Roles for the RNA polymerase III regulator MAFR-1 in regulating sperm quality in Caenorhabditis elegans

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The negative regulator of RNA polymerase (pol) III maf-1 has been shown to affect RNA pol III transcript abundance, lipid biosynthesis and storage, progeny output, and lifespan. We deleted maf-1 from the Caenorhabditis elegans genome and found that animals lacking maf-1 replicated many phenotypes from previous RNAi-based studies and discovered a new sperm-specific role. Utilizing a yeast two-hybrid assay, we discovered several novel interactors of MAFR-1 that are expressed in a sperm- and germline-enriched manner. In support of a role for MAFR-1 in the male germline, we found maf-1 null males have smaller spermatids that are less capable in competition for fertilization; a phenotype that was dependent on RNA pol III activity. Restoration of MAFR-1 expression specifically in the germline rescued the spermatid-related phenotypes, suggesting a cell autonomous role for MAFR-1 in nematode male fertility. Based on the high degree of conservation of Maf1 activity across species, our study may inform similar roles for Maf1 and RNA pol III in mammalian male fertility.

Canonically characterized as a negative regulator of RNA polymerase (pol) III, MAF1 was originally discovered and has been extensively studied in Saccharomyces cerevisiae1–3. Since its discovery, MAF1 has been identified across diverse eukaryotic clades2–7. Perturbation of MAF1 activity leads to, in addition to increased RNA pol III activity, an increase in intracellular lipid abundance8–10 and in some instances altered lifespan11,12. The majority of studies of MAF1 have been conducted in single-cell systems (as S. cerevisiae) or cultured mammalian cells4,10,13,14, but relatively few studies have probed the function of MAF1 within the context of a complete animal8,15–17. In Caenorhabditis elegans, RNA interference (RNAi) knockdown of MAF1 homolog maf-1 results in increased RNA pol III activity, increased intestinal lipid accumulation (as well as increased expression of lipid biogenesis genes), and increased expression of the vitellogenin family of lipid transport proteins8. As vitellogenesis is the process by which lipids are transferred to developing oocytes, these findings imply a role for MAFR-1 in reproduction, but this has not yet been fully investigated.

Although RNA pol III activity has, to our knowledge, not been directly implicated in fertility, many processes affected by RNA pol III activity do affect reproductive output. Drosophila with disrupted ribosome biogenesis display a Minute phenotype characterized by delayed development, short and thin bristles, and impaired fertility and viability18. Furthermore, mice lacking Zfn384, a protein whose sub-cellular location was highly correlated with that of RNA pol III in human embryonic stem cells, have fertility defects and impaired spermatogenesis9,20. While mature sperm are widely accepted to be transcriptionally and translationally quiescent21–25, as a repressor of RNA pol III activity, MAFR-1 may still be acting in developing germ cells to prevent erroneous RNA pol III transcription.

In all sexually reproducing species, sperm are produced in great excess of oocytes, and must compete with each other to successfully fertilize an oocyte. Generally, larger sperm are able to travel faster than smaller sperm, and therefore have a competitive advantage23–25. As a hermaphroditic species, C. elegans presents a unique situation: the sperm produced by hermaphrodites are capable of fertilizing oocytes but are almost completely outcompeted by male sperm, when mated26. In this work, we explore the role of MAFR-1 in maintaining male sperm quality in C. elegans, which affects sperm quality metrics such as size and activation capacity, as well as competitive advantage over hermaphrodite sperm.

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**Results**

**Characterization of a mafr-1 null mutant.** Previous studies of mafr-1 in *C. elegans* have utilized RNAi-based approaches and a gain-of-function allele of mafr-1 leaving the true loss-of-function phenotype unknown. To better examine the biological functions of MAFR-1, we assessed the impact of a true molecular null allele of mafr-1, a CRISPR-generated and sequence confirmed deletion of the entire mafr-1/C43H8.2 coding sequence from start to stop, hereafter referred to as mafr-1 (KO) (Fig. 1a). Surprisingly, a total loss of mafr-1 did not result in changes in developmental timing (Fig. 1b) or overall organismal health, as mafr-1 (KO) animals had similar lifespans to wild type (WT) controls (Fig. 1c, Fig. S1a). While mafr-1 (KO) expression was undetectable in mafr-1 (KO) animals (Fig. 1d), expression of genes downstream in the CEOP1628 operon, arch-1 and B0511.6, were unaffected (Fig S1b). Consistent with its canonical role as a negative regulator of RNA pol III activity and previously observed phenotypes for mafr-1 RNAi2,3,8,16, mafr-1 (KO) animals showed increased expression of RNA pol III transcripts, including three tRNAs: initiator Methionine, Tryptophan, and Iso-leucine (Fig. 1e), as expected from previous studies3–16. Additionally, the expression of vitellogenins, which deliver lipids from the intestine to the germline to drive reproduction, was previously demonstrated to increase in mafr-1 RNAi treated animals and diminished by mafr-1 overexpression, and was increased mafr-1 (KO) animals relative to WT controls at the transcriptional (Fig. 1f), but not the protein, level (Fig. S1c). Our previous investigations revealed that overexpression of mafr-1 can influence reproductive output8. Although the total reproductive output between mafr-1 (KO) animals and WT controls were not significantly different (Fig. 1g), peak reproductive output appeared delayed in mafr-1 (KO) animals (Fig. 1h). Taken together, these results indicate that mafr-1 (KO) animals share several of the previously documented mafr-1-associated phenotypes, without compromised overall health.

**MAFR-1 interacts with sperm-enriched proteins.** MAF1 has been shown to physically interact with components of the RNA pol III complex27–30, but other direct interactors remain elusive. In order to look for novel protein interactors with MAFR-1, we performed a yeast two-hybrid screen using MAFR-1 as bait. We identified 62 putative protein–protein interactors of MAFR-1 (Table S1), which represent GO-terms comprising multiple essential cellular processes (Fig. S2a). Among these hits, we defined a novel class of germine- or spermatid-enriched31 putative MAFR-1 interactors: SSS-1, F48C1.6, and MSP-53. Because a role for MAF1 in germ cells has not been previously described, we chose these putative interactors for further analysis. We first examined the RNA expression levels of each putative interactors in wild type and mafr-1 (KO) animals by qPCR, which revealed increased expression of sss-1 and msps-53, but not F48C1.6 relative to WT (Fig. S1b). Next, we confirmed the physical interaction of MAFR-1 with SSS-1 (Fig. 2a), and F48C1.6 (Fig. 2b) biochemically by co-expression in *E. coli* followed by affinity purification MAFR-1, which facilitate the co-purification of each interactors (Fig. 2a,b). While we observed clear enrichment for SSS-1 and F48C1.6, our ability to measure enrichment of MSP-53 was confounded by its association with the Ni–NTA resin (Fig. S2c); a quality likely associated with its known capacity to mediate multiple interactions32,33. Based on their enriched expression in spermatids, we next assessed the impact of reducing the expression of SSS-1 or F48C1.6 by RNAi on sperm quality. Sperm size is a well-established quality that influence male reproductive success through sperm competition34. *Caenorhabditis elegans* males produce sperm that are significantly larger than hermaphrodite sperm and this size difference facilitates male competitive advantage when mating occurs. As expected based on their enrichment in germ cells, RNAi of sss-1 and msps-53, but not F48C1.6, resulted in decreased spermatic size (Fig. 2c,d, Fig. S2d-e).

In addition to size, the speed of male sperm is greater than hermaphrodite-derived sperm, which also enhances male competitive advantage for fertilizing an oocyte34. To become motile, inactive spermatids must become activated by developing pseudopodia, a sophisticated process under the control of genetic and environmental factors32,34,35. In the laboratory, isolated spermatids can be activated in vitro by exposure to Pronase34,35. We also found that mafr-1 (KO) male sperm are less capable of activation upon treatment with Pronase (Fig. 3c). Thus, mafr-1 (KO) males have smaller sperm with a reduced capacity to mature, which could impact their ability to fertilize hermaphrodite oocytes. Interestingly, we found that RNAi knockdown of brf-1 did not significantly affect spermatid activation in WT or mafr-1 (KO) animals (Fig. 3d).

In order to test the physiological consequence of mafr-1 (KO) on sperm fitness phenotypes, we designed an assay to assess sperm quality35. When hermaphrodites are mated to WT males, nearly all resulting progeny are derived from male sperm36. This can be visualized if males harboring a wrmScarlet transgene are used for mating; progeny derived from male sperm express wrmScarlet while progeny stemming from hermaphrodite self-sperm.
Figure 1. *mafr-1* (KO) produces fertile, viable hermaphrodites. (a) Schematic diagram showing region deleted in *mafr-1*(syb557), a CRISPR-generated and sequence confirmed deletion of the entire *mafr-1/C43H8.2* coding sequence from the start to stop codons, hereafter referred to as *mafr-1* (KO) (Adobe Illustrator v24.3). (b) Developmental time course of WT and *mafr-1* (KO) hermaphrodites. Each line represents one population of > 100 animals. (c) Lifespan survival assay of WT and *mafr-1* (KO) hermaphrodites. Individual lines represent average of two or three biological replicates of 50-worm populations (see Fig. S1a). No significant difference was found by Log-rank (Mantel-Cox) test. (d) Quantitative PCR expression analysis of *mafr-1* and three tRNA RNA pol III transcripts: initiator Methionine, Tryptophan, and Iso-leucine. (e) Lipid abundance as measured by Nile Red staining intensity. (f) Quantitative PCR expression of *vit-2* and *vit-4*. (g) Total brood size of WT and *mafr-1* (KO) hermaphrodites (n > 10). (h) Time course showing reproductive output of WT and *mafr-1* (KO) hermaphrodites throughout reproductive span. Unless otherwise specified, statistical comparisons made by Student’s t-test (two-tailed). ns = no significance, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.
do not express wrmScarlet (Fig. 3e). Thus, the proportion of non-Red progeny corresponds to utilization of self-sperm. When WT males expressing a CRISPR-integrated, single-copy, ubiquitously-expressed wrmScarlet marker were mated to WT hermaphrodites, nearly all subsequent progeny over the reproductive span expressed wrmScarlet, and were therefore derived from male sperm acquired through mating (Fig. 3f). In contrast, when wrmScarlet-expressing \textit{mafr-1} (KO) males were mated to WT hermaphrodites, 10 of 28 individuals produced progeny that were non-Red (as opposed to 1 of 28 in WT males), which indicates a significant proportion of self-sperm-fertilized progeny, and implies impairment of these \textit{mafr-1} (KO) male sperm to outcompete WT hermaphrodite self-sperm. Taken together, these data reveal a novel role for MAFR-1 in spermiogenesis. MAFR-1 impacts sperm quality cell autonomously. One possible explanation for the discrepancy of phenotypes resulting from RNAi and genetic studies, is the lack of uniformity of RNAi across different tissues49–53. To assess whether MAFR-1 functions cell autonomously in the germline to regulate sperm quality, we restored MAFR-1 in the germline of \textit{mafr-1} (KO) animals, driving \textit{mafr-1} expression with the \textit{pie-1} promoter54–56 (Fig. S4a). Animals with germline-specific rescue of \textit{mafr-1} developed normally into fertile adults, but exhibited a slight developmental delay (Fig. S4b). Germline-specific expression of \textit{mafr-1} in \textit{mafr-1} (KO) males restored spermatid size to that of WT males (Fig. 4a, Fig. S4c), while partially restoring spermatid activation (Fig. 4b). Importantly, germline rescue of MAFR-1 also restored the competitive ability of \textit{mafr-1} (KO) male sperm in our mating assay, with only 2 of 25 Rescue-mated WT hermaphrodites producing non-Red progeny (Fig. 4c), without affecting total brood size (Fig. S4d). These data suggest a cell-autonomous role for MAFR-1 in the male germline and collectively our study reveals a role for MAFR-1 in multiple parameters of sperm quality and male reproductive fitness (Fig. 4d).

**Discussion**

In light of the conflicting phenotypes associated with the loss of MAF1 by RNA interference (RNAi), genetic mutations in metazoans8,15–17,43, and in cultured cell models10, we characterized a loss-of-function allele in a second metazoan model. \textit{mafr-1} (KO) \textit{C. elegans} display no gross defects– males have no obvious structural changes to the male copulatory organ when compared to WT (Fig. S3e-f) and are generally healthy (Fig. 1b,c),
with brood sizes comparable to WT (Fig. 1g). Nevertheless, the novel sperm-enriched interactors discovered in a yeast two-hybrid screen (Fig. 2, Fig. S2) prompted us to investigate the roles of MAFR-1 in the germline, specifically of males, which had not been previously studied. Recent work has found MAF1 expression in embryonic stem cells drives differentiation. Caenorhabditis elegans possess only one population of stem cells—the germline—which originate from a single cell and become progressively more mature as they migrate through the gonad away from the distal tip cell. In fact, previous studies have found mafr-1 enriched in the C. elegans germline, but the significance of this finding has never been studied.

While previous work documented altered vitellogenin expression at both the transcriptional and protein level in response to mafr-1 RNAi, we observed altered vitellogenin expression only at the mRNA level (Fig. 1f, Figure 3. mafr-1 (KO) males have diminished sperm quality. (a) Spermatid size in WT and mafr-1 (KO) males. See Supplemental Data Set for further statistical analysis. (b) Spermatid size of day 2 adult WT and mafr-1 (KO) males following RNAi knockdown of brf-1. See Supplemental Data Set for further statistical analysis. (c) In vitro Pronase activation of WT and mafr-1 (KO) male spermatids. (d) In vitro Pronase activation of day 2 adult WT and mafr-1 (KO) males following RNAi knockdown of brf-1. (e) Schematic diagram showing setup of male sperm competition mating assay (Adobe Illustrator v24.3). (f) Proportion of progeny fertilized by self-sperm from WT- and mafr-1 (KO)-mated hermaphrodites. Experiments done in biological triplicate. Statistical comparisons made by Student's t-test (two-tailed). ns = no significance, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.
Fig. S1c). This discrepancy could be explained by post-transcriptional regulatory pathways that govern intestinal synthesis of the vitellogenins, export from intestinal cells, or endocytosis in oocytes. Additionally, it is possible that mafr-1 RNAi results in different regulation of vitellogenins through persistent expression, as RNAi provides significant knockdown, but not complete ablation, of gene expression. RNAi is not uniformly effective across tissues, providing inefficient knockdown in the neurons, pharynx, and vulva, which could be acting cell non-autonomously to cause pleiotropic gene expression phenotypes. As reproduction was generally normal in mafr-1 (KO) animals, it is not surprising that other regulatory mechanisms might restore homeostasis. Nevertheless, our studies in males, which do not express vitellogenins, identifies a novel role of mafr-1 in proper sperm function.

Furthermore, it is likely that regulation of vitellogenin expression may be sensitive to diet, as mafr-1 expression and mafr-1-sensitive phenotypes are altered on an Escherichia coli K12/HT115 diet, in which the vast majority of RNAi studies are performed, relative to the standard E. coli B/OP50 diet. Similarly, spermatid size appears to be sensitive to diet, with HT115-fed mafr-1 (KO) males producing significantly smaller sperm than those reared on OP50 (Fig. S3b–c). MAFR-1 plays an important role coordinating regulation of biosynthetic capacity in response to stimuli from the insulin signaling pathway, which may be induced on the HT115 diet, as endogenous carbohydrates are 3- to 5-fold higher in HT115 relative to OP50.

Prior to this study, known physical interactors of MAF1 and its homologs were limited to regulatory kinases and phosphatases, as well as RNA pol III subunits and transcription factors. Although the molecular function of this interaction is not fully understood, the MSP family of proteins play multiple roles in sperm function and biogenesis. Interaction of MAFR-1 with proteins expressed in a tissue-specific manner implies that MAFR-1 could play additional roles that are unique to distinct cell types. Furthermore, restoration of WT mafr-1 expression in the germline partially rescued all measured metrics of sperm quality (Fig. 4a–c), indicating the cell-autonomous nature of the role of MAFR-1 in sperm maturation. To the best of our knowledge, this is the first instance of manipulation of any MAF1 homolog in any individual tissue of a multicellular organism.

Figure 4. Germline expression of WT mafr-1 rescues sperm quality phenotypes. (a) Spermatid size in WT, mafr-1 (KO), and germline-specific rescue males. See Supplemental Data Set for further statistical analysis. (b) In vitro Pronase activation of WT, mafr-1 (KO), and germline-specific rescue male spermatids. mafr-1 (KO)/Rescue comparison done via unpaired, one-tailed t-test. Experiments done in biological triplicate. (c) Proportion of progeny fertilized by self-sperm from WT, mafr-1 (KO), and germline-specific rescue-mated hermaphrodites. WT and mafr-1 (KO) data are same data presented in Fig. 3e. (d) Model of effects of MAFR-1 on spermatid quality. MAFR-1 interacts with MSP-53, SSS-1, and F48C1.6, and its repression of RNA pol III is required for proper sperm maturation (Adobe Illustrator v24.3). Unless otherwise specified, statistical comparisons made by Student’s t-test (two-tailed). ns = no significance, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.
Taken together, our findings suggest that MAF1 may have unique roles in unique tissues that warrant further investigation.

While mafr-1 (KO) animals have similar lifespans (Fig. 1c) and developmental rates (Fig. 1b) to WT, germ-line-rescued mafr-1 (KO) animals take approximately two hours longer than WT or mafr-1 (KO) to become gravid adults (Fig. 3b). MAF1 is activated in human cells in response to serum starvation68,69, and confers starvation rescued through perceived starvation in the germline. mafr-1 driven MAFR-1 expression may cause the observed delay in reproductive maturation of (KO) animals through perceived starvation in the germline.

MAF1 is generally viewed as a repressor of growth63. Seemingly contrary to this paradigm, spermatids from males lacking mafr-1 are smaller than those of WT males (Fig. 3a). In C. elegans, as sperm size is correlated with competitive advantage, and therefore general sperm quality24, small sperm can be an indication of poor sperm quality. RNA pol III transcripts make up an significant fraction of spermatid RNA populations71,72, and while mature spermatids are transcriptionally and translationally quiescent42, erroneous RNA pol III activity in germ cells likely results in lower quality, and therefore smaller, spermatids. Rescue of MAFR-1 expression in the germline rescued the diminished spermatid size of mafr-1 (KO) males, which suggests mafr-1 activity is required in a cell-specific manner for spermatid size (Fig. 4a). In addition, RNAs of the RNA pol III transcription factor brf-1 restored spermatid size revealing that this phenotype is linked to aberrant RNA pol III activity (Fig. 3b). Similarly, sperm activation was rescued by restoration of WT MAFR-1 in the germline (Fig. 4b), but the effects of brf-1 RNAi on activation were not significant. Based on the effect of brf-1 RNAi in WT spermatids, it appears that the loss of RNA pol III activity is perhaps pleiotropic, potentially because of the role of proteostasis in sperm activation42-45. Nevertheless, the trend was toward restoring sperm activation defects stemming from loss of mafr-1 when brf-1 is reduced by RNAi (Fig. 3d). Our results suggest that tight regulation of RNA pol III activity, through MAFR-1, is important for proper sperm function.

In all instances we employed RNAi to explore the role of a gene in regulating sperm quality, we found significant effects on sperm size but not spermatid activation (Figs. 2c,d, 3b,d, Fig. S2d-f). While this suggests that sperm size is the more sensitive assay, these results do not necessarily indicate that the genes investigated do not play a role in sperm maturation. Male gametes appear to be another tissue in which RNAi efficiency is reduced36,37, as several sperm-specific sterility phenotypes caused by genetic mutants cannot be recapitulated through RNAi7. Genetic manipulation is necessary to determine the role of these genes in spermiogenesis, but a lack of viable mutants leaves RNAi as the best currently available tool to study sss-1, F48C1.6, msp-53, and brf-1 in this context. We note that our discovery of the sperm-specific phenotypes of mafr-1 might not have been uncovered without the development of the CRISPR-generated null allele. In summary, our work defines three novel interactors of MAFR-1, and a unique role in the male germline affecting sperm maturation, likely through its regulation of RNA pol III activity (Fig. 4d).

Experimental procedures

Caenorhabditis elegans strains and maintenance. Caenorhabditis elegans were maintained at 20 °C on 6 cm plates of Nematode Growth Medium (NGM) supplemented with streptomycin and seeded with OP50-1 E. coli. For RNAi experiments, NGM plates were supplemented with 5 mM IPTG and 100 μg/ml carbenicillin and seeded with HT115 E. coli expressing dsRNA targeting gene as specified.

The following strains were used: wild type (WT) N2 Bristol, PHX557 [mafr-1(syb557) I], SPC489 [mafr-1(syb557) I; laxSi01– pie-1p::mafr-1::mafr-1 3′UTR cb-unc-119(+); J1], WBM1143 [wbmI67– eef-1A.1p::3XFLAG::wrmScarlet::unc-54 3′UTR V], SPC490 [mafr-1(syb557) I; wbmI67– eft-3p::3XFLAG::wrmScarlet::unc-54 3′UTR V], SPC491 [mafr-1(syb557) I; laxSi01– pie-1p::mafr-1::mafr-1 3′UTR cb-unc-119(+); J1], SPC492 [mafr-1(syb557) I; qut-3(sc103) II; bll-1 – vit-2::GFP rol-6(su1006) X], SPC493 [mafr-1(syb557) I; qut-1(sc103) II; bll1 – vit-2::GFP rol-6(su1006) X].

Yeast-2-hybrid screen. Bait and prey plasmids were generated by cloning mafr-1 into pLexA and a C. elegans cDNA library into pACT2.2 (Addgene), respectively. Interaction was tested on synthetic complete agar in this context. We note that our discovery of the sperm-specific phenotypes of mafr-1 might not have been uncovered without the development of the CRISPR-generated null allele. In summary, our work defines three novel interactors of MAFR-1, and a unique role in the male germline affecting sperm maturation, likely through its regulation of RNA pol III activity (Fig. 4d).

Biochemical co-purification. MAFR-1 and respective interactors were cloned into MCS1 and MCS2 of pCOLA2, respectively. Origami K-12 E. coli were grown to stationary phase and expression of pCOLA2 vector was induced using IPTG for 2 h. Cells were pelleted from 50 ml induced culture, and frozen. Proteins were isolated and purified as in Ni2–NTA Purification System protocol (Qiagen).

RNA extraction and gene expression. Worms were washed from plates using M9 containing 0.01% Triton-X100, washed twice in M9, and frozen at −80 °C in 500 μl TRI-Reagent (Zymo). Frozen worms were thawed on ice, and cuticles manually disrupted using 25G needle. RNA was extracted using Direct-zol RNA Miniprep kit (Zymo Research R2071). cDNA was synthesized using qScript reverse transcription kit (Quanta Biosciences), diluted, and quantitative PCR was performed using PerfeCTa SYBR Green (Quanta Biosciences). All genes were normalized to expression of snb-1. Primers used as previously described in Pradhan et al. and Khanna et al.64. New primers used in this study are as follows:
| Gene name | Forward primer sequence | Reverse primer sequence |
|-----------|-------------------------|-------------------------|
| msp-53    | TGCCATAATCTCACGGGAG     | TCCCTTGGGTCGGAGAAGC    |
| sss-1     | GACGGCATACCTTTTCGGG     | GAAACAGATGGCAGGCTG    |
| F48C1.6   | GGGGTAGTTTCCTTAGGCG     | AGCTCGTGGGAAGTTTG    |

Nile red lipid staining. Synchronous L4 animals were stained as in\textsuperscript{73}. Briefly, animals were washed from plate, fixed in 40% isopropanol for 3 min, stained in 0.03 mg/ml Nile Red in 40% isopropanol for 2 h, and de-stained in PBS with 0.01% Triton for 30 min. Stained worms were mounted in de-staining solution and imaged using DIC and GFP filters on Zeiss Axio Imager, with ZEN software. Fluorescence of individual worms was measured using ImageJ software (NIH).

Lifespan analysis. Lifespan data was collected as previously described\textsuperscript{40,74–76}. In brief, plates containing synchronized populations were scored daily for survival, beginning at L4 stage. Animals were moved to new plates periodically during the reproductive span to remove progeny. Each line represents the average of two (in the case of WT) or three (in the case of mafr-1 (KO)) biological replicates of 50 animals each. Animals that died of bursting, matricide, or crawling off plate were censored.

Reproduction. Self-reproduction: L1-synchronized hermaphrodites were grown to L4 and single worms were placed on individual plates. Worms were transferred to fresh plates every 24 h until egg laying ceased, and progeny were counted 48 h after hermaphrodite was removed from plate.

Mated-reproduction: Single L4 hermaphrodites were placed with virgin day 1 adult males (harboring a CRISPR-integrated, single-copy, eef-1A.1 promoter-driven wrmScarlet transgene) in a 1:1 ratio on plates seeded with 20 μl OP50, and allowed to mate overnight. Males were removed from plates, and hermaphrodites were transferred to fresh plates every 24 h until egg laying ceased. Progeny were counted and scored for wrmScarlet fluorescence 48 h after hermaphrodite was moved from plate. Animals were censored as “not sufficiently mated” if male sperm did not suppress self-progeny production to < 5% of the daily total on day 2. Hermaphrodites were counted as producing non-Red progeny if > 2% of the total progeny produced after day 1 of adulthood were non-Red (progeny produced on the first day were not included due to variance in mating efficiency).

Sperm size analysis. Spermatids were isolated as previously described\textsuperscript{35}. In brief, five virgin day 1 adult males were dissected in SM buffer containing dextrose to release spermatids. Spermatids were imaged using Zeiss Axio Imager, and size measured using ImageJ software. Unless otherwise indicated, unique WT controls were performed for each figure panel. For experiments in which sss-1, F48C1.6, and msp-53 RNAi were employed, animals were hatched on RNAi, and treated as OP50 animals—L4s were sequestered for 24 h and virgin day 1 adult males were dissected. Dissected gonads that released activated and mature sperm, instead of only spermatids were censored.

For experiments in which brf-1 RNAi was employed, RNAi was administered post-developationally: animals were hatched on OP50, and L4s were treated with RNAi and sequestered for 48 h, then virgin day 2 adult males were dissected.

Spermatid activation assay. Five virgin males were dissected in SM buffer containing BSA\textsuperscript{35}. An equal volume of SM buffer containing 400 μg/ml Pronase (Millipore Sigma) was added, and spermatids were allowed to activate for 15 min. Images were manually scored, counting spermatids with pseudopodia as “activated.” As with sperm size analysis, L4 males were sequestered for 24 h before dissection, except in the case of brf-1 RNAi, in which L4 males were isolated for 48 h.

VIT-2::GFP imaging/quantification. Synchronous animals were washed from plates and fixed in 40% isopropanol for 2 h before a 30-min wash in PBS with 0.01% Triton-X100. Fixed animals were mounted and imaged on a Zeiss Axio Imager, and fluorescence of most proximal oocyte was quantified using ImageJ software (NIH). In order to ensure animals of the same developmental stage were quantified, only animals with exactly two embryos in utero were imaged. Fluorescence intensity per animal is equal to the sum of the corrected total cell fluorescence (CTCF) of both proximal oocytes.

Developmental timing assay. Synchronous populations of L1 animals were dropped OP50, and beginning at 54 h post-drop scored for gravidity. Animals with uteruses containing at least one egg were considered to be gravid.

Data availability
All data are contained within the manuscript.

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S.P.C. designed the study; A.M.H. performed the experiments; A.M.H. and S.P.C. analyzed data. A.M.H. and S.P.C. wrote and revised the manuscript.

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