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

Kayeleigh N. Morrison, Christopher M. Uyehara, James Matthew Morrison, Israel Christopher Ragle
Department of Biology, William & Mary, Williamsburg, Virginia, USA

diane_c_shak@wm.edu

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 developing 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 meiotic divisions. Within meiotic prophase II, components which are no longer needed partition to a central midbody body (FBs). (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 ascariids ZK265.3/NSPH-2 (Nematode Specific Peptide family, group H) and C04G2.9/NSPH-3.2 with Ascaris MFP2 (NSPH-2). ZK265.3 and C04G2.9 have 41.0% identity and 52.6% sequence similarity with ZK265.3 (nsph-2) and C04G2.9 (nsph-3.2), respectively. (C) Left to right developmental sequence of developing spermatocytes and spermatids. (D) Hermaphrodite spermatogenesis within spermatids. 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=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/MSMP 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), Dividing spermatocytes (D), Mutant spermatocytes (M). 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). 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 spermatids during the post-meiotic partitioning process (Chu and Shakes 2013, Nishimura and L'Hernault, 2010, Price et al., 2018). As 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 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 developing sperm and the FBs of C. elegans spermatocytes.

To confirm that the Ascaris proteins MFP1 and MFP2 have homologs in C. elegans, we compared protein sequences between the two species. We globally aligned Ascaris MFP2 with the FBs of C. elegans spermatocytes. The two highest scoring C. elegans matches ZK265.3 and C04G2.9 (Fig. 1B). These are two nematode-specific-expressed members of a larger MFP2 domain containing nematode specific peptide family, group H (Rödelsperger 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 (nsph-2) and 38.0% identity and 52.3% similarity with C04G2.9 (nsph-3.2). ZK265.3 and C04G2.9 have 41.0% identity and 52.6% sequence similarity with ZK265.3 (nsph-2) and C04G2.9 (nsph-3.2).
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 proteins suggests that they may have similar functions and binding properties.

To determine where NSPH-2 (MFP2) and MSD-1 (MFP1) localize within C. elegans spermatoocytes and spermatoza, we performed immunocytochemistry experiments. For our analysis, we generated a rabbit polyclonal antibody against a unique NSF-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 new anti-NSPH-2 antibody, we used high-efficiency genome editing to generate CRISPR-Cas9 knockout (KO) lines through insertion of multiple premature termination codons in all three frames for both ZK265.3 (mfp-2) and C04G2.9 (mfp-3.2) (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 mfp-2 KO hermaphrodites, the anti-NSPH-2 antibody labelled distinct structures within late-stage spermatoocytes (Fig. 1C) as well as the pseudopods of spermatoocytes within the spermatheca (Fig. 1D). In mfp-2 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 spermatoocytes, both NSF-2 and MSD-1 antibodies localized to the pseudopod, and matching the known pattern in Ascaris spermatozoa. Within prophase and meiotically dividing spermatoocytes, NSF-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 NSF-2 and MSD-1 localized to FBs, we co-labelled male germlines with anti-NSPH-2 and anti-MSD-1 or anti-NSPH-2 antibodies (Figure 1H-I). Within spermatoocytes, the fully overlapping patterns revealed that NSF-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, in immature spermatozoa that still retained their FBs, the NSF-2 and MSP patterns only partially overlapped. In spermatids, the NSF-2 labelling was notably less robust which may suggest that a change in protein conformation or binding partners is blocking the antigenic site. MSNPH-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 NSF-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 spermatoocytes 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 (MSD), and MFP2 (NSPH) are encoded by multigene families. Our own individual knockout strains of mfp-2 and mfp-3.2 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 non-identical, sperm-related NSF-2 genes. Similarly, the four MSD genes encode identical 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 spermatoocytes? Alternatively, are there three 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 mitotic 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 BanHI site was included 3’ to the stop codons to facilitate genotyping. Strains JDW307 mfp-2::wdf6[CD42.9::exon 1 STOP]) and JDW308 mfp-2::wdf7[ZK265.3::exon 1 STOP]) were generated by CRISPR injection of an RNP complex (250 ng/µl of guide-RNA, 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 coinjection marker prf4 (50 ng/µl) and the relevant repair oligos (110 ng/µl) before being injected into wild-type L4 + 1 day worms grown at 20°C. After 4 days, rolling worms were plated individually, allowed to lay eggs, and the parental animal was genotyped by PCR and BanHI digestion. For candidate knock-ins the extrachromosomal array was removed by selecting non-roller progeny. Genotyping primer sequences can be provided upon request. The tracrRNA and crRNAs were obtained from Integrated DNA Technologies (IDT).

**Repair oligos (BanHI sites in bold)**

```
C04G2.9 crRNA: 5’-TACCGGGCTCGGCGGGAAGG-3’
ZK265.3 crRNA: 5’-AAGAGAAACTGATTGTGGGAGG-3’
```

**Repair oligos (BanHI sites in bold)**

```
AAGATACTTGGGCATTCCAGCCAATCGGAGCCCCCTGGGAAGTTTGTCCAGAGCAGAGGTGACTAAGTGATAA
AAGATACTTGGGCATTCCAGCCAATCGGAGCCCCCTGGGAAGTTTGTCCAGAGCAGAGGTGACTAAGTGATAA
GACAAGAATCGGCGTCAAGGACGTGGAGTTTGAACGAGCAGAGGTGACTAAGTGATAA
TACAACAAAGGCGGATGATGAAAGGAAGAATCTGATTGAGAAGGACGAGCAGAGGTGACTAAGTGATAA
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**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, P6290). Light pressure was applied to cover slips 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., 2009). Primary antibodies included: 1:150 4DS mouse anti-MSP monoclonal (Kosinski et al., 2005) and 1:200 rabbit anti-MSD-1 rabbit polyclonal (Kosinski et al., 2005). Affinity purified rabbit antiserum against MFP2/NSPH-2 (ZK265.3) was generated by YenZym Antibodies using the peptide 92-108 EWLPLKQRHENNEGIRE. As a control injection, the intracellular protein immunostaining was compared with a tricysteinic protein immunostaining using the tracrRNA and crRNAs that were obtained from Integrated DNA Technologies (IDT). A BamHI site was included 3’ to the stop codons to facilitate genotyping. Strains JDW307 mfp-2::wdf6[CD42.9::exon 1 STOP]) and JDW308 mfp-2::wdf7[ZK265.3::exon 1 STOP]) were generated by CRISPR injection of an RNP complex (250 ng/µl of guide-RNA, 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 coinjection marker prf4 (50 ng/µl) and the relevant repair oligos (110 ng/µl) before being injected into wild-type L4 + 1 day worms grown at 20°C. After 4 days, rolling worms were plated individually, allowed to lay eggs, and the parental animal was genotyped by PCR and BanHI digestion. For candidate knock-ins the extrachromosomal array was removed by selecting non-roller progeny. Genotyping primer sequences can be provided upon request. The tracrRNA and crRNAs were obtained from Integrated DNA Technologies (IDT).

**Reagents**

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

References


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 and 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 Revision received July 17, 2021 Accepted July 19, 2021 Published July 22, 2021

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Citation: Morrison, KN; Uyehara, CM; Ragle, JM; Ward, JD; Shakes, DC. (2021). MFP1/MSD-1 and MFP2/NSPH-2 co-localize with MSP during C. elegans spermatogenesis. microPublication Biology. https://doi.org/10.17912/micropub.biology.000427

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