Cholesterol and the biosynthesis of glycosphingolipids are required for sperm activation in Caenorhabditis elegans

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1. Introduction

The correct establishment and maintenance of cell polarity is essential for many biological processes, including asymmetric cell division, cellular morphogenesis and directed cell migration. Typically, actin cytoskeletons are employed to establish cell polarity and microtubules are involved in maintaining the stability of the polarized organization [1]. Nematode spermiogenesis (or sperm activation), in which round sessile spermatids differentiate into asymmetric crawling spermatids, is a symmetry-breaking process [2]. However, nematode spermatids possess neither actin nor tubulin; instead, the establishment of their polarity and cell motility depend on controlled assembly/disassembly of the major sperm protein (MSP) cytoskeleton [3,4]. After the meiosis, spermatids are transcriptionally and translationally silent and thus sperm activation, motility acquisition and fertilization are performed without new gene expression [5]. Sperm activation is initiated by vas deferens-derived trypsin-like serine protease, TRY-5 [6,7], that activates round sessile spermatid to assemble the MSP cytoskeleton and to extend a single pseudopod, which is necessary for sperm motility and fertilization competence [8,9]. In addition, PI (3,4,5)P3 signaling on the plasma membrane (PM) is involved in C. elegans sperm activation [10]. The observations that an extracellular activator functions at or within the sperm PM, and that phospholipid in the PM is required during sperm activation, indicate that the lipid environment in the PM is crucial for signal transduction in this process. Whether a specific lipid environment in the PM is essential for sperm activation remains unknown.

Increasing evidence indicates that the PM typically contains dynamic microdomains that are resistant to cold detergent extraction and are of lower buoyant density than the total PM. Such membrane microdomains or lipid rafts) in the exoplasmic leaflet of the bilayer, these microdomains are enriched with cholesterol, glycosphingolipids (GSLs) and specific membrane proteins such as GPI-anchored proteins [12]. The survival of C. elegans requires an exogenous cholesterol supply because it lacks the de novo biosynthetic pathway for cholesterol [14]. Animals deprived of cholesterol produce a reduced brood size in the second generation, and re-supplying...
cholesterol restores their fertility [15]. The requirement for trace quantities of cholesterol for the survival of *C. elegans* and its relatively low abundance in lipid extracts suggests that cholesterol might serve as a signaling molecule rather than being a structural component in most cell membranes of this species [14]. Conversely, a live cell-labeling assay with the fluorescent cholesterol analog dehydroergos-terol showed that *C. elegans* sperm are enriched with cholesterol [16], suggesting that cholesterol might be crucial for sperm function and reproductive success. However, it remains unknown whether cholesterol in the sperm plays a signaling role (e.g., as a precursor to a steroid hormone or other biologically active compound) or whether—in conjunction with GSLs—it might form lipid microdomains providing a platform mediating signal transduction during sperm activation and motility acquisition. Here we demonstrate that cholesterol is enriched in the plasma membrane of *C. elegans* spermatids and that the integrity of lipid microdomains composed of cholesterol and glycosphingolipids (GSLs) is critical during sperm activation. Disruption of sperm lipid microdomains by acute manipulation of cholesterol in vitro blocks the sperm activation. Restriction of cholesterol update also results in the abnormal sperm activation in both males and hermaphrodites. Manipulation of the integrity of lipid microdomains by targeting the biosynthesis of GSLs inhibits both the membranous organelles' fusion with PM and pseudopod extension, two events necessary for sperm activation. The inhibitory effects can be rescued by the addition of exogenous GSLs. The cleavage of glycosylphosphatidylinositol (GPI)-anchored proteins also affects sperm activation. Collectively, these findings indicate a functional role for lipid microdomains in mediating localized signal transduction during sperm activation and sperm motility acquisition in *C. elegans*.

2. Materials and methods

2.1. Nematode strains

*C. elegans* strains, provided by the *Caenorhabditis* Genetics Center, were cultured on Nematode Growth Media (NGM) or cholesterol free NGM plates at 20 °C according to Brenner [17]. The strain *him-5(e1490)V* served as the wild-type animal to facilitate the supply of males for detecting sperm function. The effect of cholesterol depletion on sperm function was performed by mating the *him-5(e1490)V* males cultured on cholesterol free NGM with normal N2 hermaphrodites or by growing the *him-5* hermaphrodites on cholesterol free NGM.

2.2. C. elegans sperm activation in vitro

Virgin males at the L4 stage were transferred to a new NGM plate to prevent mating for 2 days before the *C. elegans* sperm were dissected into sperm medium (SM) buffer containing monensin (200 nM; Sigma) for 10 min. Sperm activation and migration were recorded in real time at 20 °C using an Axio Imager M2 microscope (Carl Zeiss), equipped with a 63× and 100× differential interference contrast (DIC) objective lenses with appropriate filters. Images were captured with a charge-coupled device (CCD; Andor Technology, Belfast, Northern Ireland) and processed with MetaMorph software (Universal Imaging, Downingtown, PA).

2.3. Disruption of the integrity of lipid microdomains in *C. elegans* sperm

To extract cholesterol from the sperm, different concentrations of methyl-ß-cyclodextrin (MCD, Sigma-Aldrich, St. Louis, MO) were added into the SM buffer for 10 min along with monensin (200 nM). Filipin (Sigma-Aldrich) was used to bind the cholesterol and thus decrease its dynamic distribution. D-threo-1-Phenyl-2-decanoylamino-3-morpholino-1-propanol (D-PDMP; Sigma-Aldrich) and N-butyldeoxyxojirimycin (NB-DNJ; Sigma-Aldrich) were applied to inhibit the synthesis of glycosphingolipids in *C. elegans* sperm.

Sialoglycosphingolipids, ganglioside GM3 or GM1 (Sigma-Aldrich) was applied in the presence of D-PDMP or NB-DNJ to test the inhibitor specificity during the sperm activation.

2.4. Detection of mating behavior and sperm transfer

The cholesterol-depleted males were cultured on single plates and soaked in MitoTracker Red overnight before mating with N2 hermaphrodites for 6 h. The hermaphrodites were observed under fluorescent microscopy (Carl Zeiss).

2.5. Membrane organelle fusion assay

FM1-43 (Invitrogen, Carlsbad, CA) was used to detect fusion of membrane organelles (MOs) with the plasma membrane of *C. elegans* sperm [18] after different treatments. Sperm were incubated with FM1-43 for 1 min, rinsed twice with SM buffer. DIC and rhodamine-filtered microscopic images were obtained as described above.

2.6. Immunoﬂuorescence

Sperm were fixed with 4% paraformaldehyde (EMS, Fort Washington, PA) and 0.1% glutaraldehyde (EMS) for 15 min, rinsed three times with phosphate buffered saline and then permeabilized with 0.3% Triton X-100 for 5 min. The fixed cells were blocked with 2% bovine serum albumin (Sigma-Aldrich) for 4 h to prevent non-speciﬁc binding. An antibody against UDP-Glucosyl Ceramide Glycophosphotransferase (U GCC; 1:200; Santa Cruz Biotechnology, Santa Cruz, CA) or caveolin-1(1:100; Abgent, CA) was added and incubated overnight at 4 °C followed by staining with a secondary antibody conjugated with Alexa Fluor 488 (1:400; Invitrogen) for 2 h at room temperature. Images were acquired using a confocal microscope (Leica Microsystems, Heidelberg, Germany).

2.7. Large scale culture of *C. elegans* and preparation of sperm

The large scale culture of *him-5* worms was carried out according to a standard protocol [19]. *C. elegans* sperm cells were dissected from isolated male worms and stored at —80 °C. Sperm lysates were analyzed by SDS/PAGE and Western blotting with an antibody against UDP-Glucosyl Ceramide Glycophosphotransferase (Santa Cruz Biotechnology, Santa Cruz, CA).

2.8. Immuno electron microscopy

The *him-5* male animals at L4 stage were picked to new plates to grow until young adults. The sperm were dissected into the SM buffer or the SM buffer containing monensin on Thermax Coverslips (Electron Microscopy Sciences). The cells were allowed to adhere to the coverslip for 15 min before being fixed with 4% paraformaldehyde and 0.2% glutaraldehyde overnight at 4 °C. The fixed sperm were rinsed with SM several times before serial ethanol dehydration at 4 °C. The samples were infiltrated with serial LR-White resin (Electron Microscopy Sciences), and LR-White polymerization was carried out under UV irradiation for 48 h at 4 °C. Areas of the plastic wafer containing well preserved sperm were excised and remounted with superglue onto plastic stubs. Ultrathin (~80 nm) sections were cut on Leica ultramicrotome and transferred to 200-mesh grids. Grids containing these sections were blocked with 20% goat serum and 0.5% Tween in PBS for 1 h, followed by incubation with caveolin-1 antibody (Abgent, 1:25) for 2 h. After rinsing, the grids were transferred to droplets of 10 nm immunogold conjugated goat anti-rabbit secondary antibody (Sigma, 1:20). The grids were stained with 2% uranyl acetate for 10 min before observation on an FEI Tecnai Spirit transmission electron microscope, operating at 120 kV.
3. Results

3.1. Cholesterol is required for C. elegans sperm activation in vitro and in vivo

The low abundance of cholesterol in nematode lipid extracts suggests that there is insufficient cholesterol to play a major structural role in nematode membranes [14]. However, lipid microdomains possessing similar physical characteristics to those observed in other organisms were detected in the nervous system of C. elegans [20]. These lipid microdomains are composed of cholesterol, sphingolipids and the proteins UNC-1, UNC-8 and UNC-24, suggesting that lipid microdomains might be assembled in a small subset of cells in C. elegans. A live cell-labeling assay with the fluorescent cholesterol analog dehydroergosterol showed that C. elegans sperm are enriched with cholesterol [16]. By staining with filipin, which is highly fluorescent and binds specifically to cholesterol, we showed cholesterol was enriched in the PM of non-activated sperm (spermatids) (Fig. 1, top paired images). The enrichment of cholesterol on the PM of spermatids implies that lipid microdomains might exist in spermatids and function during sperm activation. To test whether cholesterol in C. elegans spermatids might be assembled into lipid microdomains necessary for sperm function, we manipulated the integrity of the microdomains using methyl-β-cyclodextrin (MCD), a water-soluble cyclic oligomer that extracts cholesterol from the PM [21]. When spermatids dissected from males were incubated in medium containing the cationic ionophore monensin, an artificial activator [22], the motile spermatozoa with pseudopods were generated after 10 min (Fig. 1B). In contrast, when a range of concentrations of MCD was included with monensin, the sperm activation rate in the system decreased in a dose-dependent manner (Fig. 1B and C). At 25 mM MCD, only 4.6% of sperm were activated, compared with 60.4% activation in untreated controls (P < 0.05). At 27.5 mM MCD, monensin-induced sperm activation was totally inhibited and the cells retained their round symmetrical shape. However, when the sperm were treated with a mixture of 27.5 mM MCD and 2 mM cholesterol, the cells could be activated successfully (Fig. 1B). Fluorescent staining with filipin indicates that MCD incubation largely depleted membrane cholesterol from the sperm (Fig. 1D). To confirm that the inhibition of monensin-induced sperm activation was not an artifact of membrane cholesterol depletion by MCD, we also disrupted lipid microdomains using filipin, another lipid microdomain-disrupting agent, which is structurally different from MCD and sequestrates but not depletes cholesterol from the membrane [21,23]. Monensin-induced sperm activation was consistently inhibited by treatment with 400 μg/ml filipin for 10 min (Fig. 1E). Cholesterol efflux from the plasma membrane is required for mammalian sperm capacitation in the female reproductive tract. Interestingly, though the nematode sperm are crawling cells whose motility is totally different from that in flagellated sperm found in mammals, once they are fully activated, the cholesterol level on the PM of spermatodes decreased dramatically, esp. in the leading edge (Fig. 1A, bottom paired images), as reported during mammalian sperm capacitation [24,25]. These data suggest that cholesterol efflux might have broad phylogenetic conservation for sperm activation and male reproductive success. However, our data further indicate that the cholesterol on the PM of spermatids as a component of lipid microdomain is required for the initial signal transduction events during sperm activation before the cholesterol efflux occurs, though we cannot exclude the possibility that cholesterol might also function as a signal precursor inside the sperm cell during the sperm activation.

Mature C. elegans spermatids contain specialized membranous organelles (MOs), which is a type of intracellular vesicle with similarity to lysosomes [26]. The fusion of MOs with the PM during sperm activation is necessary for spermatozoan motility and male fertility in C. elegans [18,27] and this process is known to be a regulated exocytosis. Upon spermatic activation, the heads of the MOs fuse with the PM and their contents are released into the extracellular environment, leaving a permanent invaginated pore on the PM around the cell body periphery. Using the lipophilic dye FM1-43 that stains the outer leaflet of the PM [18,28], we were able to observe fusion of MOs with the PM (bright fluorescent puncta around the periphery of the cell body) in mock-treated sperm (Fig. 1F, left paired images). The MO fusion was usually inhibited when the sperm were treated with MCD (Fig. 1F, middle paired images) or filipin (Fig. 1F, right paired images). These data indicate that MO fusion with PM during the sperm activation is dependent on the signal transduction mediated by the lipid microdomains on the sperm membrane.

To further confirm our in vitro acute cholesterol depletion data, we cultured him-5 worm on the cholesterol free NGM for several generations. Animals deprived of cholesterol produced a reduced brood size in the second generation (F2) as reported [15], although the dissected spermatids from F2 males still could be activated partially in vitro by monensin (Fig. 1G). Most of the spermatids from F3 males cultured on cholesterol free NGM could not be activated in vitro by monensin and retained their round symmetrical shape (Fig. 1G). We further tested whether the sperm from F3 males grown on cholesterol free NGM could be activated by the endogenous activators in males and/or hermaphrodite by mating F3 males grown on cholesterol free NGM with normal N2 hermaphrodites. Unlike mock-depletion control where mitotracker-labeled male sperm were activated upon ejaculation and migrated into the spermatheca after mating (Fig. 1H, upper panels), the sperm from F3 males grown on cholesterol free NGM were found in the whole uterus of N2 hermaphrodite, some near the vulva (Fig. 1H, lower panels). These data indicate that the spermatids from F3 males grown on cholesterol free NGM are insensitive to either male-derived activators or hermaphrodite-derived activators, thus fail to respond to the chemotaxis signal derived from the oocyte [29]. In C. elegans, sperm are made in both males and self-fertile hermaphrodites. Hermaphrodites produce sperm during the fourth larval stage (L4) and produce oocytes as adults. Ovulation of the first oocyte pushes the stored spermatids from the gonad into the spermatheca. Once in the spermatheca, they are rapidly activated.

**Fig. 1.** Depletion of cholesterol from the plasma membrane inhibits C. elegans sperm activation. (A) DIC (left) and fluorescence (right) images of cells labeled with filipin for visualizing membrane cholesterol in spermatids (top) or spermatozoon (bottom). Bar = 5 μm. (B) MCD treatment inhibited C. elegans sperm activation. Cells were treated with monensin and a range of concentrations of MCD for 10 min. The cells with pseudopods (indicated by arrows) are spermatids and the round ones are spermatids. Bar = 5 μm. (C) Quantification of sperm activation as shown in (B). Results represent the mean ± standard deviation (SD, n = 3 experiments). *P < 0.05, **P < 0.01. (D) Treatment with MCD decreased the amounts of cholesterol indicated with filipin. C. elegans sperm were dissected in sperm medium (SM) alone or SM containing 27.5 mM MCD for 30 min before being fixed with 4% paraformaldehyde for 1 h. The fixed cells were stained with filipin for 2 h. Bars = 5 μm. (E) Pretreatment with filipin for 10 min abolished monensin-induced sperm activation. Bar = 5 μm. (F) MO fusion was inhibited obviously when the sperm were treated with MCD and filipin. Bars = 5 μm. (G) The activation of sperm from males in F3 progeny cultured on cholesterol free NGM plates was inhibited. Spermatids were dissected from males and treated with monensin. Control, sperm from him-5 males cultured on normal NGM plates. Bar = 5 μm. (H) The sperm transfer assay showed that sperm from F3 males cultured on cholesterol free NGM plates had motility defects when mating with hermaphrodites. Upper: N2 hermaphrodites mated with normal him-5 males that were labeled with Mitotracker Red; Lower: N2 hermaphrodites mated with F3 him-5 males cultured on cholesterol free NGM plates and labeled with Mitotracker Red. Bar = 50 μm. (I) The sperm from hermaphrodites cultured on cholesterol free NGM plates showed motility defects. Upper: hermaphrodites cultured on normal NGM plates (left) and their sperm dissected (right); Lower: hermaphrodites cultured on cholesterol free NGM plates (left) and their sperm dissected (right). The arrows indicated the sperm accumulated in spermatheca. Bar in left = 25 μm, bar in right = 10 μm.
into spermatozoa. We found that most of the dissected sperm from F3 hermaphrodites grown on cholesterol free NGM were non-activated and retained their round shape (Fig. 1I, lower right), while sperm dissected from control hermaphrodites were motile with clear pseudopod extensions (Fig. 1I, upper right). Compared to control hermaphrodites (Fig. 1I, upper left), F3 hermaphrodites grown on cholesterol free NGM contained obviously less sperm (Fig. 1I, lower left), probably because the ovulation pushed the non-activated spermatids out of spermatheca and due to lack of motility they could not crawl back to the spermatheca. These data provide direct evidence that cholesterol depletion also affects self sperm activation triggered by endogenous activator(s) in hermaphrodite.
Due to their size being below the classical diffraction limit of a light microscope, lipid microdomains ranging from 10–200 nm in size have proved difficult to visualize directly. Caveolae, small surface invaginations seen in many cell types, are specialized lipid microdomains that perform a number of signalling functions. The permanent invaginated pore was formed (Fig. S1E) when MO fused with the PM during sperm activation, suggesting that caveolae-like structure might be involved for signal sorting and functions. To test this hypothesis, we employed indirect immunofluorescence microscopy and immuno-EM to examine the localization of caveolin-1 (CAV-1), a widely used marker to indicate the lipid microdomains. As shown in Fig. S1A and B, CAV-1 could be detected in both spermatid and spermatozoon by immunofluorescence microscopy. CAV-1 was localized in the subcellular compartments in the spermatid (Fig. S1A), while in the spermatozoon, the CAV-1 was clustered and enriched in the periphery of cell body, where MO fusion took place. Subcellular compartment of CAV-1 was further demonstrated using immuno-EM. Immuno-gold was exclusively localized in the non-fused or fused MOs in both spermatid (Fig. S1C) and spermatozoon (Fig. S1D). Intriguingly, the distribution pattern of the CAV-1 (Fig. S1B) is similar to that of the cholesterol in spermatozoon (Fig. 1, A, bottom paired images), indicating that lipid microdomains composed of cholesterol and CAV-1 might function during sperm activation. Taken together, these results suggest that cholesterol in the spermatids is required for sperm activation in C. elegans, and that it participates in this process mainly as a structural component of lipid microdomains.

Currently it is believed that cholesterol functions primarily in cell signaling in C. elegans, possibly as a precursor to steroid hormone(s) or to other physiologically active compounds, and instead of a structural component of the PM. This is based on the following observations: first, cholesterol constitutes only about 1% of the total lipid content of embryo extracts; second, tiny amounts of cholesterol are able to restore the development of cholesterol-depleted arrested larvae into fertile adults. Our data suggest that cholesterol functions as a structural component of lipid microdomains in C. elegans spermatids and that their integrity is important for sperm activation.

Because cholesterol and the integrity of lipid microdomains seem to be essential for C. elegans sperm activation (Fig. 1), we next examined whether other lipid microdomain components, glycosylphosphatidylinositol (GPI)-anchored proteins and glycosphingolipids (GSLs) are involved in sperm activation.

3.2. GPI-anchored proteins are required for C. elegans sperm activation

In eukaryotic cells, some cell surface proteins are anchored to the exoplasmic face of the PM via covalent attachment of a glycosylphosphatidylinositol (GPI) anchor. GPI-anchored proteins, with their unique biochemical and biophysical characteristics during the signal transduction, associate with glycosphingolipids and cholesterol to form detergent-resistant lipid microdomains in the outer leaflet of the PM. GPI-anchored proteins are often cross-linked and such cross-linking may stabilize small microdomains and, by coalescence with each other, could generate larger lipid microdomains involved in signaling [30]. The GPI anchor can be cleaved by phosphatidylinositol-specific phospholipase C (PI-PLC) [31], thus releasing the protein from the membrane surface in a soluble form. If GPI-anchored proteins are essential to signal transduction during sperm activation, cleaving these proteins by PI-PLC would result in the failure of sperm activation. This is indeed what we observed (Fig. 2A and B). Only 6.9 % of sperm were activated by monensin if the spermatids were first incubated with 25 U/mL PI-PLC for 60 min, significantly lower than that in the mock treatment (56.0%, P < 0.001, Fig. 2B). The activated sperm subjected to the PI-PLC treatment also showed an aberrant activation phenotype: i.e., the spermatids initiated sperm activation but failed to extend normal-looking pseudopods (Fig. 2A).

We also tested whether cleavage of GPI-anchored protein inhibits MO fusion during sperm activation. We were able to observe fusion of MOs with the PM (bright fluorescent puncta around the periphery of the cell body) in mock-treated sperm (Fig. 2C, left paired images). When sperm were incubated with PI-PLC, followed by monensin treatment, the fusion of MOs with the PM was dramatically inhibited and fluorescence was only detected in an even distribution around the cell body (controls (left paired images). In spermatids pretreated with 25 U/mL PI-PLC, only the PM is stained. Bars = 5 μm.
for *C. elegans* sperm activation; these proteins participate in both MO fusion and assembly of the MSP cytoskeleton during this process.

### 3.3 Glycosphingolipid (GSL) biosynthesis is essential for *C. elegans* sperm activation and sperm migration

#### 3.3.1 UDP-glucose ceramide glucosyltransferase (UGCG) is detected in *C. elegans* sperm

GSLs comprise a group of membrane components containing sugar and lipid moieties. GSLs are thought to be vertically translocated from cytoplasmic leaflet to the exoplasmic leaflet of the PM by unidentified GSL-specific flippase [32]. The localization of GSLs on the exoplasmic leaflet is important in the assembly of signaling molecules and GPI-anchored proteins into lipid microdomains during signal transduction [33]. The precursor of most GSLs, glucosylceramide, is synthesized from ceramide and catalyzed by UGCG (UDP-glucose ceramide glucosyltransferase or ceramide glucosyltransferase—CGT—in *C. elegans*) [34,35]. Strong UGCG signal was detected in *C. elegans* sperm when fused with GFP [36]. As shown by immunoblot (Fig. S2A) and immunofluorescence microscopy (Fig. S2B), UGCG was detected in the isolated sperm.

#### 3.3.2 Inhibition of GSL synthesis blocks *C. elegans* sperm activation

The content of GSLs was reduced in cells treated with D-threo-1-Phenyl-2-decanoylamino-3-morpholino-1-propanol (D-PDMP), a preferential substrate of UGCG. In the presence of D-PDMP, sphingomyelins rather than GSLs were synthesized by UGCG [37]. Incubation of *C. elegans* spermatids with D-PDMP blocked both pseudopod formation and membrane fusion during monensin-induced sperm activation (Fig. 3A–C). At 0.3 mM and 0.4 mM D-PDMP, only 8.8% and 6.9% of cells extended pseudopods, respectively; significantly lower than in controls (39.9%, P < 0.05). At 0.4 mM D-PDMP, more than 33% of the cells were arrested at the spiky stage, an intermediate phase of sperm activation in which MSP bundles project, but fail to fuse with the PM.

![Fig. 3. Inhibition of GSL biosynthesis blocked pseudopod formation and membrane fusion of *C. elegans* sperm while exogenous GSLs rescued these processes. (A) D-PDMP inhibited *C. elegans* sperm activation. Cells were treated with monensin and a range of concentrations of D-PDMP for 10 min. The white arrow indicates a spike. The rightmost panel shows that D-PDMP exhibited no cytotoxicity to *C. elegans* sperm. Activation was rescued when the cells were first treated with 1 mM D-PDMP and monensin for 10 min and then perfused with monensin. (B) Quantitative analysis of pseudopod or spike formation in *C. elegans* sperm as shown in (A). Black bars show cells with pseudopods; grey bars show cells with spikes. The values are the mean ± SD (n = 3 experiments). *P < 0.05; **P < 0.001. (C) MO fusion with the PM was inhibited by D-PDMP in a dose-dependent manner. The cells were stained with FM1-43 for 1 min before observation. (D) Exogenous GSLs rescued the D-PDMP-inhibited sperm activation. Cells were treated with monensin, 1 mM D-PDMP + monensin, 1 mM D-PDMP + 0.15 mM GM1 + monensin, 1 mM D-PDMP + 0.15 mM sialidase-treated GM1 + monensin, or 1 mM D-PDMP + 0.15 mM GM3 + monensin. (E) Exogenous GSLs rescued the D-PDMP-inhibited sperm migration. The time-course of sperm migration after perfusion with D-PDMP or GM1 in the presence of D-PDMP was recorded. The images were extracted from the movie S2. (F) Quantitative analysis of sperm migration in (E). Data represent the mean ± SD (n = 3 experiments). **P < 0.001. Bars = 5 μm.](image-url)
coalesce into a pseudopod. Pseudopod extension was totally blocked by 0.5 mM D-PDMP, while 1 mM blocked both pseudopod and spike extension. We infer that the inhibition of sperm activation by D-PDMP was caused by decreased GSLs and not by cellular toxicity, because the inhibition at 1 mM D-PDMP could be rescued by monensin perfusion, leading to pseudopod extension (Fig. 3A, rightmost panel). MO fusion during sperm activation was inhibited by D-PDMP in a dose-dependent manner and 1 mM D-PDMP treatment totally blocked fusion of MOs with the PM, as shown by FM 1–43 staining (Fig. 3C). We also found that cells treated with another UGCG inhibitor, N-butyldideoxyojirimycin (NB-DNJ) [38], manifested defects in pseudopod extension (Fig. S3A, B) and in MO fusion with the PM (Fig. S3C) in a manner similar to D-PDMP treatment during sperm activation. These results demonstrate that GSLs are crucial for *C. elegans* sperm activation, and that they function in both pseudopod formation and membrane fusion during this process.

When monensin-activated spermatozoa were perfused with D-PDMP, the MSP cytoskeleton in pseudopods disassembled and the leading edge retracted toward the cell body, resulting in rounding of the spermatozoa. Crawling capability was restored when D-PDMP was washed out (Movie S1), further suggesting that the biosynthesis of GSLs play a role in localized MSP cytoskeleton assembly in *C. elegans* sperm.

### 3.3.3. Exogenous GSLs rescue blockage including pseudopod formation and membrane fusion

When exogenous GSLs (sialoglycosphingolipid, ganglioside GM3 or GM1) were applied, we observed that they were able to rescue the inhibitory effects of D-PDMP on sperm activation (Fig. 3D). Whatever the order of application of D-PDMP, GM1 or monensin, exogenous GM1 could rescue the inhibitory effects of D-PDMP on membrane fusion and pseudopod extension during monensin-induced sperm activation (Fig. 4A). Neither pseudopods nor MO fusion could be observed in controls where exogenous GM1 was absent (Fig. 4A). Exogenous GSLs also rescued sperm activation inhibited by NB-DNJ (Fig. S3A, B). In addition, exogenous GM1 in the presence of D-PDMP rescued D-PDMP-inhibited sperm migration (Fig. 3E, F and Movie S2). To confirm that the exogenous GSLs were incorporated into the sperm PM, sperm were incubated with bodipy-GM1 for 10 min. The bright fluorescent signal of bodipy-GM1 was detected around the periphery (Fig. 4B, left paired images). Like the cholesterol staining patterns in both spermatids and spermatozoa, the bodipy-GM1 was enriched in the spermatid PM with a relatively evenly distribution pattern, while in the spermatozoon the highest fluorescent signal was detected in the periphery of cell body though with detectable signal in the leading edge PM of spermatozoon (Fig. 4B, left paired images). In the presence of 10-fold unlabeled GM1, however, the fluorescent intensity of bodipy-GM1 in sperm decreased dramatically (Fig. 4B, right paired images), indicating that GSLs had been incorporated into the sperm surface. Interestingly, when exogenous GM1 was applied in the absence of D-PDMP, the cells were activated aberrantly by monensin and failed to extend normal-looking pseudopods (Fig. 4C). It is possible that the addition of exogenous GM1 excluded other lipid microdomain-associated proteins thus blocking correct signal transduction during sperm activation. Thus, a balance of GSLs might be important for maintaining the integrity and stability of lipid microdomains required for localized signal modulation. Because the sialic acid synthesis pathway is absent in *C. elegans* [39], we used sialidase to hydrolyze terminal sialyl residues from GM1. Sialidase-treated GM1 rescued D-PDMP-inhibited sperm activation similarly to GM1 and GM3 (Fig. 3D), indicating that the lipid moiety rather than the sugar moiety of GSLs was responsible for *C. elegans*.
vesicle fusion during Ca2+-induced muscle membrane repair. Dysferline contains C2 domains characteristic of synaptotagmin, is required for the human homolog of FER-1, the protein dysferlin, containing re-
crease in the membrane
increase the biophysical properties of sperm membrane leading to an
crease. This might act as a second messenger that relays and ampli-
sification during sperm activation and motility acquisition since the leading edge of spermatozoa showed less cholesterol staining by filipin (Fig. 1A, bot-
tom paired images). However, before this occurs, the cholesterol, GSLs and GPI-anchored proteins assembling into lipid microdomain is required in mediating initial localized signal transduction during sperm activation and sperm motility acquisition. The C/PL ratio decreases should be the downstream events during the signal transduction in sperm activation. Lipid rafts were isolated and their reorganization was observed in mammalian flagellated sperm [45,46]. An interesting avenue for future investigation may be to de-
termine whether the initial signaling mediated by the lipid microdo-
mains is required for sperm maturation in other animal species besides C. elegans.

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