Filamin and phospholipase C-ε are required for calcium signaling in the *Caenorhabditis elegans* Spermatheca

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Mechanical properties of the microenvironment are fundamental in orchestrating normal tissue function, disease progression, and organismal development. Studies of mechanotransduction in cultured cells on artificial substrates have revealed underlying principles, but the in vivo roles of mechanotransduction remain unclear. We recently reported that the *Caenorhabditis elegans* spermatheca—a myoepithelial tube composed of a cell monolayer—may be mechanosensitive. Live imaging with the genetically encoded calcium indicator GCaMP revealed that oocyte-induced stretching of the spermatheca resulted in calcium oscillations and constriction of the tube. FLN-1/filamin, a mechanosensitive cytoskeletal scaffolding protein, is required to correctly trigger the calcium transients. PLC-1/phospholipase C-epsilon and ITR-1/IP$_3$ receptor are required to produce the calcium transients, and may function downstream of filamin. In addition to providing important insights into the biology of *C. elegans*, our studies offer a novel and genetically tractable model for studying mechanotransduction in a myoepithelial tissue.

Overview of the *C. elegans* Spermatheca Anatomy and Function

The spermatheca connects the oviduct with the uterus and serves as the site of sperm storage and fertilization.[1] A monolayer of 24 myoepithelial cells surrounded by a basement membrane forms the spermatheca.[2] The distal spermatheca is a narrow entryway that is contiguous with, but distinct from, the gonadal sheath cells that envelop the proximal gonad.[3] Proximally, the spermatheca is connected to the uterus by way of the spermatheca-uterine (sp-ut) valve, a four-cell syncytium.[4] Before the first ovulation the sp-ut valve contains a syncytial core cell that is displaced by the passage of the first embryo.

Oocytes mature sequentially and are pushed into the spermatheca by swift and forceful contractions of the sheath cells. Oocyte maturation and ovulation are controlled by signaling between the oocytes, sheath cells, and the sperm.[2,5] The most proximal oocyte is thought to secrete LIN-3, an epidermal growth factor (EGF)-like ligand that activates LET-23/EGF receptor on the sheath cells. LET-23 then probably activates PLC-3/phospholipase C-gamma, which generates Ins(1,4,5)P_3 and triggers calcium release via the Ins(1,4,5)P_3 receptor ITR-1.[6,7] Calcium release initiates myosin contraction within the sheath cells, and these contractions push the oocyte into the spermatheca. The ovulated oocyte is fertilized and propelled into the uterus by steady distal-to-proximal constriction of the spermatheca.

In contrast to the relatively well-understood oocyte maturation and ovulation process, the molecular details of spermatheca function remain poorly defined. In particular, it is unclear what triggers the constriction of the spermatheca. The most logical explanation is that spermathecal constriction is coupled to fertilization, but this seems unlikely given that
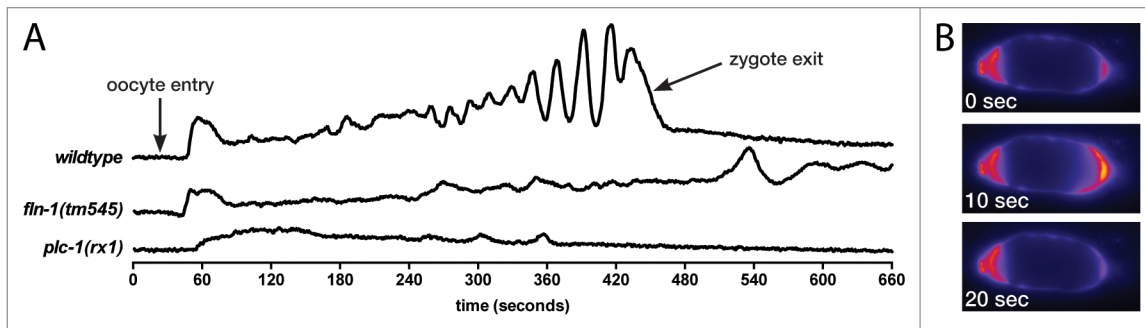
Figure 1. Oocyte entry triggers calcium signaling in the spermatheca. (**A**) Quantification of normalized GCaMP intensity in wildtype, fln-1(tm545), and plc-1(rx1) animals. The plots are aligned so that oocyte entry occurrs at 30 sec. The zygote exits at approximately 450 sec in wild-type animals, and fails to exit in fln-1(tm545) and plc-1(rx1) animals. Oocyte entry triggers a pulse of calcium in the sp-ut valve in wild-type and fln-1(tm545) animals, but not in plc-1(rx1) animals. (**B**) Representative still images of a spermatheca expressing GCaMP during one calcium pulse. The sp-ut valve is on the left, and the distal spermatheca is on the right.

mutants lacking sperm (or having incompetent sperm) are not observed retaining unfertilized oocytes in the spermatheca. Alternative explanations include the possibilities that an oocyte surface molecule binds to a receptor on the spermathecal cells, or that the ovulatory signal also activates spermathecal constriction. Finally, it is also possible that stretching of the spermatheca by an incoming oocyte triggers the constriction of the spermatheca.

One important clue came from the finding that loss of PLC-1/phospholipase C-epsilon results in embryo trapping within the spermatheca.[8] PLC-1 and PLC-3 are both phospholipase C isoenzymes that hydrolyze PtdIns(4,5)P_2 into Ins(1,4,5)P_3 and diacylglycerol (DAG), but they feature distinct regulatory domains.[8,9] The epsilon class of PLC enzymes is unusual in that it contains RasGEF and Ras association domains, in addition to the catalytic domain.[10] Experimental evidence suggests that mammalian PLC-epsilon is regulated by Rho and Ras GTPases, and may act as a GEF for Rap1 and Ras.[11-14] In *C. elegans*, PLC-1 has been shown to interact with LET-60 in a GTP-dependent manner, but the functional consequence of this interaction remains unknown.[15] Our own observations of PLC-1 localization suggest the N-terminal RasGEF domain may serve to localize PLC-1 to the membrane. PLC-gamma enzymes are generally effectors of receptor tyrosine kinases, such as the EGF receptor LET-23.[7,9] The distinct usage of PLC-3 in the sheath and PLC-1 in the spermatheca, and their different

phenotypes, suggests that the same pathway does not control both processes.

We have identified FLN-1 as essential for the function of the *C. elegans* spermatheca.[16,17] FLN-1 is a well-conserved filamin ortholog that contains an N-terminal actin-binding domain followed by a series of 20 Ig-like domains.[16,18] Filamins are best known as actin-binding proteins, but have been implicated in diverse processes and may function as molecular force sensors.[19,20] The Ig-like domains adopt globular arrangements that reveal cryptic binding sites under tension.[21,22] This tension-dependent change in binding affinity for downstream effectors may form the basis of the filamin molecular force sensor.[23] In *C. elegans*, *fln-1(tm545)*, a strong loss-of-function or null allele, results in trapping of embryos within the spermatheca, largely phenocopying *plc-1*-null mutants.[8,16]

Although *fln-1* and *plc-1* mutants both exhibit the embryo trapping phenotype, there are several important differences. In *fln-1(tm545)* animals that have not ovulated, or have ovulated few oocytes, the F-actin is essentially normal; however, as further ovulations occur, the F-actin begins to relocalize from circumferential cables to bundles coincident with cell-cell junctions.[16] In contrast, no F-actin disorganization is seen even in very old *plc-1(rx1)* animals, which may indicate that FLN-1 is required to maintain F-actin organization under stress.[16] Although the overall gonad morphogenesis is unaffected in either mutant, the sp-ut valve in *fln-1(tm545)* appears abnormally relaxed.[16]

Expression of FLN-1 in the sp-ut valve is sufficient to restore normal valve morphology, but is insufficient to rescue spermathecal function. In fact, expression of FLN-1 in the valve strikingly enhances the trapping phenotype. *plc-1*-null mutants show a normally constricted sp-ut valve, and a stronger trapping phenotype compared with *fln-1(tm545)* animals. *fln-1(tm545); plc-1(rx1)* double mutants have an intermediate trapping phenotype. These data suggest that PLC-1 may function exclusively in the spermatheca, while FLN-1 functions in the spermatheca and the sp-ut valve.

Oocyte Entry Triggers Spermathecal Calcium Transients

Because generation of IP$_3$ by phospholipases can trigger release of calcium from internal stores, in a recent study we examined calcium signaling in the spermatheca using GCaMP, a genetically encoded calcium indicator.[17] Live imaging of animals expressing GCaMP in the spermatheca revealed that a surprising and complex series of calcium transients are triggered by oocyte entry (**Fig. 1**). The calcium transients originate in the distal constriction, and travel proximally towards the uterus, in the direction of oocyte movement. A candidate RNAi screen of gap junction subunits revealed that gap junctions are required for synchronous and directional calcium oscillations in the spermatheca. The calcium oscillations are initially weak and appear restricted to the distal spermatheca, but quickly intensify.

The strongest pulses of calcium are associated with the zygote exiting into the uterus. Following zygote exit, a pulse of calcium sweeps through the spermatheca and appears to retighten the sp-ut valve, giving the embryo a final shove into the uterus.

Loss of PLC-1 completely abrogates calcium signaling in the spermatheca, which indicates that PLC-1 is probably acting as predicted to generate $Ins(1,4,5)P_3$ and trigger calcium release via ITR-1.[6,8,17] Because ITR-1 is required in the sheath cells for normal ovulations, it is not straightforward to determine whether ITR-1 is required in the spermatheca. However, a temperature-sensitive allele of ITR-1 decreased the amplitude and frequency of calcium transients.[6,17]

In contrast to the *plc-1*-null mutants that produced no calcium events, filamin-deficient animals exhibited markedly delayed calcium transients. The delayed calcium transients began well after the time a wild-type embryo would have exited the spermatheca. The calcium transients did not increase in amplitude, did not result in significant constriction of the spermatheca, and continued for a variable amount of time. *fln-1(tm545); plc-1(rx1)* double mutants completely lost calcium signaling, including the delayed transients. The delayed calcium oscillations in *fln-1(tm545)* animals suggests that filamin is required to trigger the calcium oscillations, but is not required for the production of the calcium transients per se.

A mutation in LFE-2/$Ins(1,4,5)P_3$ kinase that decreases the rate of $Ins(1,4,5)P_3$ degradation partially suppressed the *fln-1(tm545)* calcium signaling defect.[6] Similarly, a gain-of-function mutation in ITR-1 that increases its affinity for $Ins(1,4,5)P_3$ also suppressed the calcium signaling defect.[6] In addition to increasing calcium release in the *fln-1(tm545)* background, the suppressing mutations also resulted in partial exit of embryos out of the spermatheca. This functional rescue suggests that the spermatheca is able to constrict given the correct signal despite a compromised F-actin network.

plc-1-null mutations cannot be suppressed by decreasing $Ins(1,4,5)P_3$ degradation or increasing ITR-1 sensitivity, presumably due to complete absence of $Ins(1,4,5)P_3$.[8,24] Because *fln-1(tm545)* animals are eventually able to trigger calcium release, we suggest that the levels of $Ins(1,4,5)P_3$ are low, but not absent, in *fln-1(tm545)* animals possibly due to basal activity of PLC-1. The simplest interpretation of our data are that FLN-1 is required for the efficient and timely activation of PLC-1 to produce the $Ins(1,4,5)P_3$ and calcium signals, but that a secondary trigger or backup mechanism can independently activate PLC-1-dependent calcium signaling.

Our favored hypothesis is that oocyte entry into the spermatheca stretches the cells and triggers a mechanosensitive pathway that leads to calcium signaling. Filamin may serve as a direct mechanosensor, whereby a change in the filamin conformation could lead to, for example, localization of a PLC-1 activator. Because filamin binds RasGEFs and RasGAPs,[25,26] and because PLC-1 activity is regulated by Ras,[14] it is possible that PLC-1 is activated by tension-dependent binding of a GEFs and GAPs to filamin. Our unpublished observations suggest that PLC-1 is constitutively found at the membrane and that its localization is independent of FLN-1, making a direct interaction between the two proteins unlikely. Filamin has also been shown to scaffold many types of transmembrane channels, including mechanosensitive ion channels. As a scaffold, filamin may tether the transmembrane channels to the cytoskeleton, organize the transmembrane channels into specific patterns, and localize downstream effectors in close proximity, thereby serving to coordinate the timely transit of fertilized embryos through the spermatheca.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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