Significance of RNA Binding Motif Protein (RBM-39) in developmental processes in *C. elegans*

A Senior Thesis Presented to
The Faculty of the Department of Molecular Biology,
Colorado College

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Acknowledgements

First and foremost, I would like to express sincere gratitude to my advisor, Dr. Darrell Killian, for taking me into his lab and guide me through every stage of the research. His patience and immense knowledge have made this research an enjoyable and rewarding experience. I am extremely grateful for all the supports throughout the academic years. I would also like to thank Dr. Eugenia Olesnicky Killian for editing my thesis and sharing insights on RBM-39 roles in *Drosophila*. This paper would not be the same without her supports.

I thank Colorado College Molecular Biology Department for all the supports inside and outside of the lab. My thanks to Carrie Moon for all the technical and emotional supports during my research. My thanks to Dr. Jennifer Garcia, my academic advisor, for advising me on academic progression and encouragements. I want to thank the Colorado College SCoRE program for providing the opportunities and funding my research in summer 2019. I genuinely appreciate it.

I also want to thank other members from Killian's lab, Patrick Ende and Gus Park, for helping me in the lab, for all the joys and laughs, and for making up my memories of summer research. I am glad to have shared experiences with you.

Lastly, I wish to give personal thanks to my parents, who made my education possible in the first place, and my sister for constant encouragements and accompaniments throughout my life.

Abstract

RNA Binding Motif Protein 39 (or Caperα) is a conserved RBP that is known to act as an alternative splicing activator. Caper (RBM39 homolog in *Drosophila*) has been studied in the development and maintenance of the nervous system, and in cancers. Deficiency in Caper/RBM-39 compromises dendrite morphology, sensory neuron development, neuromuscular junction morphogenesis, and lifespan, mainly in *Drosophila* model. However, very few studies have been conducted in other organisms. For example, in *C. elegans*, its full phenotypic effect remains obscured due to the absence of a knockout mutant. Using *C. elegans* as a model, we have successfully generated an *rbm-39* knockout mutant *(cnj4)* via CRISPR. We found that the homozygous deletion of *rbm-39* leads to severe developmental defects, including sterility, early death, and larval arrest. We used a genetic balancer *tmC25* to maintain the recessive sterile allele in heterozygotes. Indeed, a previous study has found that Caper knockdown in *Drosophila* reduces animal life span. However, further investigation is required to understand the mechanism at the molecular level for larval mortality. Since the sterile phenotype was prominent, we decided to conduct a phenotypic analysis of germline development defects in *rbm-39* mutants to learn potential causes of mutant sterility. Our results show that *rbm-39(cnj4)* animals have either delayed or failed in oogenesis. We found that the mitotic zone length of the germ line was shorter in the homozygous *rbm-39(cnj4)* than N2 (wild type) and heterozygotes, suggesting disruptions in proliferation and/or maintenance of the germline stem cells. Gonad visualization also revealed abnormal oocytes that were seemingly endomitotic in *rbm-39(cnj4)* animals. Taken together, RBM-39 is essential for proper germline development and oogenesis. Since RBM-39 is predominantly an alternative splicing factor, genes that control aspects of germline development may be mis-spliced in the mutant. In general, our study highlights the importance of this conserved splicing factor, RBM-39, in reproductive and early development. We have established a novel mutant strain for future investigations while also expanding the knowledge of this protein's functions in *C. elegans*. Given that germline development in *C. elegans* is well studied and the processes are highly coordinated with defined signaling pathways, the mechanism of RBM-39 in germline development can be studied using the *rbm-39(cnj4)* strain. Future directions should identify the specific signaling pathways and pre-mRNA targets. Additionally, *rbm-39(cnj4)* mutants exhibited mild uncoordinated locomotion, which is strongly associated with neuronal or muscular defects. Future studies should examine the RBM-39 knockout phenotype in neurons and dendritic branches.

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Introduction

RNA Binding Proteins

RNA-Binding Proteins (RBPs) belong to a broad class of protein commonly known to regulate mRNA localization, translation, degradation, and alternative splicing. Conserved RBPs are critical for diverse developmental processes such as dendrite morphogenesis, germ cell development, and spermatogenesis (Antonacci et al., 2015; Zagore et al., 2018; Sutherland et al., 2015). For example, DAZL is an RBP that is essential for gametogenesis. In male germ cells, DAZL upregulates transcripts involved in cell cycle regulation, DNA repair, ubiquitin-ligase activity, spermatogenesis proliferation, differentiation, and survival (Zagore et al., 2018). *Drosophila alan shepard (shep)* or *sup-26* ortholog in *C. elegans*, is another conserved RBP that interacts with a series of post-transcriptional gene regulators and target mRNA in sensory neurons that are important in dendrite morphogenesis (Olesnicky et al., 2018).

RNA Binding Proteins and Alternative Splicing

High eukaryotes are known for the diverse protein variations and complex protein networks. These are the result of alternative splicing that can generate multiple proteins from a single locus, via the assistant from trans-activating factors such as RBPs. In constitutive splicing events, spliceosome formed to remove introns and ligate exons from the pre-mRNA. Alternative splicing differs from constitutive splicing in that some exons may be skipped, or introns incorporated, which generate isoforms of mRNA that could lead to variable outcomes. During the process, RBPs may act as a splicing factor, silencer, or enhancers to function to moderate RNA outcomes (Wang et al., 2015).

SR splicing factors is one large class with conserved serine-arginine-rich domain called the RS domain. The RS domain promotes protein-protein interaction while RNA-recognition motifs (RRMs) are responsible for both RNA and protein interactions. SR Splicing factors bind to the splicing enhance cis-acting elements that activates splicing events. They generally promote cassette exon inclusion by enhancing exon splice-site recognition for ribonucleoprotein complex recruitment (Van Nostrand et al., 2020; Erkelenez et al., 2012). Human Transcriptomic Screening study highlighted the complexity and flexibility of RBP mechanisms in regulating alternative splicing. When RBPs bind to an exon or flanking introns, they can regulate exon inclusion, exclusion, or moderating binding activities of 5’ or 3’ splice site. Although some RBPs have specific RNA binding motifs, a large proportion of them lack a well-defined RNA binding domain. Thus, each RBP may act on a broad range of targets, further demonstrate how diverse their functions can be (Van Nostrand et al., 2020).

Alternative splicing is essential for various cellular processes, such as early development and the nervous system. In humans, more than 95% of genes undergo alternative splicing (Pan et al., 2008). Approximately 25% of the *C. elegans* genes participate in alternative splicing, and this number is likely an underestimate of splicing events. Although with small intron size, *C. elegans* is an intron rich organism similar to mammals, thus serving as a great model for studying alternative splicing (Zahler, 2005).

Role of RNA Binding Motif Proteins 39 (RBM-39)/Caper

RNA Binding Motif Protein 39 (or Caperα) consists of one serine-arginine-rich RS domain at the C-terminus and three RNA-recognition motifs (RRMs). The RRMs are critical for both RNA and protein interactions, while the first two RRMs have distinctive functions through examining the high consensus Caper β (Dowhan et al., 2005). In humans, RBM39 is broadly expressed across tissues (Fagerberg et al., 2014). At the cellular level, it colocalizes at nuclear speckles with spliceosome complexes (Imai et al., 1993). It is also maternally expressed, and expression steadily declines across embryonic development (Levin et al., 2012). RBM-39 is a conserved RBP that is involved in alternative splicing, RNA turnover, and transcriptional regulation (Mai et al., 2016). The worm RBM-39 shares 51% identical amino acid sequence in the SR and RRMs, compared to human RBM39 (Kang et al., 2015).

RBM39 was first identified as autoantigen from hepatocellular carcinoma. It colocalized with a pre-mRNA splicing factor, SC35, and uridine-rich small nuclear RNA, supporting a function in alternative splicing (Imai et al., 1993). RBM39 is intensively involved in hundreds of alternative splicing events. In human cells, RBM39 binds to sites at the intronic region, exon, and 5’ to 3’ UTRs; most of its splicing events are cassette exons and alternative 5’ and 3’ splicing (Mai et al., 2016). Another study found that knockdown of the gene resulted in significantly more inclusion of cassette exons, suggesting a tendency of splicing activation instead of suppression. Interestingly, RBM39 also interacts with U2AF65 on the U2 snRNP protein to coordinate splicing via unelucidated mechanisms (Tari et al., 2019). All these indicate the flexibility and diversity of RBM39 mechanisms in alternative splicing.

In addition to post-transcriptional regulation, many RBPs also interact directly with chromatin and potentially regulate transcription or co-transcriptional splicing (Nafelberg et al., 2015). While most RBPs in humans intrinsically bind to single-stranded RNA, some interact with other proteins that indirectly associate with RNA sequences. For instance, human RBM39 acts as a transcriptional co-activator via direct interaction with Estrogen Receptor (ER) α, ERβ, c-Jun, and Progesterone Receptor (PR) (Jung et al., 2002; Dowhan et al., 2005). RBM39 provides a negative prognosis in several cancers, including breast cancer and leukemia, by moderating splicing isoforms of cell survival genes (Mercier et al., 2014;

Wang et al., 2019). Approximately 304 differentially expressed genes were identified in RNAi knockdown of *RBM39* in human MCF cell line. The majority of the up-regulated genes were microRNA and ribosomal genes that were predominantly associated with cancer and aminoacyl-tRNA biosynthesis, the down-regulated genes include those that are associated with neurodegenerative diseases. Overall, *RBM39* regulates gene transcripts mainly involved in the cell cycle (Mai et al., 2016).

RBM-39/Caper Functions and Mutant Phenotypes

Caper/rbm-39 plays significant roles in neuron development, morphogenesis, and mechanosensory organ development. Through an extensive screening of conserved RBP in *C. elegans*, *rbm-39* (previously known as *Y55F3AM.3*) was found to reduce the quantity of terminal dendritic branches by 19% in PVD neurons (Antonacci et al., 2015). In *Drosophila*, a reduction-of-function mutation of *caper* leads to shortened Class IV dendrite arborization neurons. The cluster of five lateral ch neurons (Ich5) were mispositioned and reduced in neuronal numbers. Hence, *caper/rbm-39* is critical for dendrite morphogenesis, neuron migration, and neuron orientation. The significance of *caper* in sensory neuron developments was reflected by gravitaxis behaviors in *Drosophila*. Both RNAi knockdown and hypomorphic alleles demonstrated progressive loss of bristle organs and disoriented gravitaxis behaviors. Interestingly, these functions were all biased toward males rather than females (Olesnicky et al., 2017).

Caper is also essential for nervous system maintenance and neuromuscular junction (NMJ) morphogenesis such that RNAi knockdown resulted in reduced locomotion, reduced life span, and supernumerary boutons (Wright, 2016). Recently, *caper* knockout in *Drosophila* motoneuron revealed defective locomotion and increased bouton numbers in NMJ innervated muscles. The extent of NMJ defects was again more severe in males. Fragile X Syndrome (FXS) also shares the hallmark of aberrant locomotor activities. Further examination found that *caper* also interacts with FMR1, an RBP that lead to FXS when in dysfunction (Titus et al. 2021). In mice, *Rbm39* directly interacts with ZFP106, another RBP that moderates a subset of alternative splicing events. Knockout of *zfp106* resulted in several neuro-muscular disorders, such as amyotrophic lateral sclerosis (ALS) and Charcot-Marie-Tooth disease (CMT), due to ectopic expression of *Nogo Rtn4* from aberrant mRNA processing (Anderson et al., 2016).

In addition to the nervous system development, RNAi knockdown of *rbm-39* in *C. elegans* revealed mild to moderate penetrance of lethality and sterility in genetic backgrounds of enhanced RNAi efficiency (Ceron et al., 2007). RBM-39 assists in nutrient-dependent metabolism and promotes growth and reproduction. In *C. elegans*, RNAi feeding at the L1 stage reduced worm life span by 30% and brood size

by 6-fold, which corroborates its participation in development and reproductive capacity (Kang et al., 2015).

RBM-39 depletion is strongly associated with mitochondrial dysfunctions (Kang et al., 2015), which are typical traits of neurological disorders. Oxidative stress could trigger apoptosis, disrupt Ca+ homeostasis, and reduce ATP production (Wu et al., 2019). Although RBM-39 depletion in murine hepatocytes did not increase apoptosis, it was found to induce autophagy, cell cycle arrest at G1/S and G2/M, superoxide activation, and transient downregulation of Nrf-2 mediated antioxidant pathways; all contribute to cell and neuron development (Kang et al., 2015).

Although *rbm-39* was implied in a myriad of developmental processes, such as disrupted dendrite morphogenesis (Antonacci et al., 2015), few studies have been conducted in the *C. elegans* model to investigate its precise functions, mechanisms, and phenotypes in neuronal development and germline development/reproduction. Given that RBPs often function redundantly or combinedly with other proteins and splicing factors on the same processes, knockdown of one RBP does not elicit a full phenotypic effect of the protein actions (Tan and Fraser, 2017).

Genome editing using CRISPR Cas9 system

To study the full effect of RBM-39 protein, we created a knockout strain using the CRISPR technique. CRISPR-Cas9 (Clustered regularly interspaced short palindromic repeats) encodes an endonuclease coupled with CRISPR RNAs (crRNAs) that base-pair with the target and a trans-activating CRISPR RNA (tracrRNA) that is required for Cas9 activation. In this study, we used two crRNAs to target the front and end of the gene for deletion. After assembly, the Cas9-RNA complex binds to a specific protospacer-adjacent motif (PAM) dinucleotide on the DNA molecule, such as NGG in the spyCas9 system. To efficiently manipulate CRISPR editing, repair strategies were selected depending on the goals. For example, non-homologous end-joining (NHEJ) is an error prone repair pathway that can be taken advantage of by inducing a Cas9 cut in the genome that may be repaired with an insertion or deletion (indel) at the cleavage site. On the other hand, Homology Directed Repair (HDR) involves an exogenous DNA repair template, enabling precise editing after a Cas9-induced cut in the genome. While NHEJ could generate loss-of-function alleles, HDR has greater flexibility in precise gene editing (Dickinson and Goldstein, 2015).

To further investigate the role of RBM-39 in *C. elegans*, this study aims to produce a full knockout mutant to establish the protein's function, using the HDR to create a precise deletion. The *rbm-39(cnj4)*

homozygotes were sterile, and some exhibited early-onset mortality. To examine the role of *rbm-39(cnj4)* in gonad development, we used DAPI stain to mark the nuclear DNA and visualized gonad under fluorescence microscopy. Through nuclear morphologies, we determined mitosis or meiotic progression of germline stem cells. Status of oogenesis entry, spermatogenesis completion, presence of transition zones, and vulva maturation was recorded for each gonad arm. Additionally, we also measured the length of the mitotic zone according to chromosome morphology. Our results highlight the significance of *rbm-39* in germ-line development.

Materials and Methods

CRISPR-Cas9 Co-Conversion and Microinjection

An overview of the CRISPR co-conversion strategy for creating a *rbm-39* deletion is shown in Figure 1A, and all crRNA, repair template, and primer sequences are provided in Table 1. *NotI* restriction site, GC'GGCCGC, was incorporated in the *rbm-39* repair template. CRISPR mix with *rbm-39* repair template was co-injected with *dpy-10* repair template at 10:1 ratio (Paix et al., 2017; Dernburg and Kohler, 2016). RNA duplex included 60 µM of trans-activating crRNA (tracrRNA), crRNA left 1, crRNA right 1, and 6 µM of *dpy-10* crRNA. Duplex mixture was incubated at 95 °C for 5 minutes, then cooled at room temperature for 5 minutes, and lastly placed at 4 °C in ice. Purified Cas9 protein (Integrated DNA Technologies) was added to the RNA duplex and incubated at room temperature for 15 minutes. Before injection, 6.25 µM *rbm-39* repair template and 0.5 µM *dpy-10* repair template was added to the previous mix. Microinjection was conducted according to the standard protocol (Dernburg and Kohler, 2016). Each Injected worm was placed on a fresh Petri-plate and stored at 22 °C for roughly two days.

Screening progeny of injected animal

Dpy-10 is a well characterized mutation with distinguishable phenotypes. Heterozygous *dpy-10* mutants exhibit roller phenotypes while homozygotes are dumpy. Co-injection of *dpy-10* constituents was a tenth lower than the target, thus effective edit of *dpy-10* should also accompany with intended *rbm-39* deletion. Animals with a dumpy phenotype were screened for *rbm-39* mutation.

Following preliminary identification of mutants, we confirmed *rbm-39* deletion via Polymerase Chain Reaction (PCR) and *Not*I restriction enzyme digestion. Primers flanked a sequence of 592 bp length in *rbm-39* deletion and 6,294 bp in un-edited gene (Table 1). PCR of 15 worm lysates were amplified at 61 °C and run on 1.5% agarose gel.10µl of lysates were incubated in 1µl *Not*I-HF (BioLab) at 37 °C for 15 minutes. *NotI* digestion of deletion amplicon expected two bands of 213 bp and 379 bp.

CRISPR Mix	Sequence
rbm-39 crRNA L1	GGCAAGGAATTGAGAAGATT
rbm-39 crRNA L2	CAAGGACAATATGGTGGATA
dpy-10 crRNA	GCTACCATAGGCACCACGAG
rbm-39 repair template	GCACCGAGAAGACGCTCGAGgcaaggaattgagaaggcggccgcATATGGCAGATATTAGgac gcttaaccacccgatttttc
dpy-10 repair template	CACTTGAACTTCAATACGGCAAGATGAGAATGACTGGAAACCGTACCGCATGCG GTGCCTATGGTAGCGGAGCTTCACATGGCTTCAGACCAACAGCCTAT
Forward Primer	agATCCCGATCAAAAGACAGGAAAAGCTCG
Reverse Primer	CGCAAAAATCCGAGCTTTTCGAATTTCG

Table 1: Sequences of CRISPR co-conversion and PCR experiment
Sequences of crRNAs, repair templates, and PCR primers used in gene editing experiments.

Balancing the mutant

To maintain the recessive sterile allele, we crossed N2 males with a balancer strain, FX30203 (Caenorhabditis Genetic Center). FX30203, *tmC25 [unc-5; myo-2::Venus],* is a genetic balancer consists of two inversions, an recessive *unc-5* mutation, and dominant Venus fluorescent in the pharynx (Dejima et al., 2018). Male heterozygous FX30203 were crossed with non-homozygous F_1 mutants, either wild-type or heterozygous. Wild type moving progeny that expressed Venus markers were isolated on individual plates (Figure 2). The subsequent non-*tmC25* progenies were screened for *rbm-39* deletion by PCR. Plates confirmed with *rbm-39* deletion were kept for strain maintenance. The deletion allele was named *rbm-39 (cnj4).*

Germline analysis and DAPI stain and microscopy

Young adults with mature vulva morphology were picked and immersed in M9. 75% ethanol was used to fix the worms for 5 minutes. Fluorescent dye, 4',6'-diamidino-2-phenylindole hydrochloride (DAPI), was used to stain and visualize DNA. DAPI (Vectashield) were added to the mixture and sit for 5 minutes (Shaham, 2006). Stained worms were mounted on 2% agarose pad on microscopic pad and visualized under Zeiss AxioCam microscope. Mitotic zone, oocytes presence, and spermatogenesis completion were compared between N2, heterozygous *rbm-39* (*cnj4*)/+, and *rbm-39* (*cnj4*) mutants (Figure 3). Images were taken for measurements. Distance from the distal mitotic cell to the transition cell using Fiji ImageJ. Fisher's Exact and Chi-square tests were performed on oogenesis and spermatogenesis between N2, heterozygous, and *rbm-39* deletion. Unpaired and non-parametric t tests (Mann-Whitney U) were performed on the mitotic zone length on Prism.

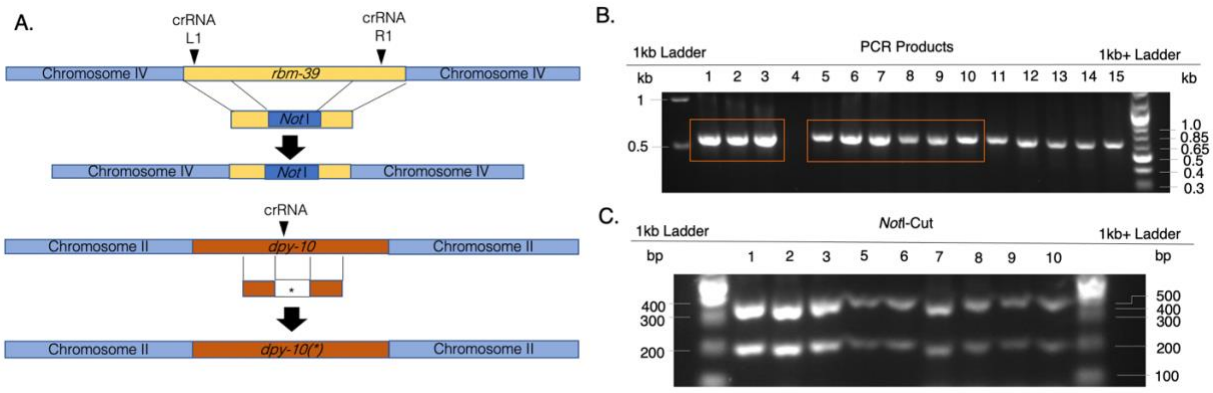

Figure 1: Genome editing strategy and detection of *rbm-39* deletion via PCR and *Not I* digestion (**A**) In co-CRISPR repair strategy, *rbm-39: dpy-10* repair template and crRNA were co-injected at a ratio of 10:1. 84 bp repair template replacing most of *rbm-39* with a *Not* I site. *dpy-10* repair template has a missense mutation. Cas-9 complex cleave sequence at crRNA sites. (**B**) PCR product of 15 worm lysates amplified with Forward (agATCCCGATCAAAAGACAGGAAAAGCTCG) and Reverse primer (CGCAAAAATCCGAGCTTTTCGAATTTCG) on 1.5% agarose gel. 10µl out of 2l product was loaded in each well. Expected 592 bp *rbm-39* deletion allele were present except in the fourth column. (**C**) PCR product with prominent bands (1,2,3,5,6,7,8,9, and 10) were selected for 15 minutes *Not* I-HF Digestion and loaded on 10µl load on 1.5% gel. Expected 213 bp and 379 bp fragments confirmed the precise editing from CRISPR.

Results

Generation of Knockout mutant *rbm-39 (cnj4)*

RBM-39 act as a transcriptional coactivator of estrogen receptors and transcriptional factor AP-1 in response to estradiol presence (Jung et al., 2002). However, it was primarily studied as a splicing factor SR family member in alternative splicing, and it is associated with many developmental events, such as dendrite morphogenesis and sensory neuron development (Olesnicky et al., 2017). Research has identified it as a target for cancer treatments such as Acute Myeloid Leukemia (Wang et al., 2019). Over the years, RBM39 was also loosely implied germ-line development and gametogenesis (Olesnicky et al., 2018; Ceron et al., 2007). Although many studies have highlighted the role of RBM39 in developments, the full effects in *C. elegans* have not been elucidated due to the absence of a knockout strain. To understand the effect of RBM-39 and how well its roles are evolutionarily conserved, we used the Co-CRISPR technique to knock out the gene in *the C. elegans*, an efficient model to study neuronal and germ line development.

Paix et al. (2017) and the Dernburg lab (2016) proposed a technique that includes co-injection of CRISPR components to introduce a *dpy-10* missense mutation. *dpy-10* gives distinguishable phenotype for screening CRISPR-edited individuals. Injecting *rbm-39: dpy-10* constituents at a 10/1 ratio increase of possibility of detecting *rbm-39* deletion from *dpy-10* mutants. We have successfully created a recessive *rbm-39* KO strain *(cnj4)*. Deletion alleles were validated by PCR and *Not*I digestion (Figure 1).

Homozygous deletion of *rbm-39* induced sterility, larval arrest, and larval death

Upon isolating a strain carrying the *rbm-39(cnj4)* deletion, several phenotypes were immediately apparent. *rbm-39 (cnj4)* mutants exhibit larval arrest, a high mortality rate, and sterility. We observed premature death at different larval stages. Few homozygous mutants reached adulthood, and no embryos from homozygous *rbm-39 (cnj4)* mutants were produced. Consistent with previous knockdown studies, *rbm-39 RNAi* caused sterility and lethality in *C. elegans* (Ceron et al. 2007). Knockdown of *rbm-39* induces lethality to varying extents on individuals. These results suggested that RBM-39 not only participates in neuronal development as previous studies indicated (Antonacci et al., 2015), but is also required in reproduction and early development.

To sustain the sterile allele in a genetically stable strain, we crossed modified animals with genetic balancer strain, FX30203. Genetic balancers are genetic constructs that maintain lethal or sterile mutations in viable heterozygotes. An excellent genetic balancer must be able to suppress recombination, yield a specific phenotype in heterozygotes that is distinguishable from homozygotes, and lastly, that phenotype must originate from the same chromosome as the target allele. Chromosomal recombination such as inversions, translocations, and transgene generate different pairing lengths in heterozygotes and efficiently prevent recombination (Edgley et al. 2006). FX30203 used in this study consists of two inversions and an *unc-5(tmIs1241)* genotype with the additional insertion of dominant fluor marker, *myo-2*::Venus, on chromosome four (Dejima et al., 2018). Homozygous strain possesses uncoordinated locomotion of curling or kinked movement due to the *unc-5* mutation. Having two inversions increased the covered region from 2.5 to 6.5 bp and the efficiency in recombination suppression (Dejima et al., 2018). The crossing strategy first obtained heterozygous *tmC25* males then crossed with *rbm-39* mutants (either wild-type or heterozygotes). Given that *myo-2::Venus* was expressed in the pharynx muscle, fluorescence were used identify the heterozygous progeny, which should also possess relatively normal locomotion. Heterozygotes were isolated to yield F_2 generation. Lastly, the non-fluorescent F_2 progeny were screened for *rbm-39 (cnj4)* allele to identify strains that incorporated the mutant allele (Figure 2).

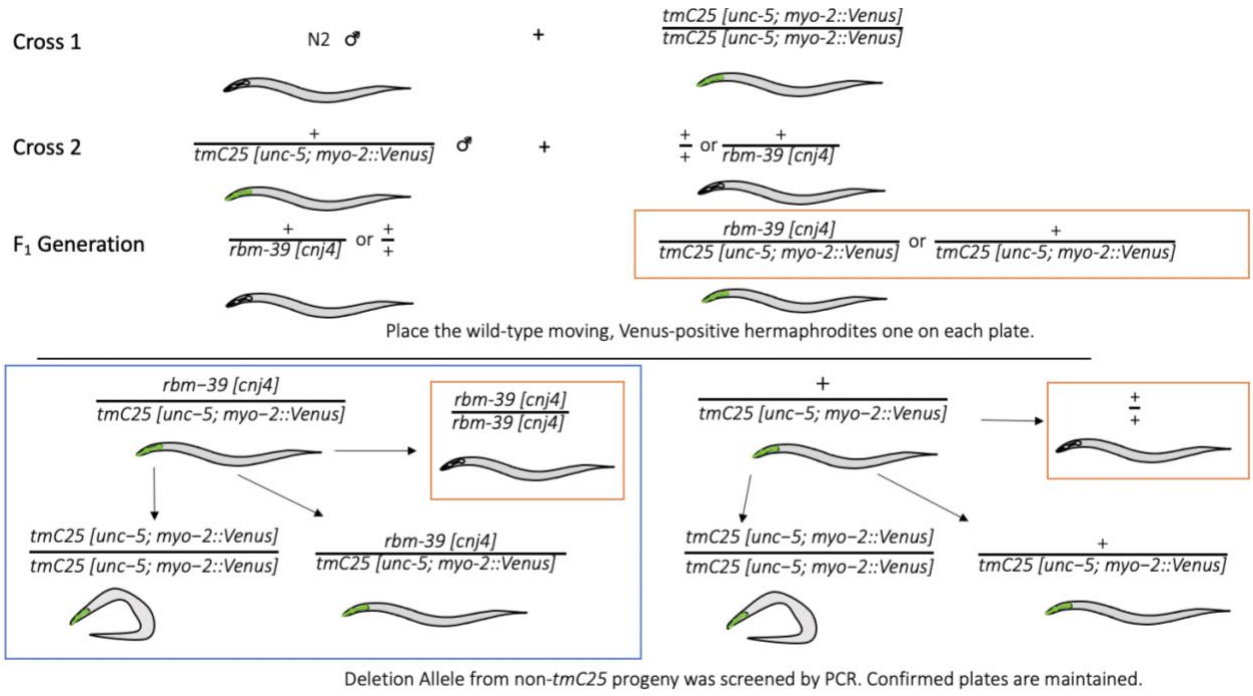

Figure 2: *rbm-39 (cnj4)* was maintained via genetic cross with balancer
tmC25 [unc-5; myo-2::Venus] was crossed with N2 males to obtain heterozygous population. Then, male heterozygous *tmC25* was crossed with mutants, either wild-type or heterozygous. Wild type moving progenies that expressed Venus markers were isolated on individual plates. Plates with non-*tmC25* progeny confirmed with *rbm-39* deletion were kept.

rbm-39 (cnj4) caused defects in gonadogenesis

Germline development in *C. elegans* is a highly orchestrated process that involves complex genetic regulatory pathways. Figure 3 provides an overview of the gonad development. Two arms of gonad develop in opposite proximal directions. Germline stem cells proliferating from the L1 stage, which will end up with approximately 200 to 250 cells in adults. By L2 state, the gonad reorganizes in the way that one Distal Tip Cell (DTC) is allocated at the distal end of each gonad arm while somatic gonad cells amass in a primordium at the center. During L3 to L4, progenitor cells from the two arms rapidly expand toward the anterior and posterior direction, respectively. Concurrently, starting from the mid-L3 stage, these stem cells commence to differentiate and enter meiosis. In the L4 stage, spermatogenesis occurs. Sperm accumulates in the pocket-like spermatheca. Subsequently, the distal sperm secrete Major Sperm Protein (MSP) hormone that signals oocyte maturation and ovulation. In adults, oocytes mature and ovulate into the spermatheca via proximal sheath cell contraction and distal spermatheca dilation, where fertilization ensues (Hansen and Schedl, 2013).

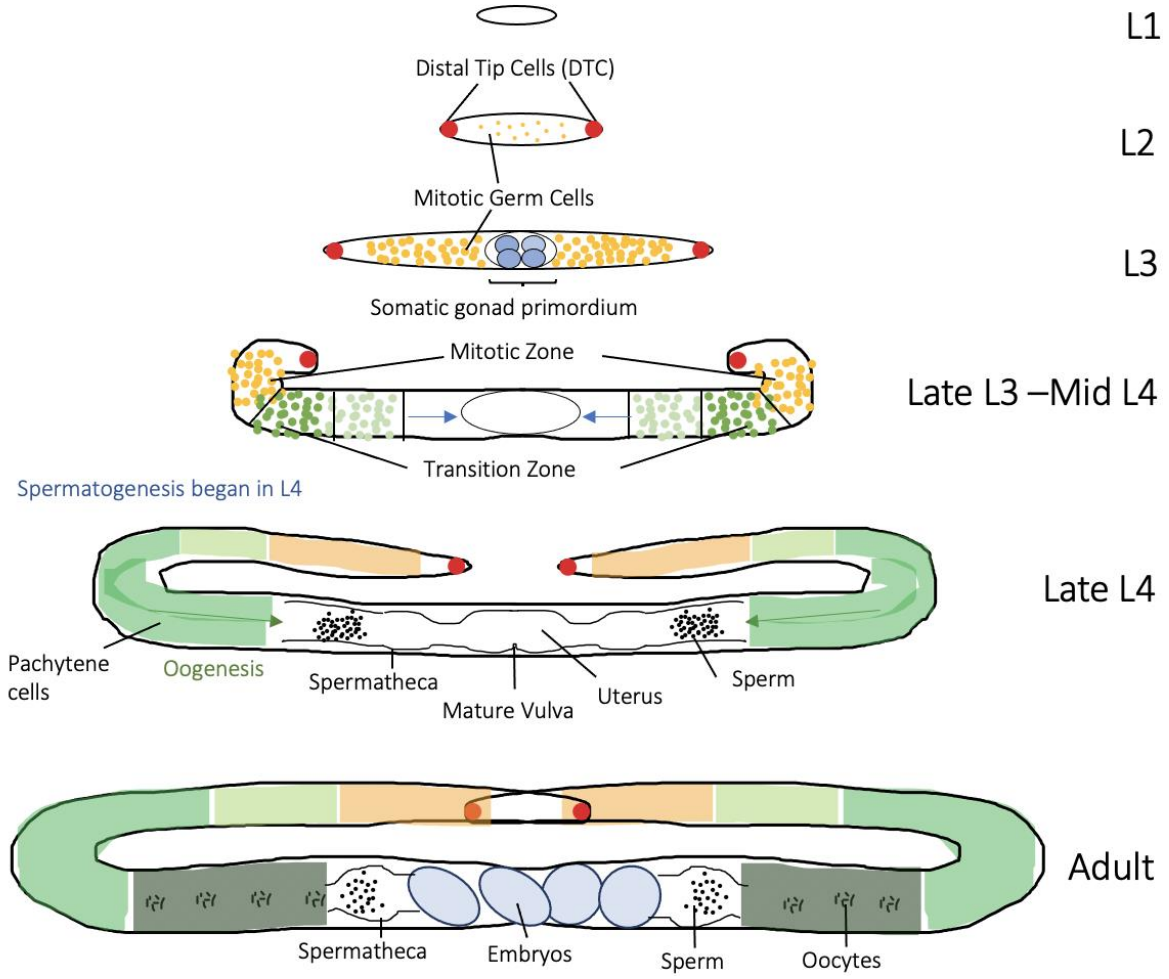

Figure 3: Overview of hermaphrodite gonadogenesis (Hubbard, 2005)
Gonadal arms extend in opposite proximal directions. Germline stem cells proliferate from L1 stage, differentiate in mid-L3 stage to form the transition zone. Spermatogenesis occurs at L4 stage. By late L4, vulva develops, sperm produced, oogenesis occurs and reaching completion. Ovulation starts and embryos formed in adult.

This switch between mitotic to meiosis cell fate has been studied intensively. In the basic model, the GLP-1/Notch receptor on the stem cells inhibited the meiotic cell fate in response to ligands from the DTC. Ligands LAG-2 and APX-1 bind to GLP-1 receptors on the germ cells, which activate LAG-1 transcriptional factor and SEL-8 transcriptional co-activators. Subsequently, nucleus complex formed to regulate gene expression that determines proliferative cell fate. A concentration gradient of ligand exists to regulate proper meiotic entry. Ligand concentration decreases in the proximal direction; thus, the cells that reach the boundary of the DTC niche enter meiosis prophase. As the Notch signaling diminished below a threshold, GLD-1 and GLD-2, in a redundant manner, promote meiotic entry. Interestingly, GLD-1 and NOS-3 in the same pathway encode for RBPs that inhibit translation via 3' UTR regulation,

highlighting the involvements of post-transcriptional modification during development (Hubbard and Schedl, 2019; Hansen and Schedl, 2013).

Indeed, many studies have proposed the function of pre-mRNA splicing in cell fate decisions. Interruptions in both constitutive and alternative splicing may, directly and indirectly, disrupt multiple regulatory programs. Throughout the splicing cascade, many splicing factors within the mitotic-to-meiotic switch pathways, such as *prp-17,* promote meiotic entry (Kerins et al., 2010).

As oocytes mature, MSP hormone is secreted from the distal sperms and interacts with the VAB-1 Eph receptor protein-tyrosine kinase on the proximal oocytes. This binding induces a signal for Mitogen-activated protein kinase (MAPK), a critical oocyte maturation pathway. During pachytene exit, MAPK is inactivated to arrest oocytes in the G_2/M. As oocytes mature, the signal from sperms reactivates MAPK that oocytes enter the M phase (Huelgas-Morales and Greenstein, 2018). Many regulatory proteins participate in the pathway. LIP-1, a MAPK phosphatase, is required to dephosphorylate MAPK that properly arrest the germ cells that exist pachytene at G2/M. LIP-1 deficiency caused endomitotic cells instead of diplotene/diakinesis (Hajnal and Berset, 2002). LIN-49 and the OMA (zinc finger RBP) proteins, together, mediate the oocyte's metaphase transition. They interact to form RNP complexes that mediate a translational modification that favors oocyte maturation (Huelgas-Morales and Greenstein, 2018).

Our preliminary examination of gonad noted that *rbm-39* KO exhibit seemingly normal spatial organization and no tumor formation. To characterize the RBM-39 roles in gonadogenesis, we captured images of gonads using fluorescence microscopy at 20X and 40X. Animals with mature vulval morphologies, indicating that they were mature adults, were selected, fixed in ethanol, and stained by DAPI to reveal nuclear morphologies.

To determine which stage of development was disrupted, completion of oogenesis and spermatogenesis were counted based on oocyte and sperms presence. Tested animals all demonstrated mature somatic development that correspond with oogenesis completion. We found that the percentage of *rbm-39* deletion (n=390) entered oogenesis was 54.36%, which was significantly lower than the control (n=48) and heterozygous (n=38), 100%. Fisher's exact test yielded a P-value smaller than 0.0001. The percentage of spermatogenesis completion was statistically insignificant, with a P-value of 0.1583 (Table 2). Compared to 100% completion in the control and heterozygous, 94.36% of *rbm-39* mutant did not complete spermatogenesis, observed with spermatocytes. These results suggested that entry of oogenesis

was disrupted. Additionally, mutants displayed abnormal oocytes that resemble Emo phenotype, endomitotic oocytes (Figure 4). Germline visualization revealed dispersed euploid or polyploid nuclei (Figure 4).

A.

	rbm-39(cnj4)	*rbm-39(cnj4)/+*	*N2*
Oogenesis	212/390 (54.36%)	38/38 (100%)	48/48 (100%)
Spermatogenesis	367/390 (94.36%)	38/38 (100%)	48/48 (100%)

B.

	N2	*rbm-39(cnj4)/+*	**Chi-square**
Oogenesis	<0.0001*	<0.0001*	<0.0001*
Spermatogenesis	0.1583	0.2476	0.0696

Table 2: *rbm-39* deletion significantly reduced the percentage of oogenesis completion
Individuals with adult vulval morphology were DAPI stained. Oocytes and Sperms were visualized and counted under Zeiss Florescent Microscopy. (**A**) Presence of oocytes and sperms between genotypes. (**B**) Fisher's Exact Test and Chi-square p values of *rbm-39(ncj4)* with the heterozygous (*rbm-39(cnj4)/+*) and N2. Variations in number of animals entered oogenesis was statistically significant between *rbm-39(cnj4)* with N2 and *rbm-39(cnj4)/+*.

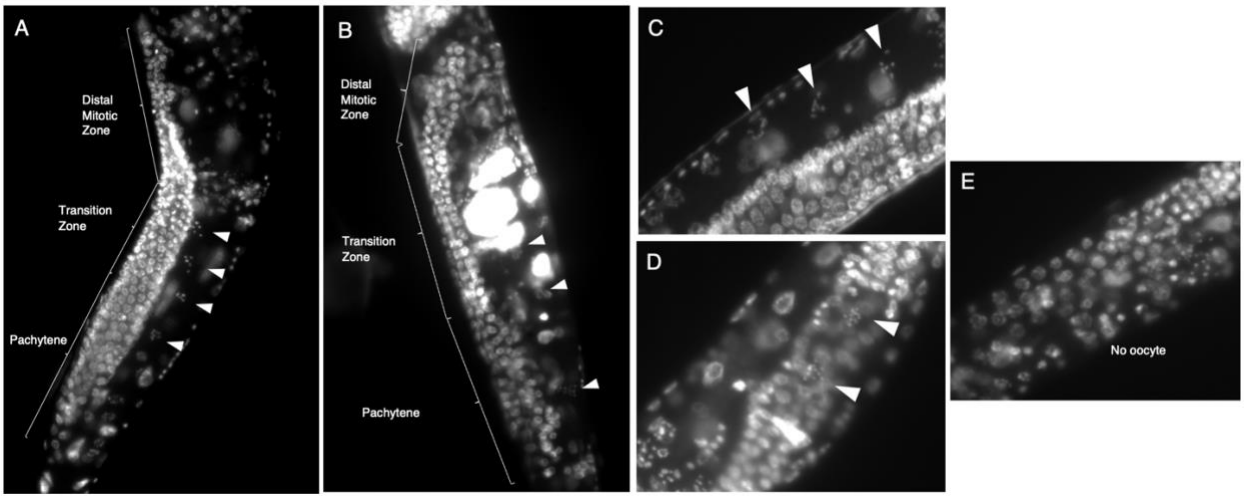

Figure 4: *rbm-39 (cnj4)* exhibit reduced Mitotic Zone Length and Endomitotic oocytes
rbm-39 (cnj4) and N2 hermaphrodites with adult vulval morphology were fixed in ethanol. Full worms were stained with DAPI and visualized under fluorescence microscopy. (**A**) N2 hermaphrodite; (**B**) *rbm-39 (cnj4)*; Scale Bar: 20 μm. Arrow heads point to oocytes. (**C**) Normal oocytes from N2; (**D**) Endomitotic oocytes from *rbm-39 (cnj4);* (**E**) Oocyte absent from *rbm-39 (cnj4).*

We examined the effects of *rbm-39 (cnj4)* in proliferation and differentiation zones by measuring the length of the mitotic zone (MZ) in micrometer (μm) via ImageJ. Frequency analysis found that *rbm-39 (cnj4)* displayed a non-normal distribution. Hence, we selected the unpaired nonparametric Mann-Whitney U test for genotype comparison. Results showed that *rbm-39 (cnj4)* has a mean mitotic zone

value of 38.23 μm (n=386), in comparison to 70.91 μm (n=35) in heterozygous and 74.63 μm (n=48) in N2 (Appendix 1). Mann-Whitney U test found that *rbm-39 (cnj4)* has a statistically significant reduction in the length of the mitotic zone, compared to heterozygous (U=519.5, p=<0.0001, Table 3) and N2 (U=612, p=<0.0001, Table 3). The unpaired t-test between heterozygous and N2 accepts the null hypothesis that there is no significant variation (U=754.5, p=0.4337, Table 3). Results found that *rbm-39 (cnj4)* displayed statistical differences in the MZ length with heterozygous and wildtype (N2), suggesting defects in the process of proliferative to meiosis switch in the cell cycle regulation (Figure 5).

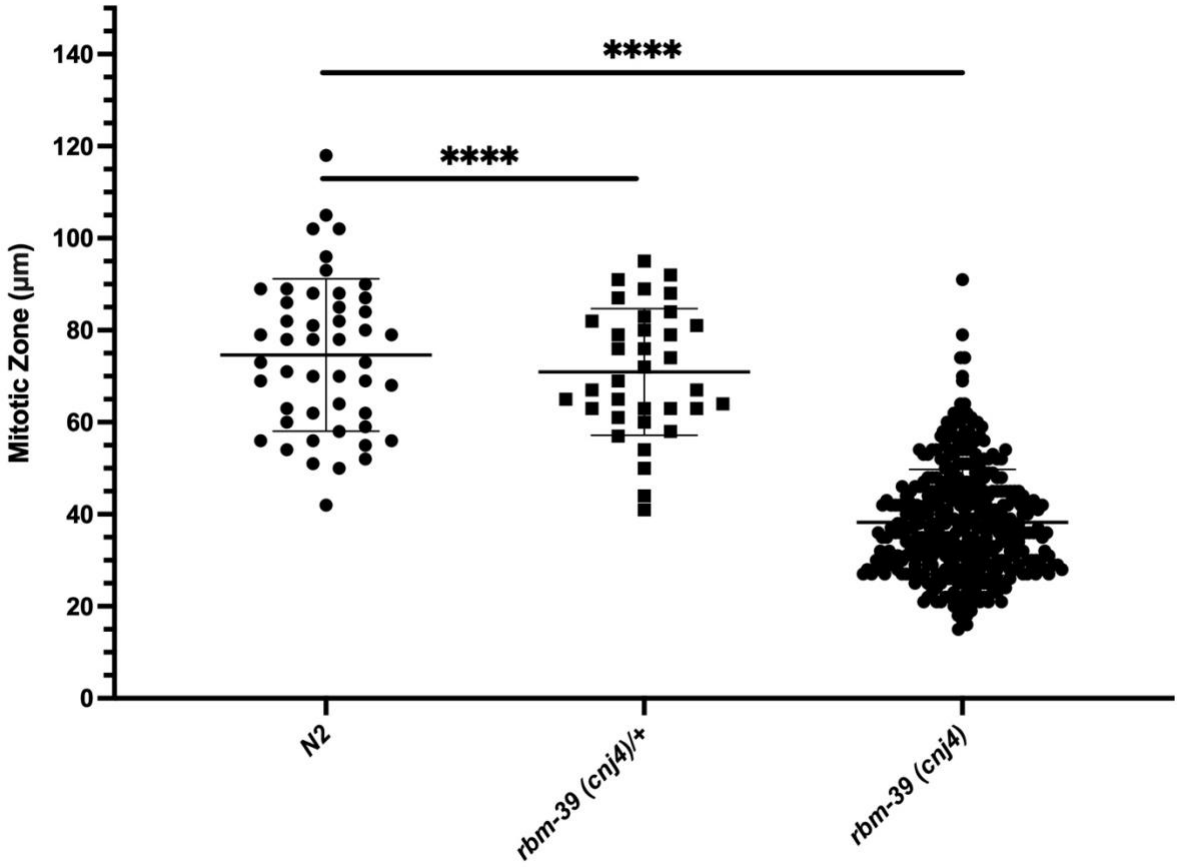

Figure 5: *rbm-39* deletion caused a smaller length of mitotic zone
DAPI stained hermaphrodites with adult vulva morphology were imaged under Zeiss Fluorescent Microscopy. The length of mitotic zone (μm) was measured on ImageJ. Differences in Mitotic Zone Length was statistically significant in *rbm-39(cnj4)* compared with *rbm-39(cnj4)/+* and N2.

Unpaired t test	P value	Mann-Whitney U	Difference between medians	One- or two-tailed P value?
rbm-39(cnj4) vs N2	<0.0001 ****	612	38.5	Two-tailed
rbm-39(cnj4) vs *rbm-39(cnj4)/+*	<0.0001 ****	519.5	32	Two-tailed
rbm-39(cnj4)/+ vs N2	0.4337	754.5	6.5	Two-tailed

Table 3: *rbm-39* deletion significantly reduced the length of mitotic zone (µm)
The length of mitotic zone in µm was measured in the ImageJ. Unpaired non-parametric t tests (Mann-Whitney U) were applied to the mitotic zone comparisons. Differences in Mitotic Zone Length was statistically significant in *rbm-39(cnj4)* compared with *rbm-39(cnj4)/+* and N2.

Discussion

Despite the fact that Caper/RBM-39 was known to participate in the neuronal development, maintenance, and has been implicated in cancers, its full phenotypic effect remains indistinct (Wright, 2016; Olesnicky et al., 2017; Anderson et al., 2016; Mercier et al., 2014; Antonacci et al., 2015). Furthermore, the absence of knockout mutants in *C. elegans* leave the gap on understanding evolutionarily conserved roles of RBM-39 homologs. Prompted by an interest to investigate *rbm-39* role in neuronal development, we have generated a *rbm-39* knockout mutant *(cnj4)* in the *C. elegans* model.

We found that the homozygous deletion leads to prominent developmental defects, including sterility, early death, and larval arrest. This was not the first time such phenotype is found to associate with RBM-39. However, previous studies found only mild phenotype possibly due to incomplete knockdown of expression (Ceron et al. 2007).

The underlying cause of lethality varies. We speculate that RBM-39 is involved in longevity relevant pathways that triggers premature death. Indeed, *rbm-39* was identified as one of the many genes that significantly shorten animal life span in the background of *daf-2* RNAi animals (Samuelson et al., 2007). It regulates the TCA (tricarboxylic acid cycle) in biosynthesis, mitochondrial transcriptional regulation, and metabolite homeostasis. Deprivation of Caper/*Y55F3AM.3* (*rbm-39*) via RNAi significantly reduces life span by 64% (Kang et al., 2015).

RBM-39 is important for germline development

The shorter mitotic zone resembles a *Glp* phenotype (germ-line proliferation defect) which is usually caused by shortened stem cell pool arise from premature meiotic entry. The underlying mechanisms, however, are many. First of all, germline cells may have slower cycling than the signaling from the growth of somatic gonad cells. The proliferative stem cells are located near the DTC and are actively

cycling. While these stem cells proliferate in proximal direction and enter meiosis, the germline stem cells must be retained within the niche of the DTC, 2 to 4 rows of cells (Crittenden et al., 2006). Another factor contributing to meiotic entry is the coordination between somatic gonad cells and germ cells. Juxtaposition of the distal sheath cells, from the gonadal sheath/spermatheca (SS) lineage, with germ cells were important for proper germ-line amplification, highlighting the temporal coordination between somatic gonad and germline. Notably, ablation of Sh1, the most distal of the SS lineage, displayed a significantly reduced proliferative zone (Killian and Hubbard, 2005). Hence, it is possible that the shorter MZ was a result of disrupted temporal interactions between SS lineage cells and germ line, which may be caused by a slower germ cell cycling than the development of SS lineage, thus leading to premature transition to meiosis.

Unlike interactions with SS lineage, a germ-line autonomous regulation of cell cycle influences the MZ length. For example, RSKS-1, served as a downstream effector of TOR (target of rapamycin) pathway, coordinates growth and metabolism. RSKS-1 promotes germline expansion such that *rsks-1(sv31)* mutants have a 50% reduction of proliferative cells. It promotes cell cycle progression in the larval germ cells that mutants exhibit delay in cycling (Korta et al., 2012). Also, loss of GLP-1, independent of the cell positions, prevents transit-amplifying division and reduces the proliferative zone (Fox and Schedl, 2015). Hence, it is possible that the RBM-39 may involve pathway genes such as *rsks-1* to supervise the decision between proliferation and meiosis.

In addition, MZ length could be influenced by nutrient up-take and metabolism. Overall, the cell cycle become slower when nutrients are inefficiently metabolized. Mutants such as *eat-2* that restrict food consumption, or *pept-1* that prevent protein digestion, act on the cell cycle and restrict proliferation (Hubbard and Schedl, 2019). Notably, RBM-39 was implicated in nutrient signaling and redox homeostasis. In mitochondria, it is induced by glucose and activates *c-Myc*, a common transcriptional regulator, to promote cell proliferation. Absence of RBM-39 induced mitochondrial superoxide and caused cell cycle arrest in G1/S and G2/S. This Caper/RBM-39 found to have high degree of conservation in target metabolites within the bioenergy pathway between *C. elegans* and mammals (Kang et al., 2015). This study supports the postulation that a shorter proliferation zone in *rbm-39 (cnj4)* was due to slower cell cycle progression.

RBM-39 deletion disrupts oogenesis and results in a sterile phenotype

What role did RBM-39 played in development? To investigate potential explanations of sterility, we used a microscopic approach to visualize the gonad and nuclear morphologies. We found less animals

completed oogenesis by late L4/adult, which represent either a heterochronic delay in the germ-line development or failure in oogenesis entry. However, it is difficult to pinpoint the exact explanation. Future research should study the oogenesis process in the mutant animals over a longer period of time. Nevertheless, this study confirms defective oogenesis, either at the pachytene exit, oocyte growth, or ovulation.

The absence of oocytes or heterochronic delay of oogenesis should have contribute to the sterile phenotype. Additionally, among animals with oocytes, many of them were observed with abnormal oocytes. Typically, they consist of more than six chromosomes, resembling endomitotic oocytes (Emo), which could also lead to inviable fertilization. However, the precise mechanisms and causes of abnormal oocytes also require further research.

There are many reasons that explain the sterility. Firstly, alteration in the sexual cell fate determination, such as feminization or masculinization of the germline, (eg. *fog-2* and *mog-1* mutations, respectively) can led to defective reproductive cells, thus induce sterility. Secondly, defects in the germ-line proliferation, such as tumor formation in *glp-1* mutants and ectopic proliferation in *lin-12* disrupt gametogenesis (Riddle et al., 1997). Thirdly, sterile phenotypes could arise from the disorders at pachytene exit. For example, *lin-41* null mutants activate CDF-1 that ultimately cause pachytene cells to enter M phase prematurely. LIN-41 positively regulates oocyte growth and meiotic progression. Spindle assembly skipped the diplotene stage and retain mitotic organization, which result in additional cycles of replication and polyploid nuclei (Spike et al., 2014). Furthermore, defects in the somatic gonad such as DTC, gonadal sheath cells, and spermatheca, may lead to defective communications with the germ cells and lead to aberrant development. The communication between germ cells and somatic gonad cells are critical in regulating the developmental processes (Killian and Hubbard, 2005). Lastly, disruptions in the cell cycle genes, as demonstrated in GLP-1 and RSKS-1, may also lead to proliferative defects. Additional explanations were studied, including vacuolated germ lines, abnormal nuclear morphologies in undifferentiated germ lines, defective oocytes, vulval abnormalities, and maternal effect (Riddle et al., 1997).

Endomitotic oocytes (Emo) are derived from ovulation failures, when oocytes undergo additional cycles of DNA replication. Many genes resulting from such phenotype. For example, *mup-2* encodes a troponin T homologue that regulates muscle contraction in the gonadal sheath cell. Its mutant (*e2346ts*) resulted in defects in the sheath cell contraction, thus leading to endomitotic oocytes (Myers et al., 1996). Another gene, *emo-1* also leads to this phenotype but through disruption protein translocation (Iwasaki et al.,

1996). Ovulation process requires proper coordination between somatic gonad cells and oocytes. DPL-1, a subunit of E2F heterodimeric transcription factor. The loss-of-function *dpl-1* caused defects in spermatheca valve dilation, thus trapped the matured oocytes in the proximal gonad (Chi and Reinke, 2009). Depletion of APX-1, a DSL ligand that is involved in LIN-21 and GLP-1 signaling, was proposed to disrupt Calcium influx pathway in both the sheath cell and spermatheca and caused ovulation defects (McGovern et al., 2018). Additionally, Emo phenotype may also stem from cytokinesis disruption. PTC-1, a membrane protein that modulates cell fate specification is required in cytokinesis. RNAi and deletion of this gene caused multi-nucleated oocytes (Kuwabara et al., 2000). Additionally, endomitotic oocytes from uncontrolled replication were observed in *vha-19 (RNAi)*, which is a protein responsible for molecule trafficking and osmotic balance in both oocytes and embryos. Knight and others (2012) proposed that VHA-19 might also regulate cytokinesis to promote oocyte development. In general, Emo phenotype could be caused by many mechanisms.

The role of RBM-39 in developmental processes

It is possible that RBM-39 modulate splicing of genes that directly or indirectly participate in many pathways relevant to oogenesis entry, maturation, and ovulation. While the exact mechanism remains elusive, there are some speculations. First of all, ovulation failed at initiation stage, as a result of defective sperms or inefficient sperm-oocyte interactions. Previous study has found a male biased phenotype in Caper dysfunction and high RNA expression in *C. elegans* sperms (Wright, 2016; Ma et al., 2014). Secondly, mutants presented defective ovulation processes due to altered protein expressions that are involved in sheath cells and spermatheca function and development. Taken together, it is possible there exists certain alteration in the sperm genes that hinder the signal transduction in oocyte maturation. Thirdly, it is very likely that all the phenotypes observed were due to the disruptions on the meiotic cell cycle. Notably, many studies have implied the RBM-39 role in regulating cell cycle, supporting the mechanisms via cell cycle regulation (Mai et al., 2016; Campbell et al., 2018). It promotes cell survival by favoring genes isoforms that are involved transcriptional network, such as HOXA9 in AML (Wang et al. 2019). Note that all these speculations may not necessarily be mutually exclusive; a splicing factor could regulate multiple processes in parallel in tissue specific manner that combined to generate the phenotypes. Although our results provided no specific explanations for the cause of sterility, they emphasize the need for future investigations on the precise mechanisms of RBM-39 function in gonad development.

Regardless of the specific mechanism of *rbm-39* in oogenesis defect, our study identified its phenotypic effect in the germline. Considering the fact that RBM-39 is also maternal supplied; even in the

homozygous mutants, there may be sufficient level of RBM-39 to survive through embryonic and early development. Since *rbm-39* is highly conserved in *C. elegans*, *Drosophila*, mouse, and human, it would be interesting to further investigate the cause of abnormal germ-line development by using protein markers or at different developmental stages.

RBM-39/Caper has significant implication in neuronal development (Olesnicky et al., 2017; Anderson et al., 2016; Mercier et al., 2014; Antonacci et al., 2015). Indeed, we observed slow and weak movements in *rbm-39* mutants which resembles uncoordinated locomotory behavior (*unc*). *Unc* phenotype is commonly caused by defects in the nervous system or muscles, such as UNC-122 (Loria et al., 2004). Previous knockdown assay in *C. elegans* resulted in incomplete phenotypes. Therefore, future research should investigate the effects of *rbm-39* deletion in the neuron development, by crossing *rbm-39 (cnj4)* with animals that possess neuronal markers.

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Appendix

Genotypes	Total count	Min. (µm)	Max. (µm)	Range (µm)	Mean (µm)	Std. Deviation (µm)	Std. Error of Mean (µm)	Coefficient of variation	Normal Distribution
rbm-39(cnj4)	386	15	91	76	38.23	11.51	0.5859	30.11%	No
rbm-39(cnj4)/+	35	41	95	54	70.91	13.77	2.328	19.42%	Yes
N2	48	42	118	76	74.63	16.56	2.391	22.2%%	Yes

Appendix 1: The length of mitotic zone (µm) in *rbm-39 (cnj4)* has a smaller mean value and not normally distributed.

Full worm DAPI staining from *rbm-39(cnj4)*, *rbm-39(cnj4)/+*, and N2 were mounted on 2% agarose pad on microscopy slide. Imaging was performed on Zeiss Fluorescent Microscope. The length of mitotic zone in µm was measured in the ImageJ. Descriptive Statistics between *rbm-39(cnj4)*, heterozygous, and N2 are compared in the table. *rbm-39(cnj4)* did not pass the normality test.