MFP1/MSD-1 and MFP2/NSPH-2 co-localize with MSP during C. elegans spermatogenesis

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Abstract

Until recently, the only verified component of Fibrous Bodies (FBs) within Caenorhabditis elegans spermatocytes was the Major Sperm Protein (MSP), a nematode-specific cytoskeletal element. Earlier studies in the pig parasite Ascaris suum had identified accessory proteins that facilitate MSP polymerization and depolymerization within the pseudopod of crawling spermatozoa. In this study, we show that C. elegans homologs of the two Ascaris accessory proteins MFP1 and MFP2 co-localize with MSP in both the pseudopods of C. elegans sperm and the FBs of C. elegans spermatocytes.

Figure 1:

(A) Overview of spermatogenesis and fibrous body–membranous organelle (FB-MO) development. (Top) Spermatocytes develop on a syncytial rachis then detach to undergo the meiotic divisions. Following anaphase II, components which are no longer needed partition to a central residual body (RB). (Bottom) Within meiotic prophase spermatocytes, fibrous bodies (FBs) develop on the cytosolic face of the Golgi-derived membranous organelle (MO). During the budding division, FB-MO complexes partition to the spermatids. MOs then dock with the plasma membrane as the FBs disassemble and release MSP (Major Sperm Protein) dimers into the cytosol. During sperm activation, MSP localizes to the pseudopod, and MOs fuse with the plasma membrane.

(B) Alignment of C. elegans ZK265.3/NSPH-2 (Nematode Specific Peptide family, group H) and C04G2.9/NSPH-3.2 with Ascaris MFP2 (MSP Fiber Protein) performed using the EMBL-EBI Clustal Omega Multiple Sequence Alignment tool (Madeira et al., 2019). Peptide for the anti-NSPH-2 antibody is underlined.

(C-D) DAPI and anti-NSPH-2 labelling in hermaphrodites with genetic knockouts of nsph-3.2 and nsph-2 respectively. (C) Left to right developmental sequence of developing spermatocytes and spermatids. (D) Hermaphrodite spermatozoa within spermatheca. No primary antibody controls were performed on nsph-3.2 knockout animals.

(E-G) Sperm spreads and individually staged cells with (E) anti-MSP labelling (n=63, 9 experiments), (F) anti-NSPH-2 labelling (n=50, 7 experiments), and (G) labelling against the MFP1 homolog, MSD-1 (Major Sperm protein Domain containing) (n=46, 3 experiments). (H-I) Co-labelling of MSP with either (H) anti-NSPH-2 or (I) anti-MSD-1. In spermatozoa (enlarged), the patterns were either fully (top) or partially overlapping (bottom). For NSPH-2/MSP n=16; for MSD-1/MSP n=14. Scale bars = 10 mm for C, D and E-G left; 5 mm for H, I and E-G right. Abbreviations: Karyosome spermatocytes (K), Metaphase spermatocytes (M), Budding Figures (B), Spermatids (S), and Spermatozoa (Z).

Description

During the process of cell differentiation, specific cytoskeletal proteins can sequentially assemble into a wide variety of diverse molecular superstructures. Nematode spermatogenesis provides a powerful system for studying these transitions since sperm-specific transcription ceases prior to the meiotic divisions and translation ceases shortly thereafter (Chu and Shakes, 2013). Therefore, structural transitions that follow the meiotic divisions must be carried out by the remodeling of already synthesized proteins. The Major Sperm Protein (MSP) is a nematode-specific cytoskeletal element whose polymerization dynamics drive the pseudopod-based motility of the activated sperm (Roberts, 2005). In C. elegans, MSP additionally functions as the extracellular signaling molecule for triggering both ovulation and oocyte maturation (Miller et al., 2003). MSP is highly abundant in sperm, where it reaches 10-15% of total and 40% of soluble cellular protein (Roberts 2005). Within developing spermatocytes, MSP is packaged into fibrous body–membranous organelle (FB-MO) complexes (Fig. 1A, Roberts et al., 1986). By assembling into paracrystalline FBs, MSP is both sequestered away from the critical meiotic processes of chromosome segregation and cytokinesis while also being packaged for efficient segregation into...
spermatozoids during the post-miotic partitioning process (Chu and Shakes 2013, Nishimura and L’Hernault, 2010, Price et al., 2012). Following the meiotic divisions and sperm individualization, FBs disassemble, and MSP disperses as dimers throughout the spermatic cytoplasm (Fig. 1A). When sperm activate to form motile spermatozoa, MSP polymerization within the pseudopod drives the motility of the crawling sperm (Chu and Shakes, 2013). Thus, MSP exists in at least three distinct molecular states: 1) in highly organized paracrystalline FBs within developing spermocytes 2) as un polymerized dimers within spermatozoa, and 3) in dynamically polymerizing filaments and fibers within crawling spermatozoa. Because MSP neither binds nucleotides nor assembles into polar filaments, its assembly and disassembly dynamics require accessory proteins. Previous biochemical studies in the pig parasite Ascaris suum identified MSP fiber proteins 1 and 2 (MFP1/2) as key regulators of MSP polymerization (Sepenson et al., 1998, Buttery et al., 2003, Grant et al., 2005). MSP assembly dynamics require MFP1 and MFP2: MFP1 acts antagonistically to slow polymerization whereas phosphorylated MFP2 enhances MSP polymerization by serving as a nucleation site for polymer extension (Buttery et al., 2003, Grant et al., 2005). However, because these studies focused on MSP dynamics within crawling sperm and in vitro biochemical assays, the localization of these proteins within developing spermatozoa remained unknown. Would they be packaged along with MSP in FBs? Would the homologs of these proteins exhibit similar localization patterns during C. elegans spermatogenesis? To confirm that the Ascaris proteins MFP2 and MFP1 have homologs in the two species we have globally aligned Ascaris MFP2 with the predicted protein sequence of the two highest scoring C. elegans matches ZK265.3 and CO4G2.9 (Fig. 1B). These are two spermatogenesis-expressed members of a larger MFP2 domain containing nematode specific peptide family, group H (Rödeloppeger et al., 2021), and will be hereafter referred to by their C. elegans gene prefix nsph. Ascaris MFP2 exhibits 51.4% identity and 66.7% sequence similarity with ZK265.3 (nsp2-2) and 38.0% identity and 52.3% similarity with CO4G2.9 (nsp3-2). ZK265.3 and CO4G2.9 have 41.0% identity and 52.6% sequence similarity with each other. Ascaris MFP1 has two isoforms, MFP1-a and MFP1-b, which when aligned with C. elegans MSD-1 (Major Sperm Protein Domain containing), show 52% identity and 70.8% sequence similarity, and 51.9% identity and 66.0% sequence similarity, respectively (Buttery et al., 2003). The sequence similarity between A. suum and C. elegans motility protein suggests that they may have similar functions and binding properties. To determine where NSPH-2 (MFP2) and MSD-1 (MFP1) localize within C. elegans spermatozoids and spermatozoa, we performed immunocytochemistry experiments. For our analysis, we generated a rabbit polyclonal antibody against a unique NSPH-2 peptide (Fig. 1B) and obtained a polyclonal antibody against C. elegans MSD-1, from David Greenstein (Kosinski et al., 2005). To test the specificity of the anti-NSPH-2 antibody, we used high-efficiency genome editing to create CRISPR-Cas9 knockout (KO) lines through insertion of multiple premature termination codons in all three frames for both ZK265.3 (nsp2-2) and CO4G2.9 (nsp3-2) (Wang et al., 2018). Although neither knockout strain on its own exhibited a phenotype, they were useful for determining the specificity of our anti-NSPH-2 antibody. In control nsp2-3 KO hermaphrodites, the anti-NSPH-2 antibody labelled distinct structures within late-stage spermatocytes (Fig. 1C). In nsp2-3 KO hermaphrodites, only background labelling was observed. We then compared the localization of anti-NSPH-2 and anti-MSD-1 with the well characterized anti-MSP pattern in male gonads (Fig. 1E-G). In spermatids, both NSPH-2 and MSD-1 antibodies localized to the pseudopod, matching the known pattern in Ascaris spermatozoa. Within prophase and meiotically dividing spermocytes, NSPH-2 and MSD-1 antibodies labelled discrete structures throughout the cytoplasm in a pattern strongly resembling FBs labelled by MSP antibodies (Fig. 1E-G). To verify that NSPH-2 and MSD-1 localized to FBs, we co-labelled male germ cells with anti-MSP and either anti-MSD-1 or anti-NSPH-2 antibodies (Figure 1H-I). Within spermocytes, the fully overlapping patterns revealed that NSPH-2 and MSD-1 are packaged along with MSP in the FBs. In spermatids, the MSP and MSD-1 were overlapping (Figure 1I). In contrast, except in immature spermatids that still retained their FBs, the NSPH-2 and MSD patterns only partially overlapped. In spermatozoa, the NSPH-2 labelling was notably less robust which may suggest that a change in protein conformation or binding partners is blocking the antigenic site. NSPH-2 does not appear to be degraded as it is present in the pseudopods of spermatozoa. All three antibodies labelled the pseudopods of spermatozoa, with the NSPH-2 and MSD-1 patterns either fully overlapping the MSP pattern (top set) or being restricted to a more central portion, adjacent to the cell body (bottom set). Until recently, it was thought that Major Sperm Protein (MSP) was the only component of Fibrous Bodies (FBs) in C. elegans. Our discovery that MSD-1 and MFP2/NSPH-2 are packaged together with MSP in the FBs of developing spermatozoids supports a model in which the FBs function to gather, concentrate, and sequester proteins that will ultimately drive or regulate pseudopod motility. While early studies of MSP dynamics in Ascaris capitalized on its assets for biochemical approaches, parallel genetic approaches in C. elegans were stymied by the fact that many key factors, including MSP, MFP1, and MSD-2 (NSPH-2) are encoded by single genes. Our own individual knockout strains of nsp2-2 and nsp3-2 exhibit no obvious phenotype. This result does not mean that MFP2/NSPH-2 is non-essential, but that exploring its function may require knocking out combinations of up to five of non-identical, sperm-related NSPH genes. Similarly, the four MSD genes encode identical MSP proteins that may need to be deleted to generate a mutant phenotype. CRISPR technologies give us the ability to knockout multiple genes and explore MSP dynamics in new ways. Beyond MSD and NSPH, will other proteins first identified in Ascaris as regulators of MSP dynamics and sperm motility (Buttery et al., 2003) also localize to the FBs of developing spermocytes? Alternatively, are there more proteins like the intrinsically disordered protein SPE-18 that localize to FBs and facilitate their growth and shaping but then are degraded shortly after the meiotic divisions (Price et al., 2021)? Further studies will reveal whether MSD-1 and NSPH-2 function together with SPE-18 to facilitate FB assembly, or if they are just conveniently packaged in FBs alongside MSP. Methods Request a detailed protocol Worm culture Worms were cultured on MYOB plates (Church et al., 1995) and inoculated with the E. coli strain OP50, using methods similar to those described by Brenner (1974). Creation of knock-out lines CRISPR/Cas9 mutagenesis was performed as previously described (Paix et al., 2014; Dokshin et al., 2018; Wang et al., 2018). Briefly, C. elegans strain N2 was gene-edited by the insertion of a 43-base-pair sequence that includes multiple stop codons in all three reading frames to disrupt translation (Wang et al., 2018). A BamHI site was included 3’ to the stop codons to facilitate genotyping. Strains JDW307 nsp2-2(n258[CO4G2.9::exon1 STOP]) and JDW308 nsp3-2(n258[ZK265.3::exon1 STOP]) were generated by CRISPR injection of an RNP complex (250 ng/µl of house-made Cas9 protein (Zuris et al., 2015), 50 ng/µl of the relevant crRNA, and 100 ng/µl tracrRNA) that was first incubated at 37°C for 15 minutes, then mixed with the co-injection nts/300 ng/µl and the relevant repair oligo (110 ng/µl). nsp2: MFP1 acts antagonistically to slow polymerization whereas phosphorylated MFP2 enhances MSP polymerization by serving as a nucleation site for polymer extension (Buttery et al., 2003) also localize to the FBs of developing spermocytes? Alternatively, are there more proteins like the intrinsically disordered protein SPE-18 that localize to FBs and facilitate their Antibodies and immunochemistry Sperm spreads were obtained by dissecting 8-15 male worms per slide in 7.5 microliters of egg buffer (Edgar, 1995) on ColorFrost Plus slides (Fisher Scientific, 12-550) coated with poly-L-lysine (Sigma Aldrich, P8290). Light pressure was applied to coverslips to flatten the samples. Samples were then freeze cracked in liquid nitrogen and fixed overnight in ~20°C methanol. Specimen preparation and antibody labeling followed established protocols (Shakes et al., 2000). Primary antibodies included: 1:1500 4D5 mouse anti-MSP monoclonal (Kosinski et al., 2005) and 1:200 rabbit anti-MSP-1 rabbit polyclonal (Kosinski et al., 2005). Affinity purified rabbit antisemur for MFP2/NSPH-2 (ZK265.3) was generated by YenZym Antibodies using the peptide RGQD-108 EWLPLKQRH/NEGIRE. In experiments, the antibody was used at a 1:200 dilution. All samples were incubated with primary antibodies for 2 hours at room temperature. Affinity-purified secondary antibodies included 1:400 Alexa Fluor Plus 555 goat anti-rabbit IgG (Invitrogen, A23732) and 1:300 Alexa Fluor 488 goat anti-mouse IgG (H+L) (Jackson ImmunoResearch, 115-545-140). Final slides were mounted with Fluoro Gel with DABCO ( Electron Microscopy Sciences #17985-02) containing DAPI. Imaging and analysis Images were acquired with epifluorescence using an Olympus BX60 microscope equipped with a QImaging EXI Aqua CCD camera. Photos were taken, merged, and exported for analysis using the program Vision. For control studies (Fig. 1C-D), image exposures were kept constant with no further image processing. For others, image exposures were optimized for individual gonads. The levels adjust function in Adobe Photoshop was used to spread the data containing regions of the image across the full range of tonalities. Reagents
### Strain | Genotype | Available from
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N2 | Wildtype *C. elegans* strain | CGC
CB4088 | *him-5(e1490) V* | CGC
JDW307 | nsph-3.2(wrd56[C0G2.9::exon 1 STOP]) | J. Ward lab
JDW308 | nsph-2(wrd57[ZK265.3::exon 1 STOP]) | J. Ward lab

### Antibody | Animal and clonality | Description
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anti-MSP | Mouse monoclonal, 4D5 | Kosinski et al., 2005
anti-MSP-1 | Rabbit polyclonal, R194P | Kosinski et al., 2005
anti-NSPH-2 | Rabbit polyclonal | Rabbit antiserum was generated by immunizing a rabbit against the aa 92-108 (EWLPLKQRHENNEGIRE) of Kosinski
Alexa Fluor Plus 555, secondary | Goat anti-rabbit IgG | Invitrogen, A32732
Alexa Fluor 488, secondary | Goat anti-mouse IgG | Jackson ImmunoResearch, 115-545-146

### Acknowledgments:
We would like David Greenstein for supplying antibodies against MSP and MSD-1.

### References
Buttery SM, Ekmam GC, Seavy M, Stewart M, Roberts TM. 2003. Dissection of the *Ascaris* sperm motility machinery identifies key proteins involved in major sperm protein-based amoeboid locomotion. Mol Biol Cell 14: 5082-8. PMID: 14565883.

Cho DS, Shakes DC. 2013. Spermatogenesis. Adv Exp Med Biol 757: 171-203. PMID: 22872478.

Dokshin GA, Ghanta KS, Piscopo KM, Mello CC. 2018. Robust Genome Editing with Short Single-Stranded and Long, Partially Single-Stranded DNA Donors in *Caenorhabditis elegans*. Genetica 210: 781-787. PMID: 30213854.

Grant RP, Buttery SM, Ekmam GC, Roberts TM, Stewart M. 2005. Structure of MFP2 and its function in enhancing MSP polymerization in *Ascaris* sperm amoeboid motility. J Mol Biol 347: 583-95. PMID: 15755452.

Italiano JE Jr, Stewart M, Roberts TM. 1999. Localized depolymerization of the major sperm protein cytoskeleton correlates with the forward movement of the cell body in the amoeboid movement of nematode sperm. J Cell Biol 146: 1087-96. PMID: 10477761.

Kosinski M, McDonald K, Schwartz J, Yamamoto I, Greenstein D. 2005. *C. elegans* sperm bud vesicles to deliver a meiotic maturation signal to distant oocytes. Development 132: 3357-69. PMID: 15975936.

Madeira F, Park YM, Lee J, Buso N, Gur T, Madhusoodanan N, Banatkar P, Tivey ARN, Potter SC, Finn RD, Lopez R. 2019. The EMBL-EBI search and sequence analysis tools APIs in 2019. Nucleic Acids Res 47: W636-W641. PMID: 30976793.

Mello CC, Kramer JM, Stinchcomb D, Ambros V. 1991. Efficient gene transfer in *C. elegans*: extrachromosomal maintenance and integration of transforming sequences. EMBO J 10: 3959-70. PMID: 1939914.

Miller MA, Ruest PJ, Kosinski M, Hanks SK, Greenstein D. 2003. An Eph receptor sperm-sensing control mechanism for oocyte meiotic maturation in *Caenorhabditis elegans*. Genes Dev 17: 187-200. PMID: 12533508.

Nishimura H, Ilerheadswal SW. 2010. Spermatogenesis-defective (spe) mutants of the nematode *Caenorhabditis elegans* provides clues to solve the puzzle of male germ line functions during reproduction. Dev Dyn 239: 1502-14. PMID: 20419782.

Paix A, Wang Y, Smith HE, Lee CY, Calián D, Lu T, Smith J, Schmidt H, Krause MW, Seydoux G. 2014. Scalable and versatile genome editing using linear DNAs with microhomology to Cas9 Sites in *Caenorhabditis elegans*. Genetics 198: 1347-56. PMID: 25249454.

Price KL, Presler M, Uyehara CM, Shakes DC. 2021. The intrinsically disordered protein SPE-18 promotes localized assembly of MSP in *Caenorhabditis elegans* spermatocytes. Development 148: dev195875. PMID: 33558389.

Roberts TM, Pavalko FM, Ward S. 1986. Membrane and cytoplasmic proteins are transported in the same organelle complex during nematode spermatogenesis. J Cell Biol 102: 1787-96. PMID: 3517007.

Roberts TM. 2005. Major sperm protein. Curr Biol 15: R153. PMID: 15753021.

Rödelsperger C, Ebbing A, Sharma DR, Okumura M, Sommer RJ, Korswagen HC. 2021. Spatial Transcriptomics of Nematodes Identifies Sperm Cells as a Source of Genomic Novelty and Rapid Evolution. Mol Biol Evol 38: 73-80. PMID: 25357182.

Rödelsperger C, Ebbing A, Sharma DR, Okumura M, Sommer RJ, Korswagen HC. 2021. Spatial Transcriptomics of Nematodes Identifies Sperm Cells as a Source of Genomic Novelty and Rapid Evolution. Mol Biol Evol 38: 73-80. PMID: 25357182.

Singson A. 2001. Every sperm is sacred: fertilization in *Caenorhabditis elegans*. Dev Biol 230: 101-9. PMID: 11165165.

Sternberg PW. 2018. An Efficient Genome Editing Strategy To Generate Putative Null Mutants in *Caenorhabditis elegans*. G3 (Bethesda) 8: 3607-3616. PMID: 30224336.

Wang H, Park H, Liu J, Sternberg PW. 2018. An Efficient Genome Editing Strategy To Generate Putative Null Mutants in *Caenorhabditis elegans* Using CRISPR/Cas9. G3 (Bethesda) 8: 3607-3616. PMID: 30224336.

Zuris JA, Thompson DB, Shu Y, Guilinger JP, Bensen JL, Hu JH, Maeder ML, Joung JK, Chen ZY, Liu DR. 2015. Cationic lipid-mediated delivery of proteins enables efficient protein-based genome editing in vitro and in vivo. Nat Biotechnol 33: 73-80. PMID: 25357182.

**Funding:** The work was supported by a grant R15GM-096309 from the National Institutes of Health and the McLeod Tyler Professorship to D.C.S, a National Science Foundation (NSF) Division of Molecular Cellular Biosciences CAREER award (1942922) to J.D.W., and an Honors Fellowship grant from the Charles Center at William & Mary to K.N.M.

**Author Contributions:** Kayleigh N. Morrison: Formal analysis, Investigation, Methodology, Validation, Visualization, Writing - original draft. Christopher M. Uyehara: Formal analysis, Investigation, Methodology, Validation. James Matthew Ragle: Investigation, Methodology, Writing - review and editing. Jordan D. Ward: Funding acquisition, Supervision, Writing - review and editing. Diane C. Shakes: Conceptualization, Funding acquisition, Supervision, Writing - review and editing.

**Reviewed By:** Anonymous

**History:** Received June 29, 2021 Revised received July 17, 2021 Accepted July 19, 2021 Published July 22, 2021

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