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Caenorhabditis elegans sperm membrane protein interactome

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Abstract

The interaction and organization of proteins in the sperm membrane are important for all aspects of sperm function. We have determined the interactions between 12 known mutationally defined and cloned sperm membrane proteins in a model system for reproduction, the nematode *Caenorhabditis elegans*. Identification of the interactions between sperm membrane proteins will improve our understanding of and ability to characterize defects in sperm function. To identify interacting proteins, we conducted a split-ubiquitin membrane yeast two-hybrid analysis of gene products identified through genetic screens that are necessary for sperm function and predicted to encode transmembrane proteins. Our analysis revealed novel interactions between sperm membrane proteins known to have roles in spermatogenesis, spermiogenesis, and fertilization. For example, we found that a protein known to play a role in sperm function during fertilization, SPE-38 (a predicted four pass transmembrane protein), interacts with proteins necessary for spermiogenesis and spermatogenesis and could serve as a central organizing protein in the plasma membrane. These novel interaction pairings will provide the foundation for investigating previously unrealized membrane protein interactions during spermatogenesis, spermiogenesis, and sperm function during fertilization.

Summary Sentence

Novel interactions between 12 *Caenorhabditis elegans* sperm membrane proteins were identified using split-ubiquitin membrane yeast two-hybrid analyses to provide insight into membrane protein order during sperm development and function.

Key words: gamete biology, gametogenesis, sperm, sperm maturation, spermatid, spermatocyte.

Introduction

Proper organization and localization of membrane proteins is necessary for sperm function. Despite the importance of membrane organization, our knowledge of how proteins are trafficked to the membrane, organized in the membrane, and reorganized during navigation of the reproductive tract is not well characterized. Both sperm and eggs contain specialized membrane regions that are necessary for function [1–7]. Multiprotein complexes have been proposed to mediate sperm function, particularly fusion [8–11]. To understand the protein complexes that form in the sperm membrane, we tested all possible interactions between 12 known sperm membrane proteins in the nematode *Caenorhabditis elegans*.

Sperm formation requires proper temporal progression of spermatogenesis and coordination of multiple individual and overlapping processes [12]. In meiosis I of *C. elegans* spermatogenesis, a primary spermatocyte divides into two secondary spermatocytes [13]. Then during meiosis II those secondary spermatocytes divide into two haploid spermatids each that are connected by a residual body [14]. The proper distribution of cellular components to the spermatids is aided by a fibrous body (FB)-membranous organelle (MO) complex [13, 14]. The MOs are Golgi-derived vesicles, as is the acrosome in mammalian sperm, that are localized near the plasma membrane of spermatids [13]. During spermiogenesis or postmeiotic sperm development, *C. elegans* sperm undergo a morphological change from a quiescent, round spermatid to an amoeboid sperm and gain motility, and the MOs fuse with the plasma membrane [13]. Membranous organelle fusion is viewed as being similar to the acrosome reaction [15]. The sperm development events that occur between anaphase II and residual body formation are analogous to spermiogenesis in other organisms, including vertebrates [12]. After spermiogenesis, the sperm are functional and can travel to the site of fertilization (the spermatheca) and fertilize the oocyte [13].

There are multiple mutationally defined, cloned, and well-characterized genes that are necessary for each developmental step of sperm development in *C. elegans*. Twelve of these genes encode proteins with predicted transmembrane domains (*wee-1.3*, *spe-4*, *spe-10*, *fer-1*, *spe-12*, *spe-19*, *spe-29*, *spe-9*, *spe-38*, *spe-41/trp-3*, *spe-42*, and *spe-45*) (Figure 1). Amongst these 12 genes, previous studies have only documented genetic interactions between three pairs: (1) *spe-38* and *spe-41/trp-3* [16], (2) *fer-1* and *spe-38* [17], and (3) *fer-1* and *spe-41/trp-3* [18]. Furthermore, only one pair of proteins, SPE-38 and SPE-41/TRP-3, has been shown to interact physically through split-ubiquitin yeast two hybrid assays [16]. Physical interactions between the remainder of the proteins encoded by these 12 genes have not been investigated. The lack of physical interaction data limits the ability to develop models of sperm membrane organization that can then be tested.

Previous high-throughput *C. elegans* interactome analyses have been conducted using yeast two hybrid assays [19, 20]. Unfortunately, transmembrane proteins are often not functional in traditional yeast two hybrid because they require insertion into a membrane; therefore, these datasets have not been helpful in developing models of the sperm membrane organization. For example, querying

the 3864 known binary protein–protein interactions in the Worm Interactome database version 8 with the 12 sperm transmembrane proteins tested results in only a single positive interaction: SPE-10 and ABU-1 [20].

To develop a model for potential sperm membrane protein interactions that would increase our understanding of sperm membrane organization during development and function, we assayed the pairwise interactions between 12 full-length sperm transmembrane proteins using split-ubiquitin membrane yeast two hybrid (MYTH) assays. Our data reveal the potential of many of the sperm membrane proteins to interact with one another and provide evidence for the complexity of membrane interactions during sperm development. Network analysis reveals a sperm membrane cluster that could play a central role in organizing proteins at different stages of sperm development. Finally, our data provide the first comprehensive dataset about physical interactions between sperm membrane proteins and generate hypotheses about sperm membrane protein interactions that can be tested further.

Materials and methods

Yeast-two hybrid

The Dualsystems Biotech (Zurich, Switzerland) DUALmembrane Yeast Two-Hybrid System was used to perform the split-ubiquitin MYTH analyses. MYTH analysis is based on a split-ubiquitin approach where two distinct fragments of ubiquitin are fused to two proteins of interest. When an interaction occurs, ubiquitin is reconstituted, recognized by a deubiquitinating enzyme, and cleaved to release a transcription factor. The transcription factor then enters the nucleus and activates a reporter gene. An advantage MYTH analysis is that allows for the detection of full-length proteins in a membrane environment; however, it does have some of the disadvantages of classical yeast two hybrid including expression, autoactivation, and false positives [21, 22]. All assays were performed as per the manufacturer's instructions.

Twelve full-length genes necessary for spermatogenesis, spermiogenesis, or fertilization (*fer* and *spe* genes) that encode proteins with at least one predicted transmembrane domain (*wee-1.3*, *spe-4*, *spe-10*, *fer-1*, *spe-12*, *spe-19*, *spe-29*, *spe-9*, *spe-38*, *spe-41/trp-3*, *spe-42*, and *spe-45*) were cloned into the appropriate expression vectors based on their predicted or reported transmembrane domain structure (Figure 2). Please see the supplementary materials for primers and plasmids used for constructing the bait and prey plasmids (Supplementary Table S1). Cloning was performed using the SfiI restriction sites for both plasmid and PCR products. All genes were assayed as both bait and prey and for homotypic interactions.

Bait expression was confirmed by the DUALmembrane expression control assay. To confirm proper expression, each bait construct is co-transformed into the yeast strain NMY51 with either pAI-Alg5 or pDL2-Alg5 prey vectors. The pAI-Alg5 vector expresses a wild-type version of the N-terminal domain of ubiquitin (NubI) and pDL2-Alg5 expresses a mutant version of the N-terminal domain
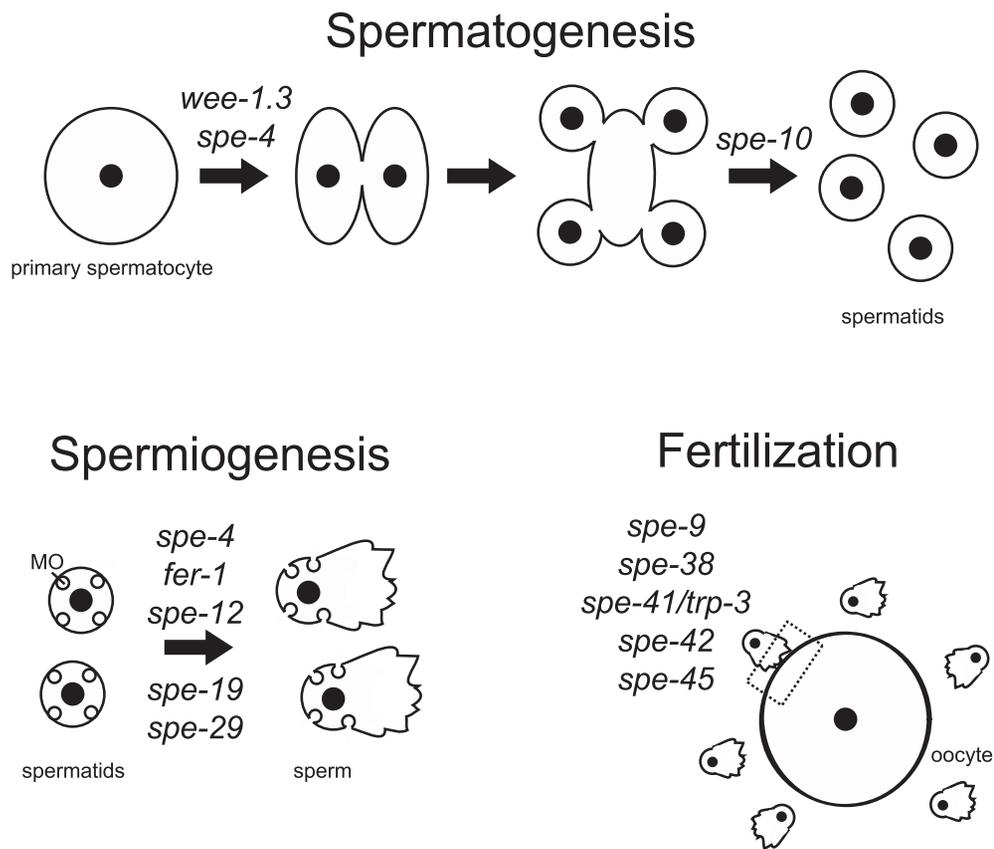
Spermatogenesis

wee-1.3
spe-4

spe-10

primary spermatocyte

spermatids

Spermiogenesis

spe-4
fer-1
spe-12

spe-19
spe-29

MO

spermatids

sperm

Fertilization

spe-9
spe-38
spe-41/trp-3
spe-42
spe-45

oocyte

Figure 1. Diagram of sperm development and function in *C. elegans*. During sperm development in *C. elegans*, primary spermatocytes divide into two secondary spermatocytes, which divide into two haploid spermatids each that are connected by a residual body [13, 14]. The membranous organelles (MOs) are Golgi-derived vesicles that are located near the plasma membrane of spermatids [13]. During spermiogenesis or post-meiotic sperm development, *C. elegans* sperm undergo a morphological change from a quiescent, round spermatid to an amoeboid sperm and gain motility, and the MOs fuse with the plasma membrane [13]. After spermiogenesis, the sperm are functional and can travel to the site of fertilization and fertilize the oocyte [13]. The approximate functional timing of the sperm genes that encode transmembrane proteins are indicated above the appropriate stage.
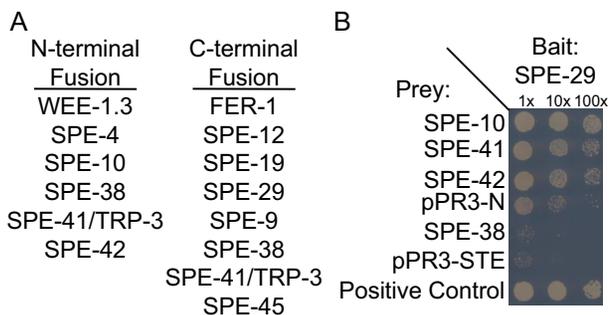
A

N-terminal Fusion	C-terminal Fusion
WEE-1.3	FER-1
SPE-4	SPE-12
SPE-10	SPE-19
SPE-38	SPE-29
SPE-41/TRP-3	SPE-9
SPE-42	SPE-38
	SPE-41/TRP-3
	SPE-45

B

Bait: SPE-29

Prey: SPE-10, SPE-41, SPE-42, pPR3-N, SPE-38, pPR3-STE, Positive Control

Figure 2. Sample results from a MYTH system to assay membrane interactions between sperm proteins. A split-ubiquitin based membrane yeast two-hybrid (MYTH) system was employed to detect interactions between SPE proteins containing a transmembrane domain. (A) Genes necessary for sperm development and function were cloned into expression vectors based on their predicted membrane topology in order to generate a protein with split-ubiquitin fused to the cytosolic terminus of the protein. (B) Yeast expressing the indicated bait and prey vectors were plated at three different dilutions for each experiment (1×, 10×, 100×). Empty prey vectors (pR3-N and pPR3-STE, respectively) were used to determine the background interactions. The positive control expresses a fusion of the yeast ER protein Alg5 to the N-terminus of ubiquitin (Nub).

of ubiquitin with an Isoleucine to Glycine substitution at position 13 (NubG). If the bait protein is properly expressed along with NubI, it will reconstitute ubiquitin and lead to activation of reporter genes and colony growth on strong selection synthetic medium lacking leucine, tryptophan, histidine and adenine and will turn blue in the X-gal assay. Colony growth after co-expression of bait and NubI indicates the bait is functional and can be used in the analysis. Co-expression of bait with pDL-2 Alg5 should not activate reporter genes and result in little or no growth on strong selection medium. Any growth that is seen after co-expression with bait and pDL-Alg5 is considered background growth. 3-Amino-1,2,4-triazole was added to the medium to reduce the number of background colonies when necessary (Supplementary Table S2). All bait constructs were properly expressed, except SPE-41/TRP-3 bait that was cloned into the pBT-3-N vector (pBT3-N-SPE-41-N). As a result, pBT3-N-SPE-41-N was not included in the analysis.

After confirming bait expression, yeast containing the confirmed bait construct were transformed with prey plasmid and selected on synthetic medium lacking leucine and tryptophan. The resulting strain harboring both bait and prey plasmids was examined for their ability or inability to turn on the reporter genes and by their growth on synthetic medium lacking tryptophan, leucine, histidine, and adenine, and the number of colonies was recorded (Supplementary Table S2). Yeast expressing the indicating bait and prey vectors were plated

Table 1. Summary of sperm membrane protein MYTH interactions.

| | | Spermatogenesis | | | Spermiogenesis | | | | Fertilization | | | | |
		WEE-1.3	SPE-4	SPE-10	FER-1	SPE-12	SPE-19	SPE-29	SPE-9	SPE-38	SPE-41	SPE-42	SPE-45
Spermatogenesis	WEE-1.3	-											
	SPE-4	-	+										
	SPE-10	-	+	+									
Spermiogenesis	FER-1	-	+	+	-								
	SPE-12	-	-	-	-	-							
	SPE-19	-	-	-	-	-	-						
	SPE-29	-	+	+	-	-	-	+					
Fertilization	SPE-9	-	-	-	-	-	-	-	-				
	SPE-38	-	+	+	+	-	+	+	+	+			
	SPE-41	-	+	+	-	-	-	+	-	+	+		
	SPE-42	-	-	+	+	-	-	+	+	+	+	-	
	SPE-45	-	-	-	-	-	-	-	-	-	-	-	+

(+ = evidence of interaction in at least two dilutions in at least two separate experiments; – = no evidence of interaction).

at three different dilutions for each experiment (1×, 10×, 100×). Yeast cells transformed with bait construct and wild-type NubI from the pAI-Alg5 prey vector serve as a positive control for each replicate. Yeast cells transformed with bait construct and mutant NubG expressed from pDL2-Alg5 vector serve as negative control. The appropriate empty vector was also used as a negative control for each prey plasmid.

Colony quantification and interaction score determination

Colonies were counted using a dissecting microscope. Experimental samples were compared to the appropriate empty vector control to determine the number of colonies counted above background. The ratio of experimental colonies to empty vector colonies was computed, and this ratio is referred to as interaction score. Experimental groups with an interaction score greater that two were considered to have evidence indicating a positive interaction. If the empty vector control exhibited no colony growth, then the absolute number of colonies for the experimental group was equated to interaction score for those samples and only groups with greater than two colonies were considered positive. To be scored as a positive interaction, pairs had to show evidence of interactions in at least two dilutions in at least two independent experiments on two dates. All colony count data were entered in to Microsoft Excel. See supplementary materials for all raw data (Supplementary Table S2)

Network analysis

The sperm membrane protein interactome was imported into Cytoscape v3.4.0 (Supplementary Table S3) [23]. Duplicate edges (ignoring edge direction) and self-loops (homotypic interactions) were removed for network analysis. The network was treated as undirected, and NetworkAnalyzer was used to generate all network attributes under default settings (Supplementary Table S3). Interaction modules were detected using the MCODE (Molecular Complex

Detection) v1.4.2 Cytoscape Application (Supplementary Table S4) [24].

Results

Twelve of the cloned *fer* and *spe* genes that are necessary for spermatogenesis, spermiogenesis, and fertilization and encode proteins with transmembrane domains were assayed using the split-ubiquitin MYTH system. Positive interactions were scored and quantified based on the number of colonies that grew on restricted medium plates (Figure 2). Of the possible 93 total heterotypic interactions between the 12 proteins, there were 21 interactions (22.6%) (Table 1). Furthermore, 6 of the 12 proteins (SPE-4, SPE-10, SPE-29, SPE-38, SPE-41/TRP-3, and SPE-45) displayed the ability to interact homotypically.

The nine proteins that did interact with other proteins heterotypically (SPE-4, SPE-10, FER-1, SPE-19, SPE-29, SPE-9, SPE-38, SPE-41/TRP-3, and SPE-42) define the sperm membrane protein interaction network or interactome (Figure 3). WEE-1.3, SPE-12, and SPE-45 did not interact with any of the other proteins tested, though SPE-45 did show the ability to interact homotypically. The resulting interactome has a density of 0.583, meaning that 58.3% of all possible interactions are realized in the network (Supplementary Table S4). The MCODE algorithm was used to identify interaction modules in the interactome. This analysis revealed that six of the interacting proteins (SPE-4, SPE-10, SPE-29, SPE-38, SPE-41/TRP-3, and SPE-42) form a cluster (Figure 3). These six proteins have 14 interactions between them and this cluster has both a density and correlation coefficient of 0.933 (Supplementary Table S4).

SPE-38 interacted with all nine proteins in the interactome, but not with WEE-1.3, SPE-12, and SPE-45 (Table 1). The high level of connectivity of SPE-38 is reflected in a betweenness centrality of 0.38, which is the highest for all interactome members (Table 2). If a member of a community has high betweenness centrality, they have a high probability of being necessary for to two neighbors to interact [25, 26]. SPE-38 is the only common interaction for all three proteins
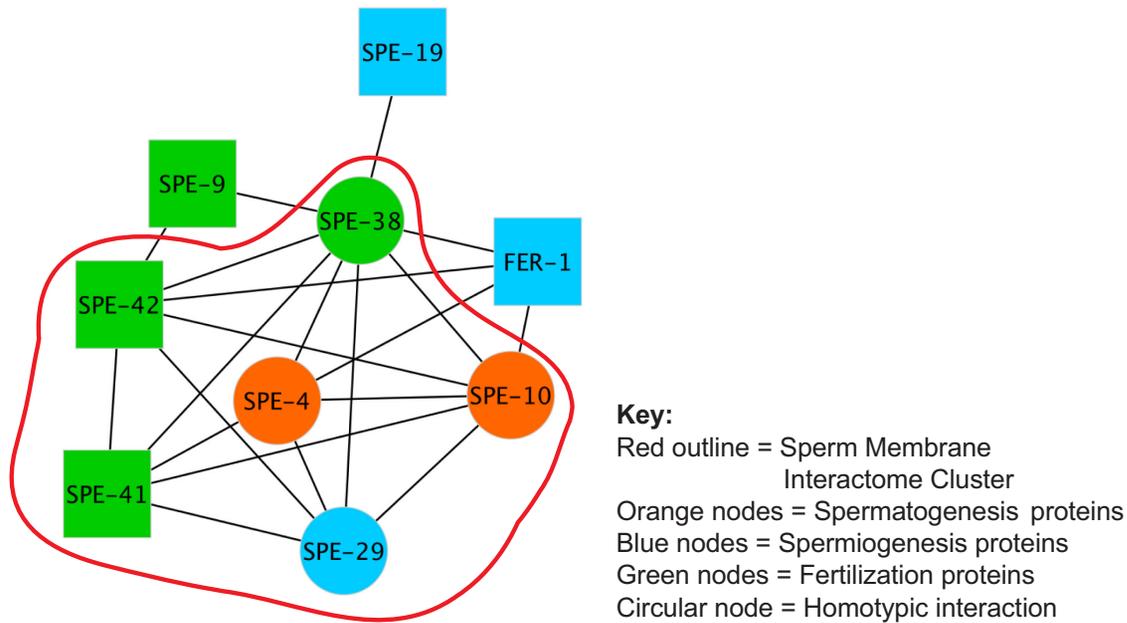
Figure 3. *Caenorhabditis elegans* sperm membrane protein interactome. Network diagram generated from MYTH interaction data. The *C. elegans* sperm membrane interactome cluster is demarcated by a red outline. [blue nodes = genes necessary for spermiogenesis; orange nodes = genes necessary for spermatogenesis; green nodes = genes necessary for fertilization; circular nodes = proteins capable of homotypic interaction (self-looping node); square node = proteins not capable of homotypic interaction].

Table 2. Summary of *C. elegans* sperm membrane protein interactome network measures.

Protein	Degree	Betweeness centrality	Closeness centrality	Neighborhood connectivity	Clustering coefficient	Topological coefficient
SPE-38	8	0.38	1	4.25	0.46	0.59
SPE-10	6	0.025	0.8	5.5	0.8	0.69
SPE-42	6	0.089	0.8	5	0.6	0.63
SPE-4	5	0.018	0.73	5.6	0.8	0.7
SPE-29	5	0.0071	0.73	6	0.9	0.75
SPE-41/TRP-3	5	0.0071	0.73	6	0.9	0.75
FER-1	4	0.0071	0.67	6.25	0.83	0.78
SPE-9	2	0	0.57	7	1	0.88
SPE-19	1	0	0.53	8	0	0

outside of the sperm membrane interactome cluster (FER-1, SPE-9, and SPE-19). The interaction of SPE-38 with these three proteins outside of the cluster results in a high neighborhood connectivity for FER-1, SPE-9, and SPE-19, despite their low number of direct interactions (Table 2). Neighborhood connectivity is a measure of how connected the neighbors are, not the individual protein itself [27].

Discussion

The sperm transmembrane protein interaction analysis revealed that the proteins examined have the potential to form multi-protein complexes in the membrane. The cluster of proteins including SPE-4, SPE-10, SPE-29, SPE-38, SPE-41/TRP-3, and SPE-42 could represent a key group of proteins that interact in the membrane at various stages of sperm development. Of these proteins in the cluster, *spe-4* and *spe-10* are necessary for spermatogenesis, *spe-29* is necessary for spermiogenesis, and *spe-38*, *spe-41/trp-3*, and *spe-42* are necessary for fertilization (Figure 1). Despite the genetic data indicating

distinct roles for these genes during sperm development, these data indicate that these proteins can interact physically.

Prior to this study, there were no published data indicating the physical interaction between any full-length sperm membrane proteins. However, previous studies by Singaravelu et al. identified an interaction between a truncated version of SPE-38 and SPE-41/TRP-3 [16]. In those studies, Singaravelu et al. used full-length SPE-38 with a ubiquitin fusion on the C-terminus and found no interaction with full-length SPE-41/TRP-3 with a ubiquitin fusion on the C-terminus [16], which we confirmed in this study. However, we did detect interactions when ubiquitin was fused at the C-terminus of SPE-38 and the N-terminus of SPE-41/TRP-3 and vice versa. This indicates that the terminal that the ubiquitin fusion is placed on can impact the detection of interaction in the membrane.

The protein–protein interaction data verify the previously reported genetic data for both *spe-38* interacting with *spe-41/trp-3* and *fer-1* interacting with *spe-38* interactions. Epistasis experiments indicated that *spe-38* and *spe-41/trp-3* interact and in *spe-38(eb44)* mutants, SPE-41/TRP-3 are mislocalized [16]. As discussed above, our data indicate that SPE-38 and SPE-41/TRP-3 interact physically.
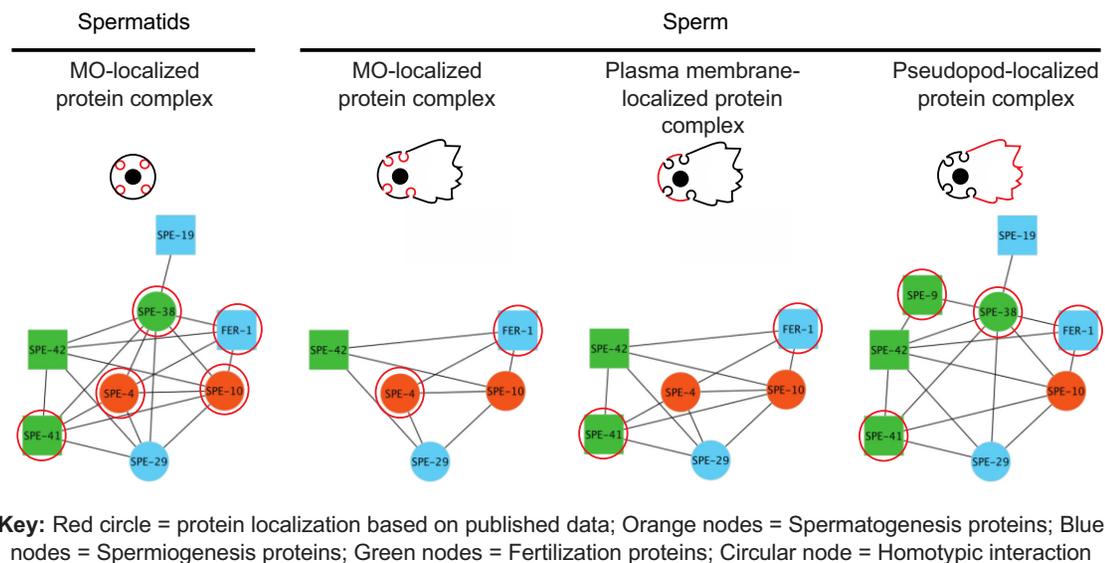
Key: Red circle = protein localization based on published data; Orange nodes = Spermatogenesis proteins; Blue nodes = Spermiogenesis proteins; Green nodes = Fertilization proteins; Circular node = Homotypic interaction

Figure 4. *Caenorhabditis elegans* sperm membrane interactions during development. Sperm protein complex composition changes depending on the stage of sperm development. In spermatids, FER-1, SPE-4, SPE-10, SPE-38, and SPE-41/TRP-3 are localized to the MO (circled in red). The data presented here indicated that these proteins interact with SPE-19, SPE-29, and SPE-42 and form a sperm membrane complex in the MO of spermatids. Similar sperm membrane complex models are presented for MO, plasma membrane, and pseudopod localization by adding the interaction data presented here with the known protein localization. [red circle = protein localization based on published data; blue nodes = genes necessary for spermiogenesis; orange nodes = genes necessary for spermatogenesis; green nodes = genes necessary for fertilization; circular nodes = proteins capable of homotypic interaction (self-looping node); square node = proteins not capable of homotypic interaction].

Therefore, the mislocalization of SPE-41/TRP-3 in *spe-38(eb44)* mutants could be because SPE-38 and SPE-41/TRP-3 can no longer interact normally in sperm. Additionally, *fer-1* has been shown to genetically interact with *spe-38* [17]. Mutations in *fer-1* prevent MO fusion in developing sperm [28], and both SPE-38 and SPE-41/TRP-3 ar e mislocalized in the sperm of *fer-1(hc1)* animals [17, 18]. Based on the results of our study, we hypothesize that SPE-38 is interacting directly with FER-1 in the MO; and that, in *fer-1(hc1)* mutants, disruption of this interaction is maintained thus preventing proper SPE-38 localization.

Unlike the interaction between FER-1 and SPE-38, our data do not indicate that FER-1 and SPE-41/TRP-3 interact physically, despite genetic data indicating an interaction [18]. We hypothesize that the failure of SPE-41/TRP-3 to relocalize from the MO to the plasma membrane and pseudopod is indirectly caused by the lack of MO fusion in *fer-1(hc1)* mutants, not the loss of physical interaction between FER-1 and SPE-41/TRP-3. In *fer-1(hc1)* mutants, SPE-38 and SPE-41/TRP-3 do not have similar localization patterns despite interacting physically. We hypothesize that the interaction between SPE-38 and SPE-41/TRP-3 is transient and regulated by additional proteins.

Of the 12 proteins tested, SPE-38 had the highest number of interactions. This is reflected in SPE-38 having the highest degree of betweenness centrality and could indicate a central role organizing proteins in the sperm membrane. SPE-38 is a novel membrane protein with unknown biochemical function [17]. The protein spans the membrane four times but lacks the CD domain of tetraspanin proteins that are known to organize proteins in the membrane [17]. *spe-38(eb44)* mutants are completely sterile, while *spe-41/trp-3* mutants [18], *spe-42* [29], and some *spe-9* mutants are weakly fertile [30]. Our data show that SPE-38 can interact with all of these proteins. *spe-38(eb44)* mutants have altered SPE-41/TRP-3 localization [16] and it is possible the SPE-9 and SPE-42 function may be defective in

these mutants. The centrality of SPE-38 to the interaction network is what could result in the complete sterility of *spe-38* mutants by indirectly disrupting other proteins in the sperm membrane.

SPE-4, SPE-10, and SPE-42 encode proteins with domains that could mediate protein–protein interactions and form a core membrane organizing cluster. *spe-4* encodes for a protein containing a presenilin domain similar to gamma-secretase [31]. Gamma-secretase cleaves intramembrane proteins with a broad range of substrates [32]. *spe-10* encodes for a protein containing a DHHC-CRD Zinc finger motif that is hypothesized to act as a palmitoyl transferase [33]. Many proteins tested in the interactome are predicted to have palmitoylation sites using CSS-Palm 2.0 predication software [34]. *spe-42* encodes a C4C4-type ring finger domain that generally mediates protein–protein interaction and could also act as a ubiquitin ligase [29]. *spe-42* also encodes for a dendritic cell specific transmembrane protein (DC-STAMP) domain [29]. DC-STAMP is a seven transmembrane protein that is necessary for osteoclast cell–cell fusion [35, 36].

MYTH studies suffer from a number of experimental challenges including issues with expression, autoactivation, and false positives [21, 22]; however, several points of data support to our results. First, we do not see broad nonspecific interactions between constructions as illustrated by the fact that WEE-1.3, SPE-12, and SPE-45 do not interact with other proteins regardless of its expression as a bait or prey. Second, there should be no false positive interactions between proteins that are not localized in the same cell type or proteins that do not share subcellular location patterns since all of the proteins tested here are expressed in sperm [37, 38] and every protein other than WEE-1.3 has nonnuclear subcellular localization [39]. Finally, we tested all interactions and both bait–prey combinations and provided all of the raw data to allow for reinterpretation based on different thresholds for interaction. Bait–prey swapping is a potential way to confirm the binary interaction between two proteins; however,

many swapping bait–prey combinations have been shown to alter the interactions detected [40, 41].

Due to the highly interconnected nature of the interactome it would be reasonable to predict that more epistasis experiments would show genetic interactions between the genes of interest. However, the lack of epistatic interaction could be due to the nature of the sperm sterile (*spe*) phenotypes examined. Analyzing an allelic series for epistatic interactions would provide more insight. In addition, the developmental stage and membrane dynamics of each stage much be taken into account. Immunofluorescence data show that FER-1, SPE-9, SPE-38, and SPE-41/TRP-3 undergo dynamic relocalization during spermiogenesis [16, 17, 42, 43]. Thus, the protein–protein interactions are not likely to be static. Our data are essential for developing a model for protein interactions that can be tested to determine how sperm membrane proteins interact during the morphological shifts associated with spermatogenesis and spermiogenesis and during sperm migration in the hermaphrodite reproductive tract.

The sperm membrane in other species is known to contain specialized domains that are modified during sperm development and transit through the reproductive tract [44]. These domains are generated during development through protein interactions with molecular chaperones, protein–protein interactions in the sperm membrane, and regulation of cholesterol in the sperm membrane. Molecular chaperones are necessary to form and maintain multiprotein complexes [45]. The disruption of the testis specific chaperones calmegin and calsperin in mice leads to the disruption of ADAM complexes in the sperm membrane and fertility defects [46–48]. The genetic deletion of single protein of multiprotein complexes can lead to the loss of other complex members on the sperm surface [49]. For example, the targeted removal of ADAM2 from sperm leads to the loss of ADAM3 from the sperm membrane [50]. In addition, the targeted elimination of sperm membrane proteins can lead to spermatogenesis arrest resulting from improper processing of proteins during spermatogenesis, as is the case with HSPA2 [44]. Based on our interactome data, a disruption of protein function or localization could influence the function or localization of multiple other proteins. This is even true for proteins such as FER-1, SPE-9, and SPE-19 that have a low number of interactions, but high neighborhood connectivity, meaning that disrupting them could influence their neighbors and additional other proteins connected to those neighbors.

The sperm membrane during spermatogenesis, spermiogenesis, and fertilization is dynamic. The *C. elegans* sperm membrane interactome data presented here provide information about the types of interactions between membrane proteins that could be occurring during spermatogenesis, spermiogenesis, and sperm function during fertilization (Figure 4). Genes that are defined as necessary in earlier steps of sperm development may also be functional at later stages. Follow-up experiments will be necessary to validate these interactions at different developmental time points. There are additional transmembrane proteins that are expressed in sperm [37] and it is likely that some are necessary sperm function. Future genetic analysis will aid in defining the functions of those genes. This work provides a means to develop hypotheses about how the sperm membrane is organized and functions and is rationale for future experimentation. The molecular genetic tools available in *C. elegans* provide an excellent opportunity to analyze allelic series of each gene and determine how the variants influence sperm function.

Supplementary data

Supplementary data are available at *BIOLRE* online.

Supplementary Table S1. Summary of the primers, primer sequences, and vectors utilized for constructing bait (a) and (b) prey plasmids used in split-ubiquitin membrane yeast two hybrid (MYTH) system.
Supplementary Table S2. Full summary of interactome and raw data from split-ubiquitin (MYTH) assays. (a) Full table of interactions bait and prey orientations. (+ = evidence of interaction in at least two dilutions in at least two separate experiments; − = no evidence of interaction). (b) Full table of colony counts and interaction scores for all experiments. [bait = bait vector plus insert; date = date of experiment (MM-DD-YY format); 3AT_conc = concentration of 3-Amino-1,2,4-triazole in synthetic medium; prey = prey vector plus insert; group = experimental group (positive = positive control, negative = negative control, empty = empty vector negative control, exp = experimental group); dilution = dilution used in experiment; colonies = colonies counted under microscope (200 colonies is the maximum value); interaction score = ratio of experimental colonies to empty vector colonies].
Supplementary Table S3. *Caenorhabditis elegans* sperm membrane interactome network file for Cytoscape import [BAIT = source node; PREY = target node; interaction = interaction type; SELF = "y" means that protein can interaction homotypically (self-looping node) and "n" means that the protein does not interact homotypically].
Supplementary Table S4. Network measures for *C. elegans* sperm membrane interactome network [(a) Cytoscape node attributes, (b) Cytoscape network analysis, (c) MCODE analysis, and (d) Cytoscape network analysis (NA) of sperm membrane interactome cluster (cluster)].

Acknowledgments

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