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Zinc availability during germline development impacts embryo viability in *Caenorhabditis elegans*

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ABSTRACT

Zinc is an essential metal that serves as a cofactor and structural regulator in a variety of cellular processes, including meiotic maturation. Cellular control of zinc uptake, availability and efflux is closely linked to meiotic progression in rodent and primate reproduction where large fluctuations in zinc levels are critical at several steps in the oocyte-to-embryo transition. Despite these well-documented roles of zinc fluxes during meiosis, only a few of the genes encoding key zinc receptors, membrane-spanning transporters, and downstream signaling pathway factors have been identified to date. Furthermore, little is known about analogous roles for zinc fluxes in the context of a whole organism. Here, we evaluate whether zinc availability regulates germline development and oocyte viability in the nematode *Caenorhabditis elegans*, an experimentally flexible model organism. We find that similar to mammals, mild zinc limitation in *C. elegans* profoundly impacts the reproductive axis: the brood size is significantly reduced under conditions of zinc limitation where other physiological functions are not perturbed. Zinc limitation in this organism has a more pronounced impact on oocytes than sperm and this leads to the decrease in viable embryo production. Moreover, acute zinc limitation of isolated zygotes prevents extrusion of the second polar body during meiosis and leads to aneuploid embryos. Thus, the zinc-dependent steps in *C. elegans* gametogenesis roughly parallel those described in meiotic-to-mitotic transitions in mammals.

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1. Introduction

Zinc is a transition metal that serves as a cofactor and structural regulator in a variety of proteins that participate in numerous cellular processes (Beyersmann and Haase, 2001; Bohnsack and Hirschi, 2004; Haase and Maret, 2010). We have shown that fluctuations in total cellular zinc levels play central regulatory roles controlling meiosis in mouse, non-human primate, and human oocytes before and after fertilization (Bernhardt et al., 2011; Duncan et al., 2016; Kong et al., 2012, 2014; Que et al., 2015; Zhang et al., 2016). In the mouse oocyte, total zinc levels increase by over 50% during meiotic maturation and this accumulation of zinc is required for the oocyte to progress properly to metaphase of meiosis II (Kim et al., 2010). Previous work has shown that fertilization and parthenogenesis initiate zinc exocytosis from zinc loaded cortical vesicles (Que et al., 2015; Kim et al., 2011) into the extracellular space through a series of coordinated events known as 'zinc sparks' (Kim et al., 2011). If zinc levels are not reduced, the egg cannot complete meiosis, and the zygote is unable to initiate the mitotic divisions. Therefore, zinc fluxes are fundamental events at several steps in the oocyte-egg-embryo transition, and are critical for mammalian reproduction. Despite these well-defined roles of zinc fluxes during meiosis (Suzuki et al., 2010a, 2010b), only a few of the genes encoding zinc receptors have been identified as mediating these switching events in mammals or other model systems to date. Zinc receptors, i.e. macromolecules defined by their ability to move or bind zinc, with known roles in meiosis include the cation transporters ZIP6 and ZIP10 and downstream signaling pathway factors, such as Emi2 (Lints and Hall, 2009d; Tian et al., 2014; Tian and Diaz, 2012, 2013). The nematode *C. elegans* would be an ideal model system for identifying pathway members, especially if readily triggered meiotic phenotypes of zinc depletion can be established. Given that zinc availability has already been established to regulate proper meiotic progression in mammalian oocytes, we test here whether a similar type of inorganic regulation of egg biology might extend further into the phylogenetic tree using the invertebrate, *C. elegans*.

C. elegans exist as two sexes, hermaphrodites and males (L'Hernault, 2006; Lints and Hall, 2009c, 2009d). These worms develop through four

Abbreviations: TPEN, N,N,N′,N′-tetrakis(2-pyridylmethyl)ethylenediamine; NGM, Nematode growth media; ZI, Zinc insufficient; TM, Ammonium tetrathiomolybdate.

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larval stages (L1–L4), entering adulthood in approximately 3 days (Corsi et al., 2015). In self-fertilizing hermaphrodites, gonadogenesis completes and meiosis begins in the L4 stage. They first produce sperm and store these gametes in a compartment called the spermatheca, but upon becoming adults, there is a switch to oocyte production (Kimble and Crittenden, 2007). At this stage, the remaining meiotic cells in the germline begin maturing into oocytes, which are then fertilized as they pass through the spermatheca, becoming embryos that are laid and hatch (Greenstein, 2005). Each hermaphrodite produces approximately 300 self-progeny before running out of sperm, but they can produce many more offspring if mated with a male and sperm availability is not limited.

Many features of this system make it ideal for evaluating the regulatory roles of zinc fluxes in reproduction. The hermaphrodite gonad has two arms and each is arranged as a production line, with a population of germline stem cells in the distal region differentiating to enter meiosis, and then forming oocytes in the proximal region, as they move toward the spermatheca (McCarter et al., 1999; Von Stetina and Orr-Weaver, 2011) (Fig. 1). Therefore, this spatial-temporal gradient means that all stages of meiosis can be visualized simultaneously within the same worm. Moreover, *C. elegans* are transparent, allowing live imaging of the meiotic and mitotic divisions of the oocyte and embryo, and they are amenable to a wide variety of experimental manipulations. Based on these advantages, we assessed whether zinc availability impacts germline development or oocyte viability in a whole animal model.

Here, we test the hypothesis that growth of the worm under zinc deficient conditions will impair oocyte function and fertility. Zinc availability to both the worm and its food, *E. coli*, can be attenuated by addition of the metal chelator N,N,N′,N′-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN) to the growth medium. TPEN has been employed in a number of studies of meiosis in isolated mammalian oocytes (Bernhardt et al., 2011; Kim et al., 2011; Suzuki et al., 2010a, 2010b). In order to establish mild zinc limitation in which there is no observable impact on the general physiological status of *C. elegans*, progeny counts were evaluated as a function of TPEN concentration in the growth medium. We found that growth of adults under mild zinc depletion leads to a statistically significant drop in the number oocytes produced. Moreover, acute zinc limitation of isolated zygotes prevents the extrusion of the second polar body and causes chromosome segregation defects, resulting in aneuploid embryos. These results establish a zinc-dependent phenotype in the reproductive axis of *C. elegans* and support the idea that zinc-regulated pathways in meiosis are evolutionarily conserved.

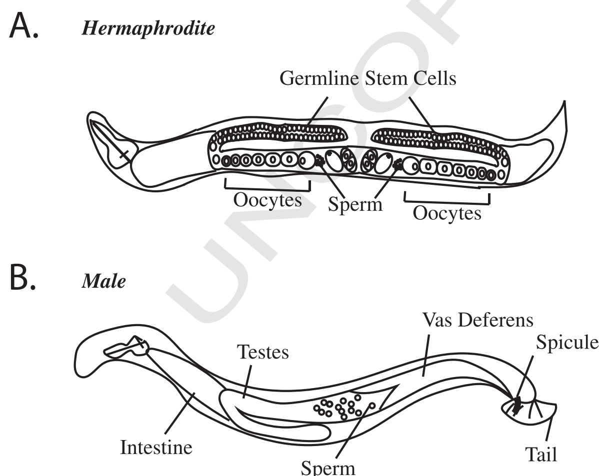

Fig. 1. *C. elegans* hermaphrodite and male anatomy. (A) Adult hermaphrodites have two gonad arms. The distal region of each arm contains germline stem cells that differentiate to enter meiosis and then move through the gonad as they progress through meiosis, forming oocytes in the proximal region of the gonad. Oocytes are then fertilized in the spermatheca, where the sperm are stored, triggering completion of the meiotic divisions and the beginning of the mitotic divisions of the embryo. (B) Males only contain sperm, and can mate with hermaphrodites.

2. Materials and methods

2.1. Worm strains

EU1067: *unc-119(ed3) ruIs32[unc-119(+) pie-1^{promoter}::GFP::H2B] III; ruIs57[unc-119(+) pie-1^{promoter}::GFP::tubulin]* was used for fluorescence imaging of the meiotic spindle, gamete count, and for generating males (Wignall and Villeneuve, 2009). N2 (Bristol) wild type strain was used in all brood size experiments, food aversion, and pharyngeal contraction experiments (Wormbase, 2016b). The mutant strain *fog-1(q253)* (Wormbase, 2016a) was used in the mating experiment.

2.2. Growth media

Animals were grown on bacterial lawns plated on 6 cm agar plates following standard methods (Stiernagle, 2006) with the following modifications. Plates containing growth media were prepared with a final concentration of 10 μM TPEN (Sigma Aldrich, St. Louis, MO) to Nematode Growth Media (NGM) (Stiernagle, 2006) (Sigma) prior to pouring the plates. After the plates solidified, 200 μl of an overnight growth of *E. coli* strain OP50 in Luria Broth (Stiernagle, 2006) was added and allowed to dry at room temperature. Alternatively, a final concentration of 10 μM of the copper-specific chelators Neocuproine (Sigma) or Ammonium tetrathiomolybdate (Sigma) were dissolved in ethanol or H_2O, respectively, and were added to the NGM as described above. For rescue experiments, plates were further supplemented with 20 μM metal salts $CuSO_4 \cdot 5H_2O$ (Sigma), $ZnSO_4 \cdot 7H_2O$ (Sigma), and $FeSO_4 \cdot 7H_2O$ (Sigma).

2.3. Brood size experiments

L4 stage hermaphrodites were picked onto control NGM or zinc insufficient NGM plates (1 worm/plate). Every 24 h, the worm was transferred to a fresh plate. Progeny were counted 48 h after the adult was removed from the plate. Progeny were scored from controls (day 1, $n = 50$, day 2, $n = 43$, day 3, $n = 40$, day 4, $n = 33$, day 5, $n = 30$ hermaphrodites) and zinc insufficient hermaphrodites (day 1, $n = 52$, day 2, $n = 52$, day 3, $n = 25$, day 4, $n = 22$, day 5, $n = 18$). Similarly, brood size experiments were conducted on plates with bacteria killed by UV irradiation, heat exposure (75 °C for 1 h) and 250 μg/ml Kanamycin (Sigma) (MacNeil et al., 2013). For DMSO vehicle: day 1, $n = 20$, day 2, $n = 19$, day 3, $n = 10$, day 4, $n = 18$, and day 5, $n = 19$. For TPEN in DMSO: day 1, $n = 25$, day 2, $n = 20$, day 3, $n = 10$, day 4, $n = 16$, and day 5, $n = 15$. For methanol vehicle: day 1, $n = 19$, day 2, $n = 19$, day 3, $n = 18$, day 4, $n = 15$, day 5, $n = 15$. For TPEN in methanol: day 1, $n = 19$, day 2, $n = 11$, day 3, $n = 20$, day 4, $n = 22$, and day 5, $n = 11$. In the case of the TPEN in the methanol group, some worms were stuck between the agar and the plate wall on day 1 and were liberated for later egg laying in the experiment, others were caught in water on the side wall and were also liberated.

2.4. Hatching experiment

N2 animals were synchronized at the L4 stage and placed on either control plates ($n = 14$ adults) or plates with 10 μM TPEN ($n = 12$ adults). Each group was incubated at 20 °C for 24 h to allow for egg laying. After incubating, the adults were removed and allowed an additional 24 h for the embryos to hatch. After this time, the number of embryos that hatched was quantified.

2.5. Eating behavior experiments

Young adult eating behavior was quantified by counting the number of pharyngeal contractions per 30 s in control ($n = 31$) and zinc

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