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The micronutrient element zinc modulates sperm activation through the SPE-8 pathway in *Caenorhabditis elegans*

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SUMMARY

Immotile spermatids produced in the testis must undergo a series of poorly understood morphological, physiological and biochemical processes called sperm activation to become motile, fertilization-competent spermatozoa. In *Caenorhabditis elegans*, the *spe-8* group contains sperm-specific genes active in both males and hermaphrodites, although their activity is required only for hermaphrodite self-sperm activation. The activating signal upstream of the SPE-8 signaling cascade remains unknown. Here, we show that the micronutrient zinc is sufficient to trigger sperm activation *in vitro*, and that extracellular zinc induces the intracellular redistribution of labile zinc. We demonstrate that other activating signals promote the similar redistribution of labile zinc, indicating that zinc might have first and/or second messenger roles during sperm activation. Moreover, zinc-induced sperm activation is SPE-8 pathway dependent. Labile zinc was enriched in the spermatheca, the normal site for self-sperm activation in hermaphrodites. High levels of zinc were also found in the secretory cells in the male gonad, suggesting that zinc might be secreted from these cells during copulation and become a component of seminal fluid, to modulate sperm activation post-copulation. These data indicate that zinc regulates sperm activation in both male and hermaphrodite *C. elegans*, a finding with important implications for understanding hermaphroditic evolution.

KEY WORDS: Sperm activation, Zinc, SPE-8 pathway, *C. elegans*

INTRODUCTION

In *Caenorhabditis elegans*, both males and hermaphrodites produce sperm, but their sperm are activated in a sex-specific manner. Although five sperm-specific genes, termed the *spe-8* group, are active in both males and hermaphrodites, their activity is only required for hermaphrodite self-sperm activation. The hermaphrodite-derived activator stimulates a signaling complex that includes the transmembrane proteins SPE-12, SPE-19 and SPE-29, and the cytoplasmic proteins SPE-8 and SPE-27. This signaling complex initiates sperm activation by repressing the activity of SPE-6, a casein I kinase downstream of the SPE-8 pathway (L'Hernault, 2009). Males also have a SPE-8-independent activation pathway involving TRY-5, a sperm-activating protease found in male seminal fluid (Smith and Stanfield, 2011). SWM-1, a somatically expressed trypsin inhibitor-like protein, promotes male reproductive success (Stanfield and Villeneuve, 2006) by inhibiting TRY-5 activity. This inhibition prevents ectopic sperm activation in the male reproductive tract and improves sperm transfer efficiency (Singson, 2006). However, *try-5* mutant males also display premature sperm activation, indicating that another activating signal insensitive to SWM-1 exists in males. The loss of both *try-5* and *spe-8*-group function leads to complete infertility for males (Smith and Stanfield, 2011), indicating that the unidentified activating signal is SPE-8 pathway dependent. Therefore, males appear to use two activating signals (SPE-8-independent TRY-5 and a SPE-8-dependent factor) and hermaphrodites use only one signal (the SPE-8-dependent factor) to induce sperm activation. The activating signal that functions upstream of the SPE-8 signaling cascade remains unknown.

The micronutrient element zinc is essential for sperm motility in various animal species (Clapper et al., 1985; Saaranen et al., 1987; Sørensen et al., 1999; Lishko et al., 2010). Although zinc deficiency and excess are both deleterious for *C. elegans* development (Roh et al., 2012), whether zinc plays a role in *C. elegans* sperm motility has not yet been determined. Here, we show that zinc is sufficient to trigger *C. elegans* sperm activation *in vitro*, and that zinc-mediated sperm activation is SPE-8 pathway dependent. Labile zinc is detected in the reproductive tract of both hermaphrodites and males, indicating that females might have acquired the zinc pathway from males to modulate sperm activation during the evolutionary origin of hermaphroditism.

MATERIALS AND METHODS

Worm strains

C. elegans strains were grown on nematode growth medium (NGM) plates at 20°C according to standard protocols (Brenner, 1974). All strains were derived from Bristol N2 strains and obtained from the *Caenorhabditis* Genetics Center. The following genes and mutations were used in this study: *fer-1(hc24)*I, *spe-8(hc50)*I, *spe-8(hc53)*I, *spe-12(hc76)*I, *spe-27(it132)*IV, *spe-29(it127)*IV, *him-5(e1490)*V, *fog-2(q71)*V.

In vitro sperm activation

Virgin L4 males were placed on fresh NGM plates for 48-72 hours at 20°C. The spermatids were dissected and released into a drop of sperm medium (SM) (Nelson and Ward, 1980) or divalent cation-free SM, with or without activators [200 μg/ml Pronase, 100 nM monensin, 1 mg/ml trypsin or different concentrations of $ZnCl_2$ or $ZnSO_4$]. Activation was scored and performed as described (Shakes and Ward, 1989).

MO fusion assay

A MO fusion assay was performed as described previously (Washington and Ward, 2006). Briefly, spermatids or spermatozoa were perfused with FM 1-43 in SM buffer at 5 μg/ml for 3 minutes to visualize the plasma membrane and fused MO using a confocal microscope (Leica).

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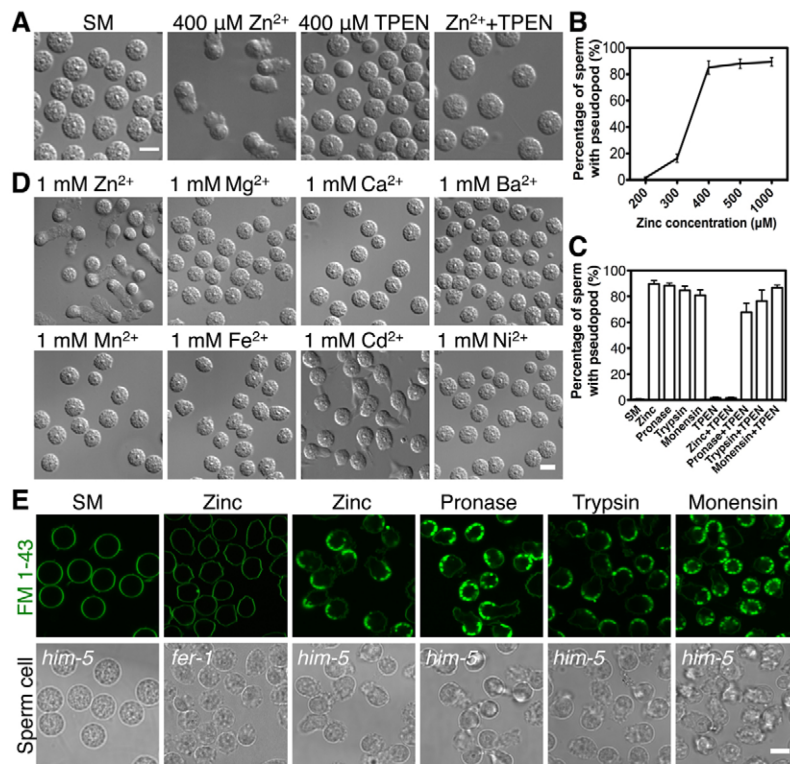

Fig. 1. Extracellular zinc triggers *C. elegans* sperm activation *in vitro*. (**A**) *C. elegans* male spermatids were round in sperm medium (SM). Spermatids extended pseudopods 10 minutes after 400 μM $ZnCl_2$ treatment. The zinc chelator TPEN (400 μM) abolished zinc-induced sperm activation. (**B**) Concentration dependence of zinc on sperm activation. Results are given as the mean±s.e.m. (n>10). (**C**) Quantification of TPEN inhibition on zinc-, Pronase-, trypsin- and monensin-induced sperm activation. Results are presented as mean±s.e.m. (n>10). (**D**) Other divalent cations failed to trigger sperm activation. (**E**) Zinc-treated sperm displayed bright fluorescent puncta of FM 1-43 in their rear edge as seen in the spermatozoa activated by other *in vitro* activators. Scale bars: 5 μm.

Zinpyr-1 and MitoTracker fluorescent imaging

Spermatids or spermatozoa activated with Zn^{2+} or monensin were incubated with 10 μM zinpyr-1 (Molecular Probes) and 10 μM MitoTracker Red (Invitrogen) in SM for 10 minutes at room temperature. In the chelating assay, zinpyr-1-labeled sperm were washed with 100 μM TPEN (Sigma-Aldrich) in cation-free SM buffer for 5 minutes. For the zinpyr-1 staining of male gonads or *fog-2* gonads, dissected male gonads or *fog-2* gonads were exposed to 10 μM zinpyr-1 with or without 100 μM TPEN in M9 buffer (Stiernagle, 2006) for 5 minutes. The fluorescent images were captured using a confocal microscope (Leica).

Autometallography (AMG)

Adult virgin males were dissected in a drop of SM. The released sperm or gonads were fixed in NeoTimm Solution (Danscher, 1981) at 4°C for 48 hours. For controls, the dissected sperm or gonads were pretreated with zinc chelators diethyldithiocarbamate (DEDTC) or EDTA for 30 minutes and fixed as described above. Zinc-specific autometallography was developed and enhanced by silver as described (Danscher, 1981). The samples were post-fixed in osmium tetroxide followed by serial dehydration, infiltration and embedding in Embed 812 resin. Thin sections were cut, stained with uranyl acetate and lead citrate and then examined with a Tecnai Spirit transmission electron microscope (FEI).

RESULTS AND DISCUSSION

Extracellular zinc triggers *C. elegans* sperm activation *in vitro*

When exposed to extracellular zinc (400 μM), round spermatids broke symmetry and extended a single pseudopod (Fig. 1A; supplementary material Movie 1). The resulting spermatozoa exhibited amoeboid cell motility (supplementary material Movie 1) (Roberts and Stewart, 2000). Sperm activation by zinc is concentration dependent, reaching a plateau at 400 μM with >80% activation (Fig. 1B). Treatment of the spermatids with the zinc-selective chelator TPEN specifically blocked the sperm activation induced by zinc (Fig. 1A,C), but not by other *in vitro* activating signals (Fig. 1C). To determine whether the sperm activation is a specific response to zinc, we also tested the bioactivity of other divalent cations; however, Mg^{2+}, Ca^{2+}, Ba^{2+}, Mn^{2+}, Fe^{2+} and Ni^{2+} did not trigger sperm activation (Fig. 1D). Cd^{2+}-treated spermatids only extended spiky projections, an activation intermediate (L'Hernault, 2009) that differs significantly from functional pseudopods.

The fusion of the membranous organelle (MO) with the plasma membrane during sperm activation is essential for sperm motility and male fertility (Washington and Ward, 2006). MO fusion can be monitored fluorescently by staining sperm with the lipophilic dye FM 1-43, which partitions into the outer membrane leaflet of cells. Similar to spermatozoa stimulated by other activators, zinc stimulation led to the formation of clear fluorescent puncta at the rear edge in activated spermatozoa (Fig. 1E). For either non-treated sperm or the MO fusion-defective mutant *fer-1* sperm treated with zinc, MO fusion failed and the plasma membrane of sperm cells was stained evenly with FM 1-43 (Fig. 1E). Thus, zinc alone is sufficient to induce sperm activation *in vitro*.

Extracellular zinc induces the exocytosis of vesicular labile zinc during sperm activation

Zinc, like other neurotransmitters, has been shown to act as a transmembrane signal in neurons. Synaptically released zinc from presynaptic neurons enters the adjacent postsynaptic neurons and glial cells through voltage-gated zinc or calcium channels and functions as a cell-to-cell signaling modulator (Pan et al., 2011). Zinc has also been shown to be a novel second messenger for signal transduction (Yamasaki et al., 2007). To explore the mechanism by which zinc induces sperm activation, we compared the intracellular zinc distribution pattern before and after sperm activation using a membrane-permeable fluorescent probe of zinc, zinpyr-1 (K_d=0.7±0.1 nM). Surprisingly, we found that in non-activated spermatids zinc ions were highly enriched in vesicular structures even in the absence of the exogenous zinc (Fig. 2A).

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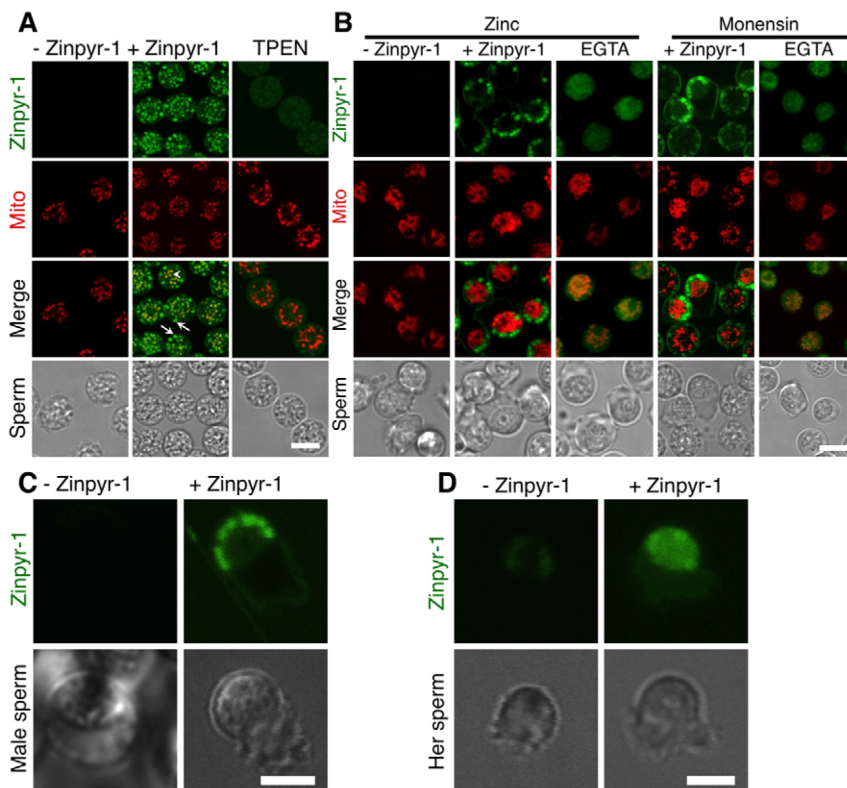

Fig. 2. Re-distribution of labile zinc during sperm activation. (**A**) Fluorescent images of spermatids co-stained with zinpyr-1 (green) and MitoTracker (Mito; red). In spermatids, zinpyr-1 labeled the mitochondria (arrowhead), as indicated by MitoTracker staining, and the vesicular structure when both fluorescent probes were added in (arrows, middle panels). As a control, only MitoTracker fluorescence could be detected when zinpyr-1 was not administered (left-hand panels). The zinc chelator TPEN diminished the zinpyr-1 fluorescence intensity significantly (right-hand panels). (**B**) The redistribution of labile zinc occurred during zinc or monensin-induced sperm activation. Zinpyr-1 fluorescence was detected at the rear edge of zinc or monensin-induced spermatozoa. After activation, the fluorescence intensity of zinpyr-1 in the mitochondria markedly decreased, whereas the fluorescence intensity of zinpyr-1 from the fused MOs increased. Zinpyr-1 fluorescence was eliminated by the addition of 10 mM EGTA in divalent cation-free SM buffer. (**C**) Zinpyr-1 staining of male-derived sperm dissected from a mated *fog-2* female uterus. (**D**) Zinpyr-1 staining of spermatozoa dissected from *him-5* hermaphrodites. Scale bars: 5 μm.

Labile zinc was also observed in the mitochondria in spermatids as indicated by the colocalization of zinpyr-1 and MitoTracker (Fig. 2A). Treatment with the zinc-specific chelator TPEN before zinpyr-1 staining resulted in a significant decrease of zinc-associated fluorescence, confirming the specificity of the zinpyr-1 staining for zinc (Fig. 2A, right-hand panels). We investigated further the distribution of labile zinc in spermatids at an ultrastructural level by autometallographic (AMG) analysis. Transmission electron microscopy (TEM) of Timm's-stained spermatid sections revealed Timm's silver precipitates in the mitochondria and in the MOs, especially the head of MOs and the tubular network in the MOs (supplementary material Fig. S1A-C), consistent with the observations of labile zinc distribution in spermatids revealed by fluorescent microscopy. Sperm activation by extracellular zinc or monensin led to a marked decrease in zinpyr-1 staining in the mitochondria; a weak zinc signal was observed along the leading edge of spermatozoa and considerable increase in zinpyr-1 staining in the MOs (Fig. 2B). To determine whether zinpyr-1 staining of the MOs in the spermatozoa is caused by the efflux of zinc from the MOs to the extracellular space or by the influx of zinc from the MOs to the cytoplasm during sperm activation, we performed a perfusion assay with EGTA, a membrane-impermeable divalent cation chelator. We found that the zinpyr-1 signals detected at the MO and the leading edge of the plasma membrane were eliminated by EGTA perfusion in either zinc- or monensin-induced spermatozoa (Fig. 2B), indicating that vesicular zinc is exocytosed when the MO fuses with the plasma membrane upon activation. However, because of technical limitations, the exocytosed zinc observed by zinpyr-1 staining in the fused MOs could not be fixed and detected by AMG (supplementary material Fig. S1D).

We also stained the male-derived spermatozoa dissected from the uteruses of *fog-2* females and found that the pattern of zinpyr-1 staining (Fig. 2C) was similar to that of *in vitro* zinc-induced spermatozoa. For hermaphrodite-derived spermatozoa, zinc could also be detected in a similar way (Fig. 2D). The small differences in the pattern of zinpyr-1 staining between male sperm (Fig. 2C) and hermaphrodite sperm (Fig. 2D) might reflect the length of time that has passed since first activation, or perhaps the amount of zinc in the environment that might have been taken up as part of the signaling process. Together, our findings demonstrate that vesicular zinc is exocytosed into the extracellular space during sperm activation induced by zinc or other activating signals.

Zinc is a potential physiological modulator for sperm activation in *C. elegans*

Zinc is not required for trypsin-induced sperm activation (Fig. 1C), suggesting that zinc and trypsin (or TRY-5) might activate sperm through distinct signaling pathways. Indeed, we found that zinc-induced sperm activation was dependent on the SPE-8 signaling cascade. Sperm from *spe-8* group mutant males (we analyzed *spe-8*, *spe-12*, *spe-27* and *spe-29*) were rarely activated by zinc [in *spe-8(hc53)*, *spe-12(hc76)* and *spe-29(it127)*] or not activated at all [in *spe-8(hc50)* and *spe-27(it132)*] (Fig. 3A,B). The *spe-8(hc50)* and *spe-12(hc76)* mutants are genetically null and have no functional gene product, whereas the *spe-8(hc53)*, *spe-27(it132)* and *spe-29(it127)* mutants are hypomorphic. Consistent with previous studies (Nance et al., 1999), the spermatids from null mutants of *spe-8(hc50)* failed to activate in response to the exogenous zinc, whereas the spermatids from hypomorphic *spe-8(hc53)* partially activated, though the activation rate was low. Unlike the response of spermatids of *spe-8(hc50)* to zinc, the spermatids of the null mutant *spe-12(hc76)* partially activated and the activation rate was comparable to that of hypomorphic *spe-8(hc53)*. As *spe-8* is predicted to encode a non-receptor tyrosine kinase (Smith, 2006), the loss of this activity might be more important than the loss of other *spe-8* group genes. Together,

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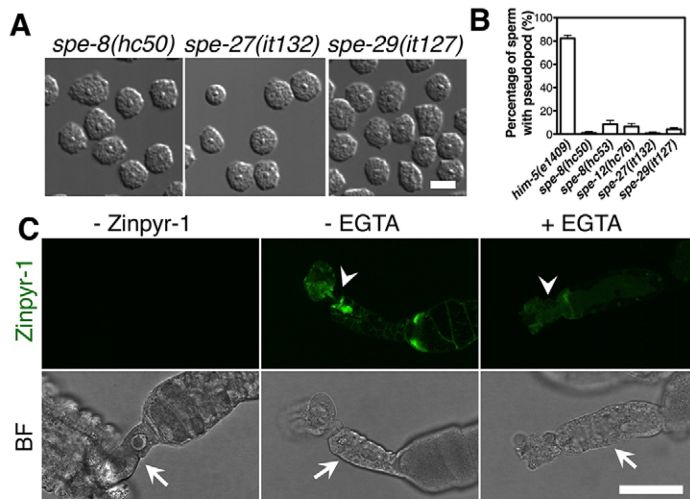

Fig. 3. Zinc functions through the SPE-8 pathway and localizes in the spermatheca of hermaphrodite. (**A**) Zinc failed to activate spermatids from *spe-8* group mutant males. Scale bar: 5 μm. (**B**) Quantification of zinc-induced sperm activation in *spe-8* group mutant male spermatids. Results are shown as means±s.e.m. (n>10). (**C**) Fluorescent images of dissected spermatheca stained with zinpyr-1 (middle panels). Zinpyr-1 fluorescent intensity around the spermatheca-uterine valve markedly decreased with the addition of EGTA (right-hand panels). Arrows in brightfield (BF) images point to the position of the spermatheca. Arrowheads in fluorescent images point to the spermatheca-uterine valve. Scale bar: 50 μm.

these data suggest that zinc might modulate sperm activation dependent on the SPE-8 pathway.

If zinc is an endogenous hermaphrodite sperm activator, labile zinc should be detected in the spermatheca, where self-sperm activation takes place in hermaphrodites. Therefore, we dissected *fog-2* female gonads and stained them with zinpyr-1. Bright zinpyr-1 staining could be detected in the spermatheca, especially in the spermatheca-uterine valve area (Fig. 3C, middle panels), in an EGTA-dependent manner (Fig. 3C, right-hand panels). Spermathecal cells, however, still showed some zinpyr-1 staining in the presence of EGTA. Together, these data demonstrate that zinc-induced sperm activation is SPE-8 pathway dependent and that labile zinc is found in the spermatheca.

Zinc accumulates in the seminal vesicles of the male gonad

Because high levels of zinc have been found in the seminal fluid of various animal species, we hypothesized that labile zinc might be enriched in the seminal fluid in *C. elegans*. To determine the distribution of zinc in *C. elegans* male gonads, we stained them with zinpyr-1. A bright signal was found in the inner secretory layer (Kimble and Hirsh, 1979), with a granular appearance similar to that observed when these cells are viewed with differential interference contrast (DIC) optics (Fig. 4A, left-hand panels). This signal could not be detected either in the presence of the zinc-selective chelator TPEN or in the absence of zinpyr-1 (Fig. 4A, middle and right-hand panels). To confirm the distribution of labile

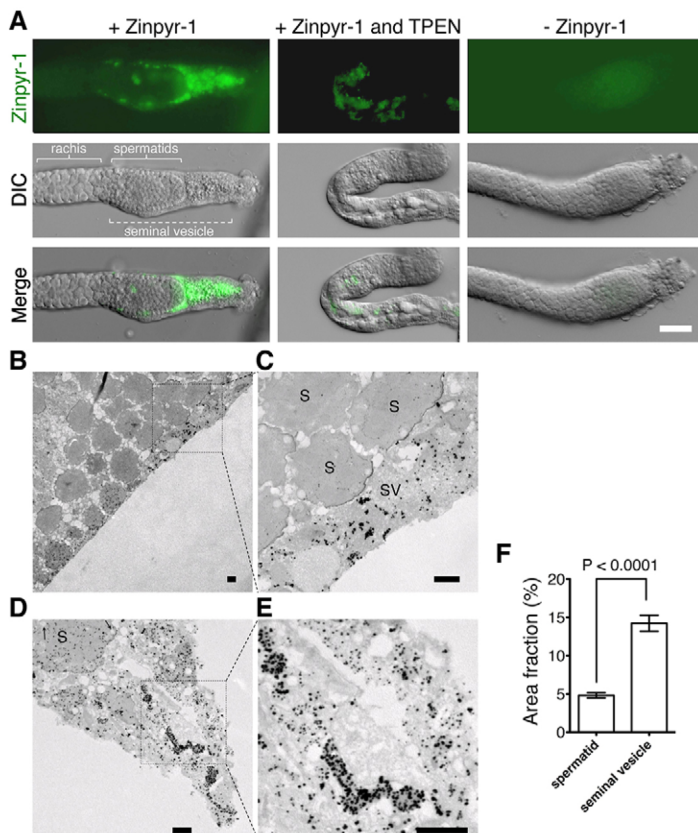

Fig. 4. Zinc storage in the seminal vesicle of male gonads. (**A**) Zinpyr-1 staining in dissected male gonads. (**B**) Autometallographic (AMG) staining for zinc in the seminal vesicle. These cells showed a distinct pattern of black, granular precipitates, indicating the presence of chelatable zinc ions. The AMG grains were predominantly distributed in the seminal vesicle cells. (**C**) High-magnification picture of the framed area in B. S, spermatids; SV, seminal vesicle. (**D**) AMG grains were densely accumulated in the secretory cells of the seminal vesicle. Arrows, MOs; S, spermatid. (**E**) High-magnification picture of the framed area in D. (**F**) The area fraction of black granules in the spermatids and seminal vesicle cells. n=10; P<0.0001, Student's t-test. Error bars represent s.e.m. Scale bars: in A, 50 μm; in B-E, 1 μm.

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zinc at ultrastructural levels in these areas, we performed AMG analysis. Consistent with the zinpyr-1 staining above, silver-enhanced zinc grains were found in distal seminal vesicle cells (Fig. 4B,C) and were highly concentrated in the secretory cells in the seminal vesicle (Fig. 4D,E). A relatively low level of grains could also be detected in the spermatids at ultrastructural levels by AMG, consistent with the observations described above. Our analysis showed that zinc grains were significantly more abundant in the seminal vesicle cells than in the spermatids (Fig. 4F). The localization of zinc in the secretory cells of the seminal vesicle suggests that zinc might be secreted from these cells to become a component of the seminal fluid during copulation to modulate sperm activation post-copulation.

Here, we have revealed that exogenous zinc is sufficient to activate *C. elegans* sperm in a SPE-8-dependent manner. Furthermore, we have found that labile zinc is enriched in the reproductive tract of hermaphrodites and males, indicating that zinc might function as a potential physiological sperm activator for *C. elegans* in both males and hermaphrodites. Androdioecious species like *C. elegans* evolved from gonochoristic ones in which individuals are either males or females. Double mutations of the sex determination gene *tra-2* and the sperm activator suppressor gene *swm-1* were sufficient to create a fertile hermaphroditic organism in the gonochoristic *C. remanei* (Baldi et al., 2009). In hermaphroditic *C. elegans*, both TRY-5 and SWM-1 are dispensable for hermaphrodite fertility but *spe-8* hermaphrodites are sterile (Stanfield and Villeneuve, 2006; Smith and Stanfield, 2011), implying that hermaphrodites might have pathways other than proteolysis to activate their sperm. The fact that both males and hermaphrodites share the zinc pathway for sperm activation suggests that during the hermaphroditic evolutionary processes, females might have co-opted this male pathway to modulate sperm activation.

Extracellular zinc is required for sperm motility and the acrosome reaction in sea urchins (Clapper et al., 1985). Zinc also plays essential roles in the regulation of the rate and duration of sperm motility in Japanese eels (Yamaguchi et al., 2009). In humans, high levels of zinc are found in the seminal plasma (Sørensen et al., 1999). However, the zinc in the seminal plasma actually plays an inhibitory role for sperm motility by inhibiting voltage-gated proton channels on sperm flagellum (Lishko et al., 2010). Zinc also plays an inhibitory role for human sperm motility by its involvement in the formation of seminal coagulation. This physically traps the sperm until they are functionally mature (Yoshida et al., 2008). Thus, zinc affects sperm motility in various animal species but whether this is inhibitory or stimulatory appears to be species specific. Although nematode sperm are crawling cells, morphologically distinct from flagellated sperm, and the molecular machineries for motility in amoeboid and flagellated sperm differ from each other (Roberts and Stewart, 2000), both types of sperm might utilize the micronutrient element zinc to modulate their motility. These observations also indicate that some pathways that regulate germ cell development appear to be broadly conserved, even though sperm from different species have diverged dramatically during evolution.

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Competing interests statement
The authors declare no competing financial interests.

Supplementary material
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