 7.0) or calcium-free SM buffer (50 mM Hepes, 25 mM KCl, 45 mM NaCl, 5 mM EGTA, 10 mg/mL PVP, pH 7.0). For the Control group, sperm cells were treated with the same final concentration of DMSO.

2.2. Worm strains

C. elegans strains were grown on nematode growth medium (NGM) plates at 20 °C according to standard protocols [23]. All strains were derived from Bristol N2 strains and obtained from the *Caenorhabditis* Genetics Center at the University of Minnesota. To increase the frequency of males, *him-5(e1490)* males were used as a source of male spermatids in our experiments. Strains used in this study were as follows: *spe-8(hc50)*I, *spe-8(hc53)*I, *spe-12(hc76)*I, *spe-27(it110)*IV, *spe-29(it127)*IV, and *him-5(e1490)*V.

2.3. Microscopy

Sperm were observed using differential interference contrast (DIC) microscopy. Samples were observed using an Axio Imager M2 (Carl Zeiss) equipped with a charge-coupled device (CCD) (Andor) under the control of MetaMorph software (Universal Imaging). Fluorescent imaging was obtained using a Leica HCS A confocal microscopy (Leica) controlled with LAS AF MATRIX M3 automation software. Images were processed using Image J and Photoshop (Adobe Systems).

2.4. In vitro sperm activation and inhibition assays

Virgin L4 males were placed in fresh NGM plates without hermaphrodites for 48–72 h at 20 °C. Spermatids were dissected from male gonads and released into a drop of SM buffer with or without activators (200 μg/mL Pronase, 1 mg/mL trypsin, 1 mM $ZnCl_2$ or $ZnSO_4$) at room temperature. Live sperm cells were maintained in chambers formed by mounting a 22 × 22-mm glass coverslip onto a glass slide with two parallel strips of two-sided sticky tape and examined with the Axio Imager M2 microscope (Carl Zeiss) equipped with a 100× DIC objective lens with appropriate filters. After 15 min exposure to the in vitro activators, sperm activation rate was scored based on the pseudopod extension. For AEBSF-induced sperm activation, spermatids were treated with 100 μM–20 mM AEBSF for 3 min as described above, and then the remnant AEBSF was washed out by perfusion with SM buffer lacking AEBSF.

For the inhibition of sperm activation, spermatids were bathed with various inhibitors at different concentrations in SM buffer for 10 min, then perfused with a mixture of inhibitor and activator, and finally scored for the rate of sperm activation. For each sperm inhibition assay, the toxicity of inhibitors was determined by rescuing the inhibitor-treated sperm with perfusion of activator-containing SM buffer.

2.5. MO fusion assay

To visualize the fusion of sperm specific membranous organelle (MO) with the plasma membrane, sperm cells were treated with 5 μg/mL FM 1-43 for 3 min. The lipophilic fluorescent dye FM 1-43, which partitions into the outer membrane leaflet of cells, can monitor the MO fusion during sperm activation. At the points of MO fusion, permanent membrane invaginations are generated, which allow FM 1-43 entering into the fused MO displaying a bright fluorescent puncta pattern at the rear edge of the cell body. For spermatids in which MO fusion did not occur, FM 1-43 distributed evenly on the plasma membrane of sperm cells. The images were captured using a confocal laser-scanning microscope (Leica).

2.6. Immunofluorescence

The antibody MAPK-YT (derived from rabbit, against the *dp*MPK-1 in *C. elegans* sperm, Sigma) was used at 1:100 dilution to detect the diphosphorylated MAPK using indirect immunofluorescence [24,25]. The MO was visualized with monoclonal antibody 1CB4 (derived from mouse, a gift from Steven W. L'Hernault, Emory University, Georgia) used at 1:200 dilution. To detect the ERK1/2 phosphorylation in spermatids or spermatozoa, we used the phospho-ERK1/2 (Y204, ImmunoWay) polyclonal antibody at 1:200 dilution.

Spermatids and spermatozoa were fixed with 4% paraformaldehyde and 0.01% glutaraldehyde in SM buffer at room temperature for 10 min. Fixed samples were permeabilized with 0.5% Triton X-100 in PBS. Blocked with 2% BSA in PBS at room temperature for 6 h, samples were incubated with the primary antibodies (diluted in PBS with 2% BSA) overnight at 4 °C. After being washed with PBS three times, samples were stained for 1 h with Alexa Fluor 488-conjugated goat anti-rabbit (for MAPK-YT antibody and phospho-ERK1/2 polyclonal antibody) or rhodamine-conjugated goat anti-mouse (for 1CB4 antibody)

secondary antibodies at room temperature. Images were obtained with a confocal laser scanning microscopy (Zeiss).

2.7. Statistical analysis

The results were presented as the mean ± s.e.m. For each sperm activation assay or inhibition assay, more than 10 males were dissected to obtain enough spermatids for independent experiments. For statistical analysis of sperm activation rate, more than 1000 spermatids or spermatozoa were counted. The data were expressed as the percentage of sperm with pseudopods and analyzed with GraphPad Prism software.

3. Results

3.1. Calcium signaling is required for zinc-, Pronase- or trypsin-induced sperm activation in C. elegans

Our previous studies have demonstrated that extracellular zinc at the micromolar level triggers sperm activation in a SPE-8 pathway dependent manner and that the labile zinc can be detected in the reproductive tracts of both male and hermaphrodite, positioning zinc as a physiological activator in *C. elegans* [12]. In mammalian cells, extracellular zinc ions have been demonstrated to induce the release of intracellular calcium and activation of the MAPK cascade during zinc-induced cell proliferation [14,15]. Genetic analyses in *C. elegans* also showed that the

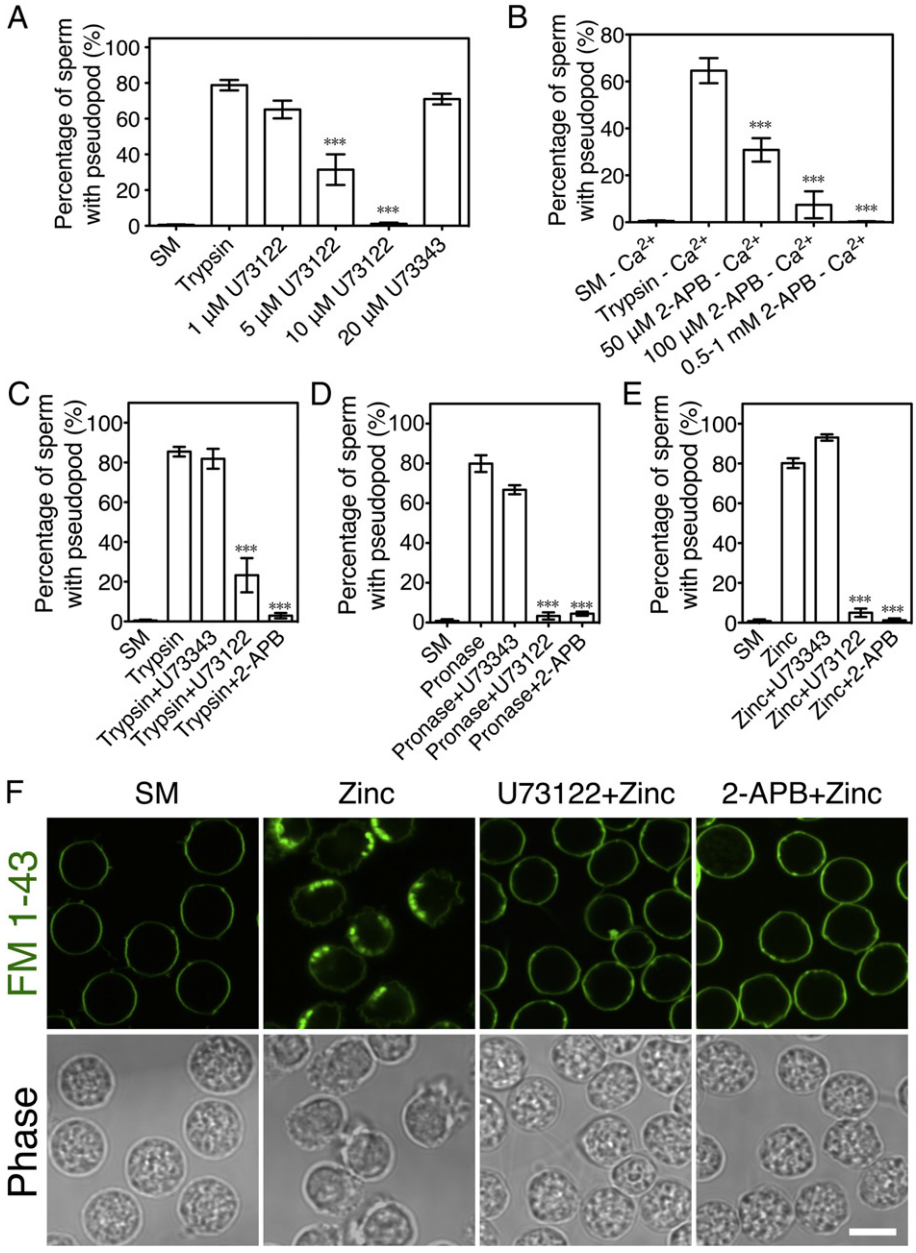

Fig. 1. Calcium signaling is involved in trypsin-, Pronase- or zinc-induced sperm activation. (A) U73122-treated spermatids fail to be activated by trypsin. SM: untreated spermatids maintained in SM buffer. Trypsin: sperm treated with 1 mg/mL trypsin. U73122: PLC inhibitor. U73343: inactive analog of U73122. (B) Trypsin fails to activate spermatids pretreated 2-APB, a selective IP_3R antagonist. - Ca^{2+}: Cells incubated in calcium-free SM buffer. (C) The effects of 10 μM U73343, 10 μM U73122 and 100 μM 2-APB on trypsin-induced sperm activation. (D) The inhibition of 10 μM U73122 and 100 μM 2-APB on Pronase-induced sperm activation. (E) The inhibition of 10 μM U73122 and 100 μM 2-APB on zinc-induced sperm activation. In panels A, B, C, D and E, results are presented as mean ± s.e.m. ***$P < 0.001$. (F) The inhibitory effects of 10 μM U73122 and 100 μM 2-APB on MO fusion-induced by zinc during sperm activation. The MO fusion is revealed by the fluorescent puncta of membrane probe FM 1-43. Phase: phase contrast images. Bar: 5 μm.

calcium-sensing protein FER-1 is required for calcium-mediated vesicle fusion with the plasma membrane during sperm activation [22]. To determine whether the intracellular calcium signaling is involved in sperm activation induced by various activators such Pronase, trypsin and zinc, we treated spermatids with a phospholipase (PLC) inhibitor (U73122) and an IP_3R antagonist (2-APB), both of which are known to dramatically disturb calcium signaling in human cells [14,26,27]. At the concentrations ranging from 1 to 10 μM, U73122 blocked trypsin-induced sperm activation in a dose-dependent manner (Fig. 1A). The IC_{50} of U73122 on trypsin-induced sperm activation was less than 5 μM, similar to its IC_{50} in human cells (at 0.2–7 μM [28,29]) and fruit fly spermatocytes (at 5 μM [30]). At the concentration of 10 μM U73122, sperm activation was significantly blocked (Fig. 1A and C). As a control, U73343, an inactive analog of U73122, had no effect on trypsin-induced sperm activation even at 20 μM (Fig. 1A and C). U73122 at 10 μM also significantly inhibited Pronase- and zinc-induced sperm activation (Fig. 1 D and E). We found that the inhibitory effects of U73122 on sperm activation could be rescued by perfusion of treated sperm with either $ZnCl_2$ (Supplementary material Fig. S1) or Pronase (data not shown) in SM buffer lacking U73122, excluding the toxicity of U73122 on sperm cells. In the typical calcium signaling, PLC cleaves the PIP_2 into DAG and IP_3. IP_3 is released as a soluble secondary messenger molecule into the cytoplasm and diffuses through the cytoplasm to bind to IP_3R, the calcium channel in smooth endoplasmic reticulum (ER). This causes the intracellular calcium release and a cascade of intracellular response. To determine whether IP_3R is required for *C. elegans* sperm activation, we checked the effect of 2-APB on trypsin-induced sperm activation. Considering that the nonspecific actions of 2-APB might exist and that its antagonistic effect is on the extracellular calcium entry instead of calcium release, we performed the inhibition experiments using a calcium-free SM buffer avoiding the extracellular calcium entry into sperm cells. Trypsin-induced sperm activation was significantly blocked by 100 μM to 1 mM 2-APB (Fig. 1B and C), approximately the same concentration to inhibit IP_3R in human cells (the IC_{50} for 2-APB as the IP_3R antagonist is 42 μM [31]). 2-APB at 100 μM also blocked the sperm activation induced by Pronase or zinc (Fig. 1D and E). Collectively, our experiment results indicate that calcium signaling is a shared downstream signaling in both zinc- and protease-induced sperm activation.

Another characteristic of sperm activation is the fusion of the sperm specific intracellular membranous organelle (MO) with the plasma membrane (termed MO fusion), a regulated exocytosis process critical for sperm motility and male fertility [32,33]. The lipophilic fluorescent dye FM 1-43, which partitions into the outer membrane leaflet of cells, can monitor the MO fusion during sperm activation. At the points of MO fusion, permanent membrane invaginations are generated, allowing FM 1-43 to enter the fused MO and stain the MO membrane leaflet resulting in a bright fluorescent puncta pattern at the rear edge of the cell body. For spermatids in which MO fusion did not occur, FM 1-43 distributed evenly on the sperm surface (Fig. 1F). Zinc stimulation led to the formation of clear fluorescent puncta at the rear edge in activated spermatozoa (Fig. 1F; [12]). FM 1-43 staining of sperm treated by zinc in the presence of calcium signaling inhibitors, including U73122 and 2-APB, showed that MO fusion was completely blocked (Fig. 1F). These results suggest that intracellular calcium signaling is an important event for MO fusion during zinc-induced sperm activation, which is consistent with previous genetic studies that showed intracellular calcium plays a critical role in MO fusion [22]. Collectively, intracellular calcium signaling is crucial for zinc-, Pronase- or trypsin-induced sperm activation, indicating that the in vivo sperm activators zinc and TRY-5 may share the same downstream IP_3-Ca^{2+} signaling for sperm activation.

3.2. The MAPK cascade is involved in the sperm activation of C. elegans

For mammalian cells, extracellular zinc stimulates the receptors on the plasma membrane resulting in a rise of the intracellular calcium, which further activates the downstream mitogen-activated protein kinase (MAPK) cascade linked to optimal activation, proliferation, and downstream gene expression [15,34]. Therefore, we examined whether MAPK activity is involved during sperm activation in *C. elegans*. U0126, a widely used MEK1/2 inhibitor, inhibited trypsin-induced sperm activation at concentrations ranging from 50 to 200 μM (50 μM < IC_{50} < 100 μM, Fig. 2A and C). U0126 at 200 μM also inhibited sperm activation induced by zinc and Pronase (Fig. 2B and C). U0126 also significantly blocked the zinc-induced MO fusion (Fig. 2E). To verify the effect of U0126 is specific on MEK/ERK and involvement of the MAPK cascade in sperm activation, we also checked the effect of PD169316 (a cell-permeable selective ERK kinase inhibitor with IC_{50} > 8.9 μM for human cells), FR180204 (a selective, cell permeable and ATP-competitive ERK and p38 inhibitor, IC_{50} = 0.51 μM for ERK1/2, IC_{50} = 10 μM for p38), SB203580 (a selective inhibitor of p38 MAPK) and PD98059 (a highly selective inhibitor of MEK1 activation and the MAP kinase cascade, IC_{50} values for inhibitory activity are around 5–10 μM) on in vitro sperm activation. We found that 25 μM PD169316 inhibited trypsin-, zinc- or Pronase-induced sperm activation (Fig. 2A, B and D) and blocked zinc-induced MO fusion (Fig. 2E). FR180204 also showed the inhibitory effect on in vitro sperm activation (Supplementary material Fig. S2), while SB203580 and PD98059 failed to inhibit the sperm activation induced by zinc-, trypsin- or Pronase (data not shown). These data suggest that the MAPK activity is indispensable during sperm activation in *C. elegans*.

3.3. The activation of JNK/p38 is sufficient to trigger sperm activation in C. elegans

The inhibitory effect of U0126, PD169316 and FR180204 on in vitro sperm activation indicates that the MAPK cascade is involved in *C. elegans* sperm activation. However, considering the fact that the off-target binding of these inhibitors might be present in *C. elegans* sperm, we also applied some MAPK cascade activators to trigger sperm activation to verify the role of the MAPK cascade during sperm activation. Strikingly, we found that treating the spermatids with 500 μM to 20 mM AEBSF, a JNK/p38 activator, is sufficient to induce sperm activation to generate pseudopod and MO fusion (Fig. 3A, C and Supplementary material Movie S1). AEBSF is also widely used as a serine protease inhibitor (commonly used at 0.1–1 mM in mammalian cells) with the same specificity as PMSF (commonly used at 0.1–1 mM in mammalian cells). To determine that AEBSF works as a JNK/p38 activator for sperm activation, instead of protease inhibitor, we tested the capacity of other widely used protease inhibitors, including PMSF, aprotinin (commonly used at 1 mM in mammalian cells), E-64 (commonly used at 10–100 μM in mammalian cells), pepstatin A (commonly used at 1 μM in mammalian cells) and bestatin (commonly used at 27 μM M in mammalian cells). No sperm activation was observed after treatment with PMSF at concentrations ranging from 1 to 20 mM (Fig. 2A) or other protease inhibitors (Supplementary material Fig. S3), indicating that AEBSF functions as a JNK/p38 agonist, instead of a protease inhibitor to trigger sperm activation in *C. elegans*. To confirm the role of JNK/p38 in sperm activation, we also checked the effect of another JNK/p38 agonist, Anisomycin, which has been well documented to strongly activate the stress kinases SAPK/JNK and p38 MAPK. At concentrations above 1 mM, Anisomycin alone also triggered spermatids to extend pseudopods and induced MO fusion (Fig. 3B and C), similar to the effect of AEBSF on sperm activation. Collectively, these data indicate that the MAPK cascade is necessary for sperm activation and that the activation of JNK/p38 is sufficient to activate sperm without additional input from upstream signals.

3.4. JNK/p38 plays dual functional roles in promoting sperm activation and blocking sperm motility

Though AEBSF alone was sufficient to trigger pseudopod extension, the continuous presence of AEBSF caused the formation of abnormal-

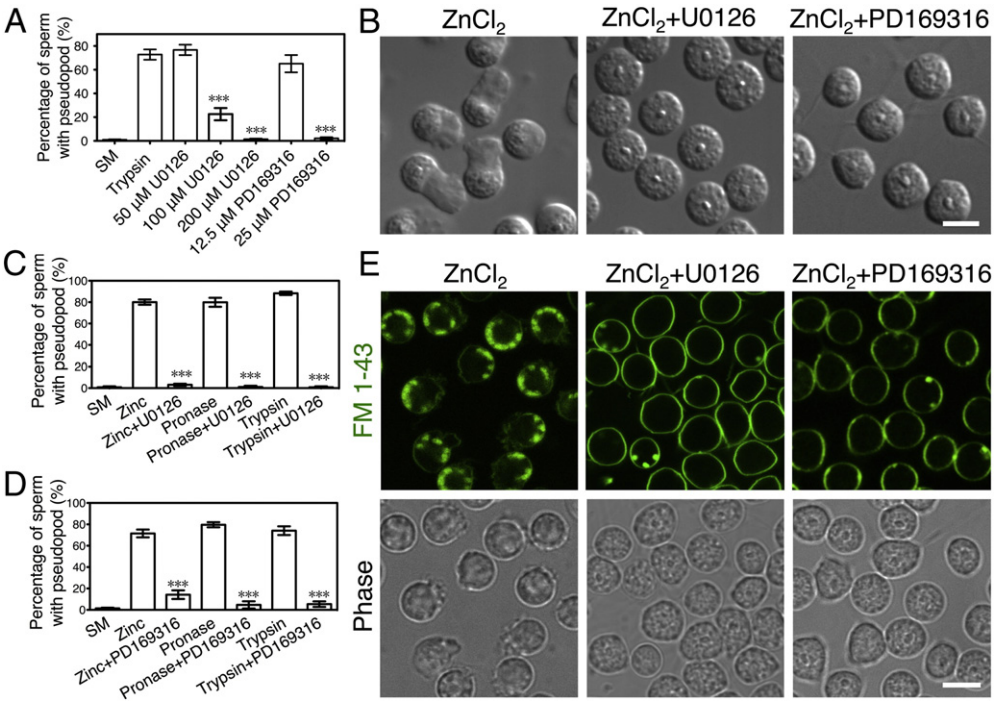

Fig. 2. The MEK/ERK activity is required for *C. elegans* sperm activation. (A) Blocking the activities of MEK1/2 and ERK causes trypsin-induced sperm activation failure. SM: untreated spermatids maintained in SM buffer. Trypsin: sperm treated with 1 mg/mL trypsin. U0126: MEK1/2 inhibitor. PD169316: ERK inhibitor. (B) The MEK/ERK activity is required for zinc-induced sperm activation. Sperm pretreated with 200 μM U0126 or 25 μM PD169316 fail to respond to zinc to extend pseudopods, while sperm treated with zinc alone protrude pseudopods to form spermatozoa. (C) Quantification of the inhibitory effect of 200 μM U0126 on zinc, Pronase or trypsin-induced sperm activation. (D) Quantification of the inhibitory effect of 25 μM PD169316 on zinc, Pronase or trypsin-induced sperm activation. In A, C and D, results are presented as mean ± s.e.m. ***$P < 0.001$. (E) U0126 and PD169316 inhibit the MO fusion induced by zinc. Bars in B and E: 5 μm.

looking pseudopod displaying less dynamics of MSP cytoskeleton (Fig. 3D and Supplementary material Movie S1). To obtain a normal-looking pseudopod carrying spermatozoa, the AEBSF-treated sperm cells (treated with 1–20 mM AEBSF for no more than 3 min) were subsequently perfused with SM buffer to wash away the remnant AEBSF. The abnormal-looking pseudopods began to show the MSP-based cytoskeletal dynamics and therefore the normal-looking, motile pseudopods were formed (Fig. 3D). If the functional pseudopod of trypsin, Pronase or zinc-induced spermatozoa were treated with 1 to 20 mM AEBSF, then the normal-looking pseudopod immediately disassembled and retracted back to the cell body (data not shown). During the process of sperm activation induced by Anisomycin, the treated sperm also exhibited the similar abnormal-looking pseudopods as AEBSF-treated sperm (Fig. 3E and Supplementary material Movie S2). Hence, JNK/p38 plays dual roles during sperm maturation in *C. elegans*, in which the activation of JNK/p38 is sufficient to induce sperm activation, while the deactivation of JNK/p38 is essential for the dynamics of MSP cytoskeleton. Therefore, the temporal and spatial regulation of JNK/p38 activity is linked with both sperm activation and MSP-based cell migration.

3.5. The calcium signaling and SPE-8 pathway are not necessary for the AEBSF-induced sperm activation

To determine whether the intracellular calcium signaling is required for AEBSF-induced sperm activation, we examined the effect of U73122 and 2-APB on AEBSF-induced sperm activation. Spermatids pretreated with 10 μM U73122 or 100 μM 2-APB could be activated by 1 mM AEBSF (Fig. 4A), indicating that the calcium signaling is not necessary for AEBSF-induced sperm activation. However, pretreated with 200 μM U0126, spermatids just protruded spike-like structures, the activation intermediates [2], which are distinguished from the functional pseudopods (Fig. 4A). These data suggest that MEK and JNK/p38 must cooperate to complete the whole processes of sperm activation, including spike protrusion and pseudopod extension. We propose that the activation of JNK/p38 initiated the spike formation and the activity of MEK is required for the formation of functional pseudopod.

SPE-8 group proteins are essential for self-sperm activation and fertility in hermaphrodites. Our previous studies have shown that the labile zinc is enriched in the reproductive tract of both male and hermaphrodites as an endogenous sperm activator and that zinc-induced sperm activation is dependent on SPE-8 pathway [12]. To verify the role of SPE-8 group proteins in AEBSF-induced sperm activation, we treated the *spe-8(hc50)*, *spe-8(hc53)*, *spe-12(hc76)*, *spe-27(it110)* and *spe-29(it127)* male spermatids with AEBSF and found that AEBSF activated *spe-8* group spermatids with an activation rate of about 60% (Fig. 4B and C). Together, these data suggest that the activation of JNK/p38 bypasses the requirements of upstream SPE-8 signaling cascade to initiate sperm activation.

3.6. The phosphorylation level of ERK and MPK-1 is elevated in C. elegans spermatozoa

Our pharmacological studies as shown above indicate that intracellular calcium signaling and the MAPK cascade are involved during *C. elegans* sperm activation. However, we are cautious about the limitation of our inhibitor assays because these drugs are well characterized in the context of mammalian proteins. The off-target binding to other cellular components in nematode sperm might exist to cause sperm activation failure. U0126 has been used to promote the sperm fate during hermaphrodite sperm/oocyte decision (at the concentrations ranging from 20 μM to 100 μM), inhibiting the Ras-ERK signaling of the *C. elegans* hermaphrodite germ line [35], confirming its specificity in *C. elegans*. To further substantiate the significance of the MAPK cascade during sperm activation, we examined these proteins in *C. elegans* sperm by the immunofluorescence staining. We screened dozens of antibodies against the human homologs of *C. elegans* proteins and found that the phosphorylation of ERK (detected with the phospho-ERK1/2

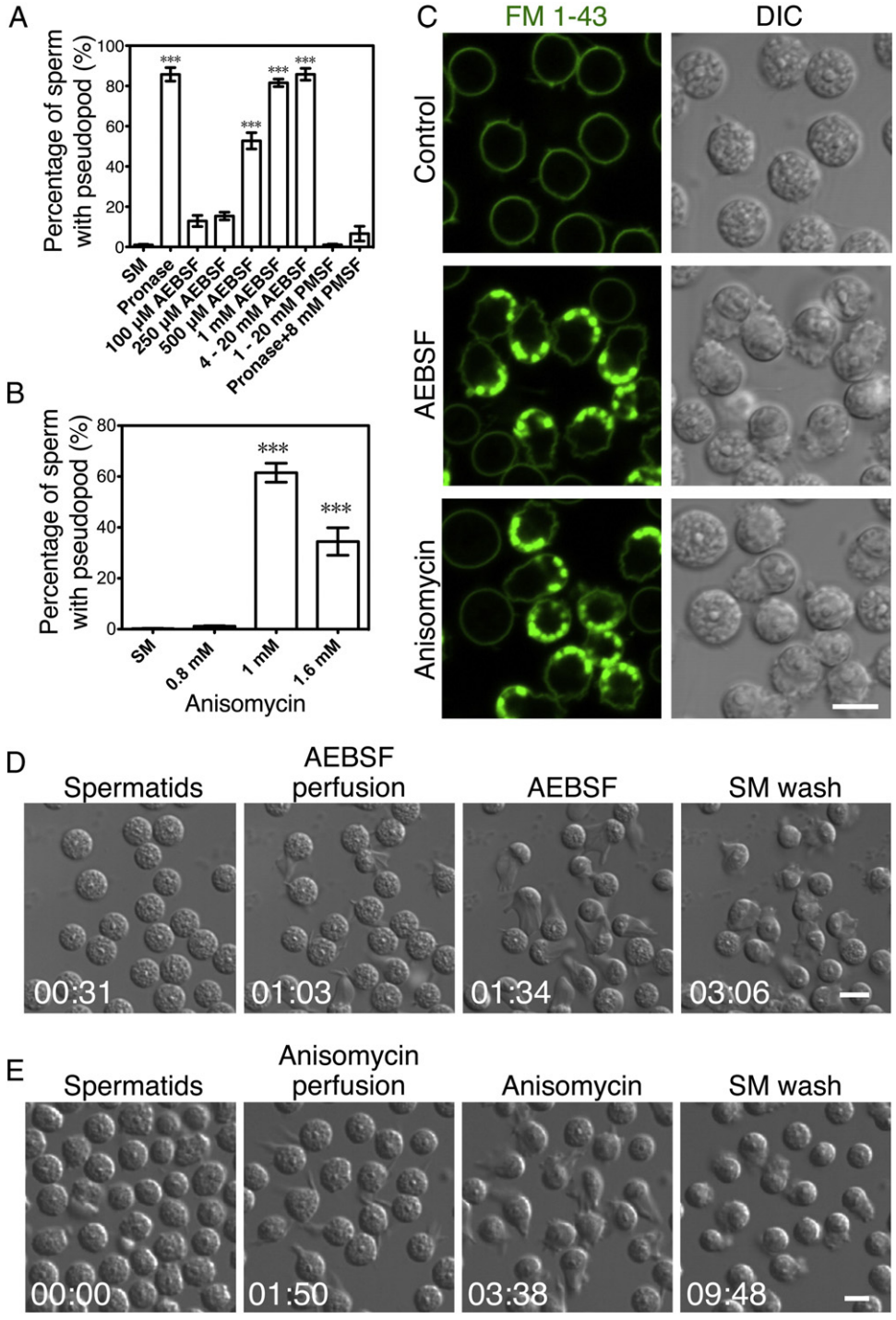

Fig. 3. The activation of JNK/p38 is sufficient to initiate sperm activation. (A) Quantification of AEBSF or PMSF-induced sperm activation. Spermatids are treated with AEBSF or PMSF for 3 min, and then perfused with SM buffer to remove AEBSF or PMSF. AEBSF: JNK/p38 activator. PMSF: Protease inhibitor. (B) Quantification of Anisomycin-induced sperm activation. Spermatids are treated with Anisomycin for 3 min, and then perfused with SM buffer to remove the applied Anisomycin. Anisomycin: JNK/p38 agonist. In A and B, sperm cells establishing the polarities with clear pseudopods are scored as activated spermatozoa. Results are presented as mean ± s.e.m. ***$P < 0.001$. (C) FM 1-43 staining and DIC micrographs show that 1 mM AEBSF or 1 mM Anisomycin triggers sperm to extend pseudopods (DIC) and causes MO fusion with the plasma membrane (green fluorescent puncta). Control: untreated spermatids. (D) Serial still images of sperm cells perfused with 4 mM AEBSF and then washed with SM buffer. The time points indicate the relative time in consistent with the time labeled in the Supplemental Material Movie S1. (E) DIC micrographs show the process of *C. elegans* sperm activation triggered by 1 mM Anisomycin and then perfused with SM buffer (See the Supplemental Material Movie S2). Bars in C, D and E: 5 μm.

(Y204) polyclonal antibody, Fig. 5A) and MPK-1 (detected with the antibody MAPK-YT, against the *dp*MPK-1 in *C. elegans* sperm [24,25], Fig. 5C) were obviously elevated in the activated spermatozoa as compared with the non-activated spermatids (Fig. 5B and D). An interesting phenomenon is that the phosphorylated ERK and MPK-1 are localized around the plasma membrane of the pseudopod. The level of phosphorylated ERK and MPK-1 is much lower in the cytoplasm, especially for *dp*MPK-1. However, we did not detect an increase of the phosphorylation level of MEK, JNK and p38 in the spermatozoa (data not shown).

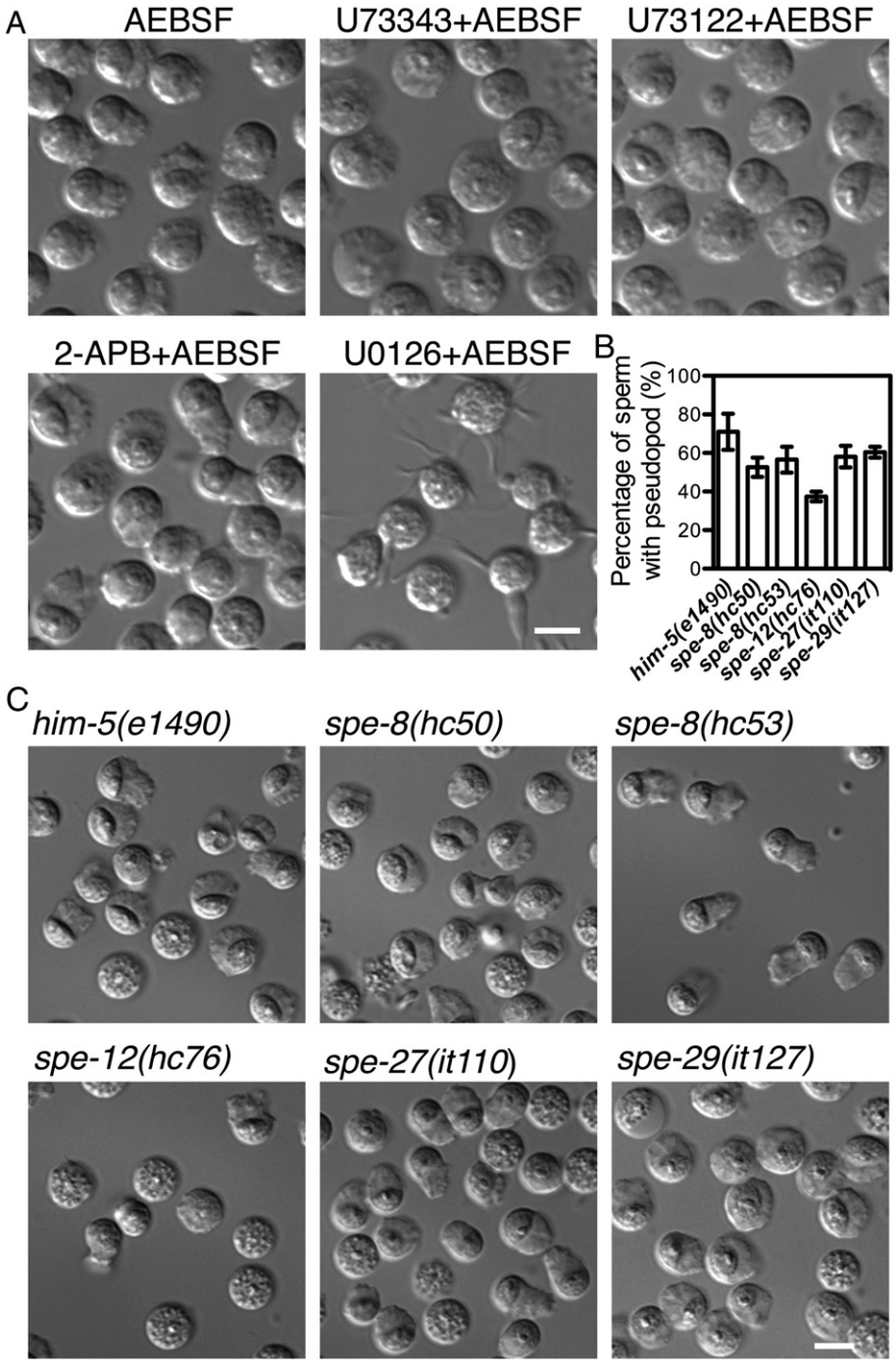

Fig. 4. Intracellular calcium signaling and SPE-8 group proteins are not necessary for AEBSF-induced sperm activation. (A) The effects of 10 μM U73122, 100 μM 2-APB and 200 μM U0126 on AEBSF-induced sperm activation. Spermatids are pretreated with 10 μM U73122, 100 μM 2-APB or 200 μM U0126 for 10 min, and then the treated sperm are perfused with 4 mM AEBSF in the presence of 10 μM U73122, 100 μM 2-APB or 200 μM U0126, respectively. After treated with the mixture for 3 min, sperm cells are perfused with SM buffer ensuring the formation of normal-looking pseudopod. U73122 and 2-APB do not inhibit the sperm activation induced by AEBSF, while U0126 partially inhibits the AEBSF-induced sperm activation. (B) Quantification of AEBSF-induced activation of sperm derived from *spe-8*, *spe-12*, *spe-27* and *spe-29* males. Results are presented as mean ± s.e.m. (C) AEBSF triggers the activation of sperm from *spe-8* group mutant males. Bars in A and C: 5 μm.

4. Discussion

We have demonstrated that the calcium signaling and the MAPK cascade are required during *C. elegans* sperm activation, the process by which nematode sperm cells acquire cellular polarity and motility. Based on our pharmacological and genetic analyses, we propose that the various in vitro sperm activators share a common calcium signaling and that the MAPK works downstream of calcium signaling and SPE-8 pathway. By immunofluorescence microscopy, we have observed that the phosphorylation level of ERK and MPK-1 is increased and phosphorylated ERK and MPK-1 are accumulated around the plasma membrane of the pseudopod.

Previous molecular genetic studies show that *C. elegans* sperm activation is regulated in a sex-specific manner [3,36]. Our recent studies demonstrate that the labile zinc, enriched in the reproductive tract of both males and hermaphrodites, initiates sperm activation in a SPE-8 dependent manner [12]. Males, however, have another activator, TRY-5, a trypsin-like serine protease secreted from the vas deferens during

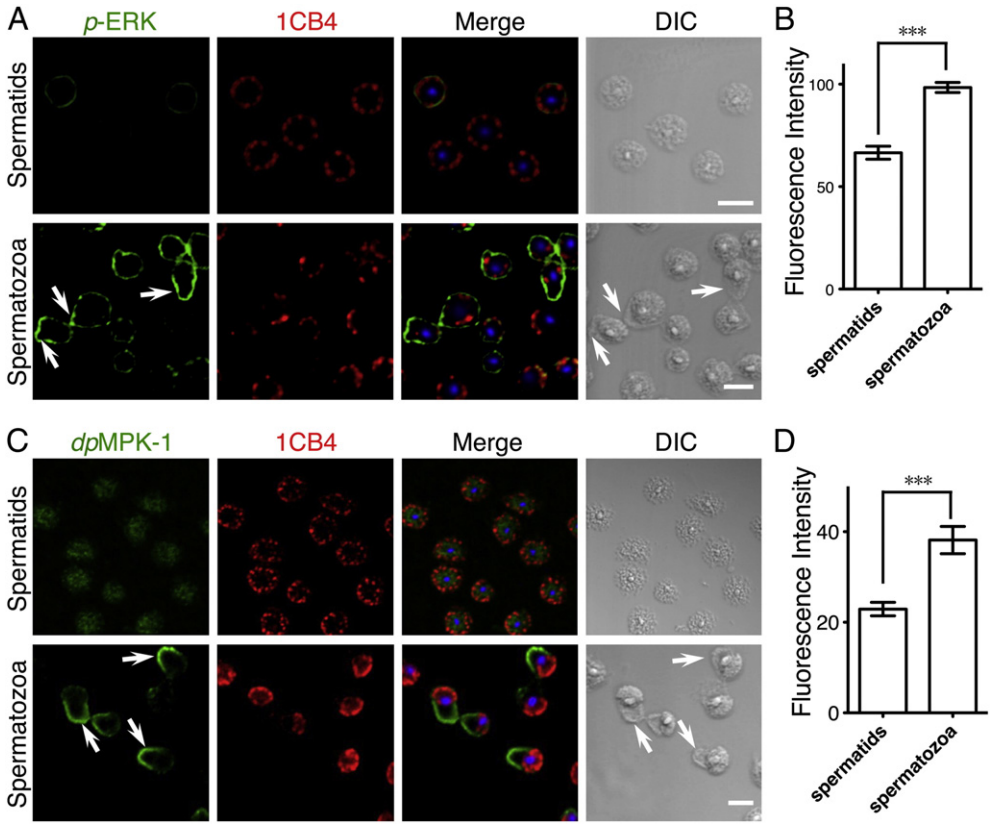

Fig. 5. The phosphorylation of ERK and MPK-1 is elevated in spermatozoa. (A, C) Immunostaining of spermatids or Pronase-activated spermatozoa with phospho-ERK1/2 (Y204) polyclonal antibody or MAPK-YT antibody. The antibody 1CB4 (red) labels the sperm specific organelles (MOs). Arrows indicate visible pseudopod. Bars in A and C, 5 μm. (B, D) Fluorescence intensity is quantified between spermatids and spermatozoa for *p*-ERK (B) and *dp*MPK-1 (D). Results are presented as mean ± s.e.m. ***P < 0.001.

copulation [8]. Ejaculated TRY-5 activates male sperm rapidly via a SPE-8 independent pathway in the hermaphrodite uterus, functioning redundantly with the labile zinc to guarantee the immediate activation of male sperm, avoiding male sperm being swept from the reproductive tract as laying eggs pass by. TRY-5 also trans-activates the spermatids from *spe-8* group hermaphrodite mutants [8]. Our data demonstrate that though the activators (the labile zinc ions and TRY-5) initiate the sperm activation using different molecular and genetic pathways, both pathways lead to a common downstream signaling cascade including intracellular calcium signaling and the MAPK cascade. Our discovery of calcium signaling and the MAPK cascade provides a mechanistic explanation for previous results linking extracellular sperm activators with sperm activation in *C. elegans*. Taken together, these data suggest a model where the extracellular activators initiate a signaling cascade including calcium signaling and MAPK pathway to stimulate sperm activation and motility acquisition (Fig. 6).

For flagellated sperm, calcium modulates various steps of sperm maturation and sperm–oocyte fertilization, including sperm capacitation, hyperactivation, chemotaxis, acrosome reaction and sperm–oocyte recognition [37–39]. The function of calcium during *C. elegans* sperm activation and sperm–oocyte recognition and fusion is also complicated [40]. Chelating extracellular calcium with EDTA or EGTA does not affect sperm activation, suggesting that calcium from the extracellular environment may not be necessary [10]. By contrast, intracellular calcium depletion with BAPTA-AM (a cell membrane permeable calcium chelator) results in the MO fusion defect, which indicates that intracellular calcium is necessary for MO fusion during sperm activation [22]. FER-1, a ferlin family protein that has calcium-sensing C2 domains mediating calcium-assisted events, responds to the intracellular calcium signaling and facilitates the fusion of MOs with the plasma membrane [22]. Two types of calcium channels, including constitutively active calcium-permeable channel (CAC) and store-operated channel (SOC) have been implicated in the changes of intracellular calcium level in *C. elegans* sperm [41–43]. In mature spermatozoa, the SOC activity is mediated by TRP-3/SPE-41 channel. However, spermatids exhibit a much lower SOC activity than that in mature sperm and very little CAC activity was found in spermatids. The calreticulin (named CRT-1 in *C. elegans*), a calcium binding molecular chaperone, is necessary for fertility and is detected in *C. elegans* sperm [44]. In vitro activated *crt-1* mutant spermatozoa display shorter pseudopods than wild-type spermatozoa. In addition, another calcium binding protein, Calmodulin, is also involved in sperm activation [9]. Various Calmodulin inhibitors, such as trifluoperazine (TFP), chlorpromazine (CPZ) and naphthalenesulfonamide (W7), initiate sperm activation in vitro [9], indicating that Calmodulin likely functions as an inhibitory factor for sperm activation. However, the complete transformation from spermatids to spermatozoa needs these chemicals to be washed away following the initial treatment, indicating that those drugs induce sperm activation normally, but then block the pseudopod motility [9]. These effects of Calmodulin inhibitors on sperm cells are similar to those results from treatment with JNK/p38 activators (AEBSF and Anisomycin) used in our experiments. One of the downstream targets of calcium/Calmodulin is calcineurin (a Ser/Thr phosphatase), whose mutant sperm displays a smaller pseudopod in size [45]. All those observations suggest the possible role of calcium in the initial onset of sperm activation and the acquisition of sperm motility [46]. Although intracellular calcium is necessary during sperm activation, it is not sufficient to initiate sperm activation by artificial elevation of the intracellular calcium by treating spermatids with calcium ionophores [10]. Here, our findings that PLC inhibitor and IP_3R inhibitor block sperm activation in vitro partially illustrate the signaling pathway responding to intracellular calcium-dependent sperm activation.

The involvement of intracellular calcium in sperm activation raises the question of how calcium homeostasis in *C. elegans* sperm cells is

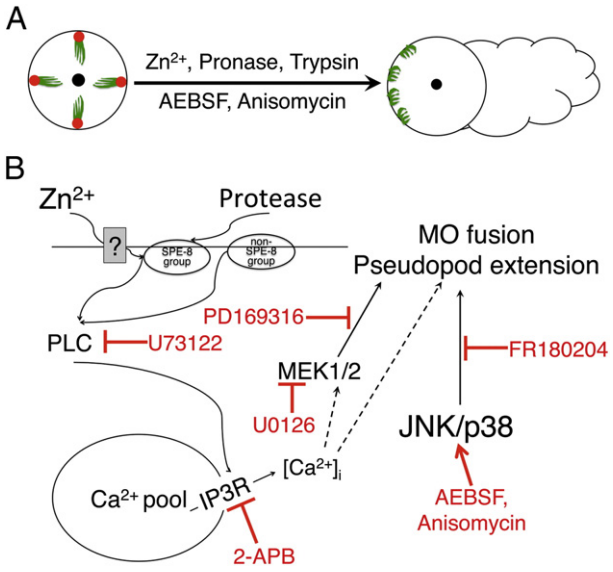

Fig. 6. A proposed model for the calcium signaling and the MAPK cascade during sperm activation in *C. elegans*. (A) Zinc, Pronase, trypsin, AEBSF and Anisomycin are able to trigger sperm activation, which is characterized with pseudopod extension and MO fusion with the plasma membrane. Red: the head of MO underneath the plasma membrane of spermatid. Green: the tail of MO. (B) Calcium signaling and the MAPK cascade are involved in sperm activation. Zinc and proteases (Pronase and trypsin) are dependent on SPE-8 pathway to trigger sperm activation. PLC activity is required for both zinc- or protease-induced sperm activation. PLC hydrolyzes PIP_2 to form the second messengers IP_3 and DAG. IP_3 binds the IP_3R releasing calcium from the calcium pool and elevating the cytoplasmic calcium level. The increment of intracellular calcium activates the MAPK cascade to complete the whole process of sperm activation. AEBSF or Anisomycin activates JNK/p38 and triggers sperm activation directly, independent of SPE-8 pathway and/or intracellular calcium increment. Red letter: pharmacological agents (U73122, U0126, 2-APB, PD169316, FR180204, AEBSF and Anisomycin) used in this study. Red line with arrow head represents activation while red lines with bar heads represent inhibition. Black solid lines with arrowheads: the confirmed signal pathways. Black dash lines with arrowheads: the postulated signal pathways.

regulated. As terminally differentiated cells, *C. elegans* sperm have jettisoned most cellular components such as actin, tubulin and ribosome into an anucleate residual body during sperm development [2]. Considering that the highly specific sperm lack endoplasmic reticulum (ER), it is not likely that the calcium store is maintained in the ER like in most eukaryotic cells. Given that the nematode sperm-specific organelles, MO, are derived from RE/Golgi and related to lysosome [47], it is likely that MOs function as the intracellular calcium stores during sperm development. Consistent with this idea, we observed the accumulation of calcium around some of the MOs in spermatids and spermatozoa using the cell permeable calcium probe Fluo 4-AM (data not shown).

During the production of flagellated sperm, MAPK cascades are not only involved in the regulation of transcription and germ cell specialization in testis, but also regulate mature sperm motility, hyperactivation and acrosome reaction [20]. It is reported that ERK and p38 act as the downstream of PKC in flagellated sperm motility. PKC activators, PMA and OAG, activate ERK and stimulate sperm motility and hyperactivation [21]. The specific MEK1/2 inhibitors U0126 and PD98059 decrease both motility and hyperactivation during flagellated sperm maturation. Additionally, the p38 inhibitors, SB203580 and PD169316, increase sperm motility, suggesting that p38 is a negative regulator of sperm motility [21]. For nematode species, spermatozoa are lacking flagella displaying classic features of amoeboid motility with pseudopods, powered by the dynamics of the cytoskeleton composed of Major Sperm Protein (MSP) rather than conventional actin. It is proposed that the assembly of the MSP cytoskeleton is regulated by the phosphorylation and de-phosphorylation circles of cytoskeletal accessory proteins at the leading edge of the pseudopod in nematode spermatozoa [48,49]. Consistent with previous hypotheses, our studies showed that the phosphorylation of ERK and MPK-1 is specifically localized at the leading edge of the pseudopod.

GPR39, a G protein-coupling receptor functioning as a zinc-sensing receptor, has been identified for zinc-triggered epithelial repair [50,51]. GPR39 responds to the increase of extracellular zinc levels by triggering the release of intracellular calcium and ERK-dependent activation of Na^+/H^+ exchanger [15]. The zinc-induced sperm activation in *C. elegans* also requires the intracellular calcium-activated MEK signaling pathway, indicating that a GPR39-like receptor might exist on the sperm surface and serve as a mediator for signal transduction during sperm activation in vivo.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamcr.2013.11.001.

Conflict of interest

The authors declare no competing financial interests.

Acknowledgments

We are grateful to Dr. Layne Zhao for editing the manuscript. This research was supported by grants from the National Basic Research Program of China [2012CB945002 and 2010CB912303 to L.M.] and by the National Natural Science Foundation of China [30971648 and 31171337 to L.M., 31000597 to B.W., and 31071180 and 31371357 to Y.Z.]. L.M. was supported by the Chinese Academy of Sciences 100-Talents Program.

Reference

[1] H. Nishimura, S.W. L'Hernault, Spermatogenesis-defective (*spe*) mutants of the nematode *Caenorhabditis elegans* provide clues to solve the puzzle of male germline functions during reproduction, Dev. Dynam. 239 (2010) 1502–1514.
[2] S.W. L'Hernault, The genetics and cell biology of spermatogenesis in the nematode *C. elegans*, Mol. Cell. Endocrinol. 306 (2009) 59–65.
[3] A. Singson, Sperm activation: time and tide wait for no sperm, Curr. Biol. 16 (2006) R160–R162.
[4] J. Nance, A.N. Minniti, C. Sadler, S. Ward, *spe-12* encodes a sperm cell surface protein that promotes spermiogenesis in *Caenorhabditis elegans*, Genetics 152 (1999) 209–220.
[5] B. Geldziler, I. Chatterjee, A. Singson, The genetic and molecular analysis of *spe-19*, a gene required for sperm activation in *Caenorhabditis elegans*, Dev. Biol. 283 (2005) 424–436.
[6] J. Nance, E.B. Davis, S. Ward, *spe-29* encodes a small predicted membrane protein required for the initiation of sperm activation in *Caenorhabditis elegans*, Genetics 156 (2000) 1623–1633.
[7] A.N. Minniti, C. Sadler, S. Ward, Genetic and molecular analysis of *spe-27*, a gene required for spermiogenesis in *Caenorhabditis elegans* hermaphrodites, Genetics 143 (1996) 213–223.
[8] J.R. Smith, G.M. Stanfield, TRY-5 is a sperm-activating protease in *Caenorhabditis elegans* seminal fluid, Plos Genet. 7 (2011).
[9] D.C. Shakes, S. Ward, Initiation of spermiogenesis in *C. elegans*: a pharmacological and genetic analysis, Dev. Biol. 134 (1989) 189–200.
[10] G. Nelson, S. Ward, Vesicle fusion, pseudopod extension and amoeboid motility are induced in nematode spermatids by the ionophore monensin, Cell 19 (1980) 457–464.
[11] S. Ward, E. Hogan, G.A. Nelson, The initiation of spermiogenesis in the nematode *Caenorhabditis elegans*, Dev. Biol. 98 (1983) 70–79.
[12] Z. Liu, L. Chen, Y. Shang, P. Huang, L. Miao, The micronutrient element zinc modulates sperm activation through the SPE-8 pathway in *Caenorhabditis elegans*, Development 140 (2013) 2103–2107.
[13] E.H. Pan, X.A. Zhang, Z. Huang, A. Krezel, M. Zhao, C.E. Tinberg, S.J. Lippard, J.O. McNamara, Vesicular zinc promotes presynaptic and inhibits postsynaptic long-term potentiation of mossy fiber-CA3 synapse, Neuron 71 (2011) 1116–1126.
[14] M. Hershfinkel, A. Moran, N. Grossman, I. Sekler, A zinc-sensing receptor triggers the release of intracellular Ca^{2+} and regulates ion transport, Proc. Natl. Acad. Sci. U. S. A. 98 (2001) 11749–11754.
[15] H. Azriel-Tamir, H. Sharir, B. Schwartz, M. Hershfinkel, Extracellular zinc triggers ERK-dependent activation of Na^+/H^+ exchange in colonocytes mediated by the zinc-sensing receptor, J. Biol. Chem. 279 (2004) 51804–51816.
[16] S.Q. Wang, M.X. Zhu, E. Carafoli, Ca^{2+}: a versatile master key for intracellular signaling cascades, Sci. China Life Sci. 54 (2011) 683–685.
[17] M. Hershfinkel, W.F. Silverman, I. Sekler, The zinc sensing receptor, a link between zinc and cell signaling, Mol. Med. 13 (2007) 331–336.
[18] A.M. Adamo, M.P. Zago, G.G. Mackenzie, L. Aimo, C.L. Keen, A. Keenan, P.I. Oteiza, The role of zinc in the modulation of neuronal proliferation and apoptosis, Neurotox. Res. 17 (2010) 1–14.

[19] J.R. Nuttall, P.I. Oteiza, Zinc and the ERK kinases in the developing brain, Neurotox. Res. 21 (2012) 128–141.
[20] T. Almog, Z. Naor, The role of mitogen activated protein kinase (MAPK) in sperm functions, Mol. Cell. Endocrinol. 314 (2010) 239–243.
[21] T. Almog, S. Lazar, N. Reiss, N. Etkovitz, E. Milch, N. Rahamim, M. Dobkin-Bekman, R. Rotem, M. Kalina, J. Ramon, A. Raziel, H. Brietbart, R. Seger, Z. Naor, Identification of extracellular signal-regulated kinase 1/2 and p38 MAPK as regulators of human sperm motility and acrosome reaction and as predictors of poor spermatozoan quality, J. Biol. Chem. 283 (2008) 14479–14489.
[22] N.L. Washington, S. Ward, FER-1 regulates Ca^{2+}-mediated membrane fusion during *C. elegans* spermatogenesis, J. Cell Sci. 119 (2006) 2552–2562.
[23] S. Brenner, The genetics of *Caenorhabditis elegans*, Genetics 77 (1974) 71–94.
[24] S. Arur, M. Ohmachi, S. Nayak, M. Hayes, A. Miranda, A. Hay, A. Golden, T. Schedl, Multiple ERK substrates execute single biological processes in *Caenorhabditis elegans* germ-line development, Proc. Natl. Acad. Sci. U. S. A. 106 (2009) 4776–4781.
[25] M.A. Miller, V.Q. Nguyen, M.H. Lee, M. Kosinski, T. Schedl, R.M. Caprioli, D. Greenstein, A sperm cytoskeletal protein that signals oocyte meiotic maturation and ovulation, Science 291 (2001) 2144–2147.
[26] C. Dettbarn, P. Palade, Effects of three sarcoplasmic/endoplasmic reticulum Ca^{2+} pump inhibitors on release channels of intracellular stores, J. Pharmacol. Exp. Ther. 285 (1998) 739–745.
[27] W. Maret, Crosstalk of the group IIa and IIb metals calcium and zinc in cellular signaling, Proc. Natl. Acad. Sci. U. S. A. 98 (2001) 12325–12327.
[28] A. Zapf-Colby, J. Eichhorn, N.J.G. Webster, J.M. Olefsky, Inhibition of PLC-gamma 1 activity converts nerve growth factor from an anti-mitogenic to a mitogenic signal in CHO cells, Oncogene 18 (1999) 4908–4919.
[29] S.A. Saeed, H. Rasheed, F.A.W. Fecto, M.I. Achakzai, R. Ali, J.D. Connor, A.U.H. Gilani, Signaling mechanisms mediated by G-protein coupled receptors in human platelets, Acta Pharmacol. Sin. 25 (2004) 887–892.
[30] R. Wong, I. Hadjiyanni, H.C. Wei, G. Polevoy, R. McBride, K.P. Sem, J.A. Brill, PIP_2 hydrolysis and calcium release are required for cytokinesis in *Drosophila* spermatocytes, Curr. Biol. 15 (2005) 1401–1406.
[31] A.M. Hagenston, N.D. Rudnick, C.E. Boone, M.F. Yeckel, 2-Aminoethoxydiphenyl-borate (2-APB) increases excitability in pyramidal neurons, Cell Calcium 45 (2009) 310–317.
[32] Y. Zhao, W. Sun, P. Zhang, H. Chi, M.J. Zhang, C.Q. Song, X. Ma, Y. Shang, B. Wang, Y. Hu, Z. Hao, A.F. Huhmer, F. Meng, S.W. L'Hernault, S.M. He, M.Q. Dong, L. Miao, Nematode sperm maturation triggered by protease involves sperm-secreted serine protease inhibitor (Serpin), Proc. Natl. Acad. Sci. U. S. A. 109 (2012) 1542–1547.
[33] E.L. Batchelder, G. Hollopeter, C. Campillo, X. Mezanges, E.M. Jorgensen, P. Nassoy, P. Sens, J. Plastino, Membrane tension regulates motility by controlling lamellipodium organization, Proc. Natl. Acad. Sci. U. S. A. 108 (2011) 11429–11434.
[34] K. He, E. Aizenman, ERK signaling leads to mitochondrial dysfunction in extracellular zinc-induced neurotoxicity, J. Neurochem. 114 (2010) 452–461.
[35] C.T. Morgan, M.H. Lee, J. Kimble, Chemical reprogramming of *Caenorhabditis elegans* germ cell fate, Nat. Chem. Biol. 6 (2010) 102–104.
[36] D.C. Shakes, For male *Caenorhabditis elegans*, sperm activation is a "just-in-time" event, Plos Genet. 7 (2011) e1002392.
[37] Y. Kirichok, B. Navarro, D.E. Clapham, Whole-cell patch-clamp measurements of spermatozoa reveal an alkaline-activated Ca^{2+} channel, Nature 439 (2006) 737–740.
[38] U.B. Kaupp, N.D. Kashikar, I. Weyand, Mechanisms of sperm chemotaxis, Annu. Rev. Physiol. 70 (2008) 93–117.
[39] M.E. Teves, H.A. Guidobaldi, D.R. Unates, R. Sanchez, W. Miska, S.J. Publicover, A.A.M. Garcia, L.C. Giojalas, Molecular mechanism for human sperm chemotaxis mediated by progesterone, Plos One 4 (2009).
[40] G. Singaravelu, A. Singson, Calcium signaling surrounding fertilization in the nematode *Caenorhabditis elegans*, Cell Calcium 53 (2013) 2–9.
[41] X.Z.S. Xu, P.W. Sternberg, A *C. elegans* sperm TRP protein required for sperm–egg interactions during fertilization, Cell 114 (2003) 285–297.
[42] A.H. Kahn-Kirby, C.I. Bargmann, TRP channels in *C. elegans*, Annu. Rev. Physiol. 68 (2006) 719–736.
[43] R. Xiao, X.Z.S. Xu, Function and regulation of TRP family channels in *C. elegans*, Pflugers Arch. Eur. J. Phy. 458 (2009) 851–860.
[44] B.J. Park, D.G. Lee, J.R. Yu, S.K. Jung, K. Choi, J. Lee, J. Lee, Y.S. Kim, J. Il Lee, J.Y. Kwon, J. Lee, A. Singson, W.K. Song, S.H. Eom, C.S. Park, D.H. Kim, J. Bandyopadhyay, J. Ahnn, Calreticulin, a calcium-binding molecular chaperone, is required for stress response and fertility in *Caenorhabditis elegans*, Mol. Biol. Cell 12 (2001) 2835–2845.
[45] J. Bandyopadhyay, J. Lee, J. Lee, J.I. Lee, J.R. Yu, C. Jee, J.H. Cho, S. Jung, M.H. Lee, S. Zannoni, A. Singson, D.H. Kim, H.S. Koo, J. Ahnn, Calcineurin, a calcium/calmodulin-dependent protein phosphatase, is involved in movement, fertility, egg laying, and growth in *Caenorhabditis elegans*, Mol. Biol. Cell 13 (2002) 3281–3293.
[46] Y. Shang, L. Chen, Z. Liu, X. Wang, X. Ma, L. Miao, Cytosolic Ca^{2+} as a multifunctional modulator is required for spermiogenesis in *Ascaris suum*, Protein & Cell 4 (2013) 456–466.
[47] G.D. Zhu, G. Salazar, S.A. Zlatic, B. Fiza, M.M. Doucette, C.J. Heilman, A.I. Levey, V. Faundez, S.W. L'Hernault, SPE-39 family proteins interact with the HOPS complex and function in lysosomal delivery, Mol. Biol. Cell 20 (2009) 1223–1240.
[48] T.M. Roberts, M. Stewart, Acting like actin: the dynamics of the nematode Major Sperm Protein (MSP) cytoskeleton indicate a push–pull mechanism for amoeboid cell motility, J. Cell Biol. 149 (2000) 7–12.
[49] J.C. Wu, A.C. Go, M. Samson, T. Cintra, S. Mirsoian, T.F. Wu, M.M. Jow, E.J. Routman, D.S. Chu, Sperm development and motility are regulated by PP1 phosphatases in *Caenorhabditis elegans*, Genetics 190 (2012) 143–157.
[50] H. Sharir, A. Zinger, A. Nevo, I. Sekler, M. Hershfinkel, Zinc released from injured cells is acting via the Zn^{2+}-sensing receptor, ZnR, to trigger signaling leading to epithelial repair, J. Biol. Chem. 285 (2010) 26097–26106.
[51] E. Chorin, O. Vinograd, I. Fleidervish, D. Gilad, S. Herrmann, I. Sekler, E. Aizenman, M. Hershfinkel, Upregulation of KCC2 activity by zinc-mediated neurotransmission via the mZnR/GPR39 receptor, J. Neurosci. 31 (2011) 12916–12926.