

CHARACTERIZING THE  
PROTEASOMAL SUBUNIT RPN-6.2  
IN *C. ELEGANS*

by

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## ABSTRACT

Spermatogenesis is the process whereby sperm cells develop into specialized reproductive cells capable of fertilizing an oocyte. The proteins that tightly regulate sperm cell development are in turn regulated by the ubiquitin proteasome system (UPS). Due to the proteasomes role in regulating meiosis, disruption of the proteasome during spermatogenesis may result in decreased sperm counts and other fertilization defects. We have identified a proteasomal subunit, RPN-6.2, exclusively expressed in sperm. RPN-6.2 surrounds the DNA in spermatids and the motile spermatozoa, while also appearing as dense puncta scattered around the cytoplasm. RPN-6.2 does not localize to membranous organelles or the mitochondria in spermatids or spermatozoa. *rpn-6.2* knock-down or RPN-6.2 mutant *C. elegans* produce less progeny during their reproductive period. *rpn-6.2* mutant *C. elegans* produce fewer sperm suggesting the decrease in progeny may be directly linked to a defect in spermatogenesis caused by dysfunctional proteasome.

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## CHAPTER I: INTRODUCTION

Defects in spermatogenesis or spermatid homeostasis are known to cause fertility issues in men. The lack of knowledge regarding sperm counts recently made national news. The New York Times published an article in 2017 highlighting a meta-analysis showing sperm counts in western men has dropped 50% since 1973 (Levine *et al*, 2017). There is no concrete evidence accounting for the drastic decrease in sperm counts, making sperm studies valuable to the scientific community. The difficulty of studying spermatogenesis in human sperm has led scientists to pursue alternate models. The hermaphroditic nematode, *Caenorhabditis elegans* (*C. elegans*), are particularly useful to study spermatogenetic defects as they share conserved mechanisms with mammals and provide a simple model to observe biological events during spermatogenesis.

The meiotic stages during spermatogenesis are tightly regulated by targeted protein degradation via the ubiquitin-proteasome system (UPS). Studies in *C. elegans*, have implicated roles for the proteasome during meiosis, but these studies mostly focus on oogenesis. The proteasome degrades proteins targeted by the anaphase-promoting complex (APC) (Shakes *et al*, 2003) and also appears to be required for the pairing of homologous chromosomes during meiosis in the oocyte (Ahuja *et al*, 2017). However, there are key differences in when the meiotic cycles of egg and sperm begin and are completed. Oocytes begin meiosis and are halted at prophase I until fertilized by a sperm. Spermatogenesis is completed prior to fertilization, resulting in the production of multiple haploid sperm which go on to fertilize an oocyte. Given the differences in cellular dynamics between the development of sex cells, it is important to study each cell independently. The role of the proteasome during these processes remains to be explored.

In this study, we focus on spermatogenesis, specifically the role of the sperm specific proteasome subunit, RPN-6.2.

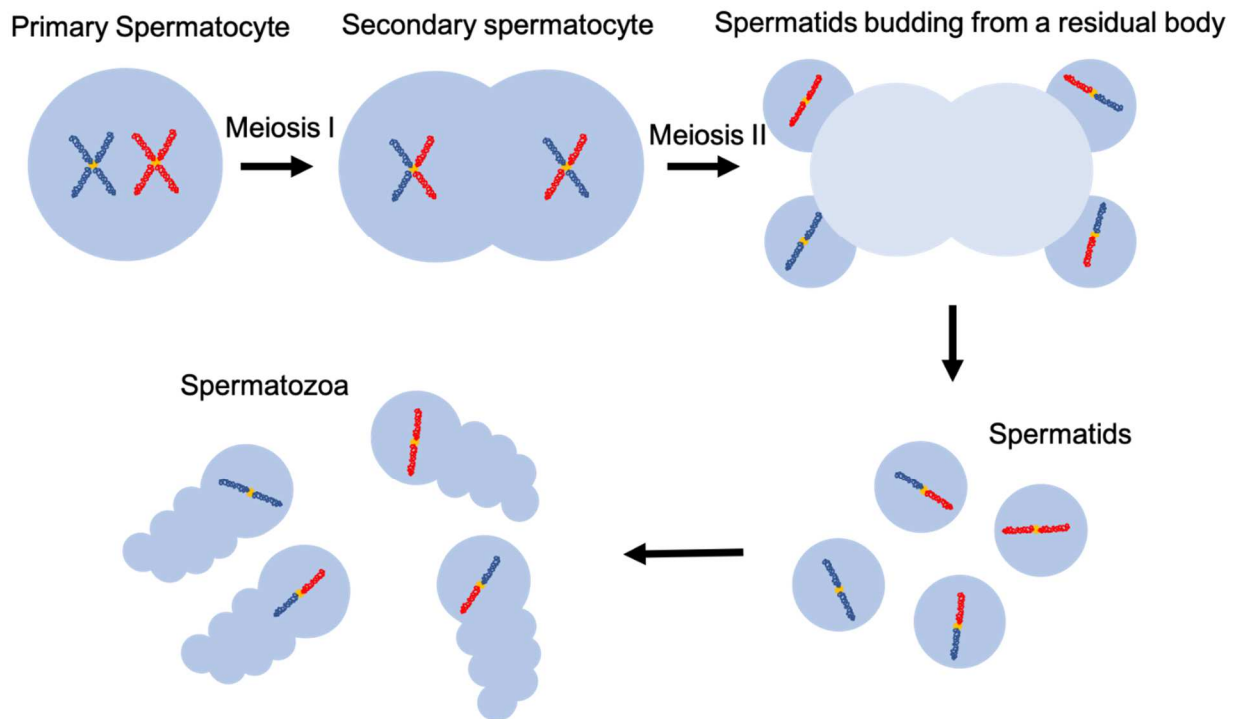
### **Spermatogenesis**

Spermatogenesis is the specialization of germ cells resulting in haploid spermatozoa. Spermatogenesis is the counterpart to oogenesis and is a common process among animals that undergo sexual reproduction. *C. elegans* is a hermaphroditic species. In *C. elegans* hermaphrodites, spermatogenesis precedes oogenesis. Once oogenesis begins, spermatogenesis stops, and sperm production is halted. The sperm are inactive until they enter the spermatheca near the beginning of ovulation. Spermatogenesis begins with a single diploid primary spermatocyte that will undergo meiosis I forming a secondary spermatocyte (Figure 1). The secondary spermatocyte undergoes a second round of meiosis, forming 4 haploid spermatids and leaving behind a residual body containing non-essential organelles and transcriptional machinery. These haploid spermatids contain the proteins necessary for further development into spermatozoa, the mature, motile sperm cell. Remarkably, the physical presence of paternal DNA in the spermatozoa is not required for the fertilization of an oocyte (Sadler *et al*, 2000). Anucleate sperm fertilize and activate the oocyte, initiate polarization of the embryo, but fail to form a functional embryo.

Dysfunctional spermatogenesis may result in polyspermy, sterilization, and decreased fertilization (Tapanainen *et al*, 1997). Although spermatogenesis is a widely studied process, little is understood of the underlying mechanisms of spermatogenesis resulting in functional sperm. Interestingly, increased proteasomal activity in the nucleus

is associated with the spermatid formation in humans (Tipler *et al*, 1997) indicating a role for the ubiquitin-proteasome system during spermatogenesis.

The ubiquitin-proteasome system is involved in the penetration of the vitelline coat during fertilization in mammals, echinoderms, and ascidians, which may help to explain the increase in proteasome activity in spermatids (Morales *et al*, 2013), (Chakravarty *et al*, 2008). On the other hand, the nematode *C. elegans* lacks the acrosomal fertilization mechanism yet appears to have proteasomes in *C. elegans* sperm (Boyd lab), suggesting an undiscovered function for the proteasome in sperm.



**Figure 1: Spermatogenesis**

Diploid primary spermatocytes undergo meiosis I to form secondary spermatocytes. Secondary spermatocytes undergo meiosis II forming haploid spermatids that will bud off of a residual body. Spermatids are then activated to form motile spermatozoa with pseudopods, capable of fertilizing an oocyte.

## **The Ubiquitin-Proteasome Pathway**

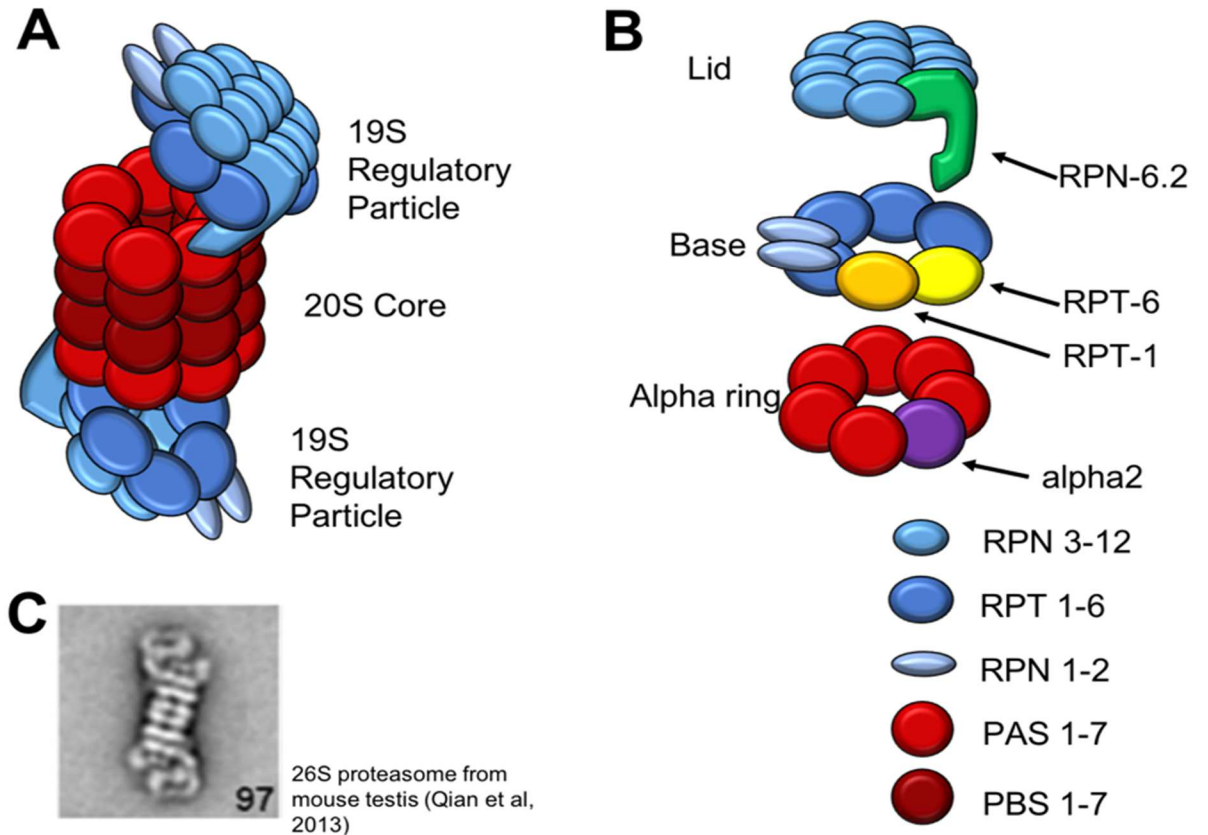
Proteasomal activity is required for fertilization in multiple models such as pig, mouse, human, ascidian, and echinoderm (Reviewed by Zimmerman *et al*, 2009). Disruption of the UPS using protease inhibitors on echinoderm (Yokota *et al*, 2007), cow (Rawe *et al*, 2008), and pig sperm (Sun *et al*, 2004) prevents spermatozoa from penetrating the vitelline coat of the oocyte. Pronuclear development and sperm aster formation in human sperm are also dependent on proteasome activity, specifically the chymotrypsin-like activity of the 20S core (Rawe *et al*, 2008). These studies focus on proteasome involvement during and after penetration of the egg, but do not address the involvement of proteasome prior to capacitation.

The proteasome is best known for its role in the ubiquitin-proteasome system (UPS) where it acts to cleave misfolded or excess proteins into smaller peptides, which are further degraded to amino acids, helping to maintain proteostasis and prevent the formation of toxic protein aggregates. In addition to this primary function, the UPS is also essential for other cellular processes which will be discussed later.

The proteasome is recruited to a substrate by its recognition of a post-translation modification known as ubiquitination. Ubiquitin is covalently attached to proteins through enzymatic reactions forming mono or polyubiquitin chains, which then act as a signals for the cell. Ubiquitin is a 76 amino acid protein containing 7 lysine residues and a N-terminal methionine and is able to form specific polyubiquitin chains by conjugating ubiquitin moieties to any of these 8 residues. Ubiquitination of proteins results from the coordination of E1, E2, and E3 enzymes. The E1 ubiquitin activating enzyme adenylates the C-terminal glycine of ubiquitin in an ATP dependent reaction, forming a thioester bond between the

E1 cysteine residue and the now activated glycine residue of ubiquitin. The activated ubiquitin is then transferred to the E2 conjugating enzyme through transthiolation. E3 ligases bind specific proteins and mediate the transfer of ubiquitin from the E2 enzyme to protein. This process can be repeated to form a specific type of polyubiquitin chain, known as K48 chains, that act as a signal for proteasomal degradation.

The proteasome is a large 700 KDa protein complex composed of a 20S core and normally a pair of 19S caps (Figure 2A). The proteasome can exist as doubly capped (19S-20S-19S), singly capped (19S-20S), or without a cap (20S) (Figure 2A). The 20S core is made of four stacked homologous rings, two  $\alpha$ -rings and 2  $\beta$ -rings that form a central pore (Figure 2A). Each of the rings are composed of seven different protein subunits 20-30 KDa in size. The outer  $\alpha$ -rings act as a gate into the 20S core, regulating the access of substrates to the core. Three of the seven  $\beta$ -subunits have proteolytic activities; caspase, trypsin, and chymotrypsin activities used to breakdown polypeptide chains into smaller 6-7 amino acid oligopeptides. The 19S regulatory cap can be further divided into a non-ATPase lid structure and a ring-like base attached to the base (Figure 2B). The base is important for the recognition of ubiquitin chains and the ATP dependent unfolding of substrates. The 19S ring helps to feed the linear amino acid chain into the 20S core. The ring of the 19S regulatory particle also helps to stimulate the 20S core in an ATP dependent manner to initiate protein degradation (Groll *et al*, 2000).



**Figure 2: Structure of the 26S proteasome**

(A) The 26S proteasome. (B) The regulatory particle (cap) composed of a lid and a base. RPN-6.2 predicted location on the lid shown in green, RPT-1 of the base shown in orange, RPT-6 shown in yellow, alpha2 shown in purple. (C) Cryo-electron microscopy image of the 26S proteasome from mouse testis.



### **Proteasome in sex cell determination**

The proteasome plays a major role in the sex cell determination pathways in *C. elegans*. Both sperm and oocytes differentiate from a common germ-cell progenitor. Whether a cell differentiates into sperm or oocyte depends on the expression of the terminal regulator *tra-1*. Expression of TRA-1 leads to a female fate in the *C. elegans* gonad. Prior to oogenesis, TRA-1 levels are repressed by the expression of FEM-1, FEM-2, and FEM-3, consequently leading to a male fate (Starostina *et al*, 2007). Also, the sperm-oocyte switch in *C. elegans* is controlled by the negative regulation of *fem-3* (Ahringer and Kimble, 1991). Disruption of any of these FEM proteins leads to a feminized gonad (FOG) (Schwarzstein and Spence, 2006) lacking sperm. FEM-1, 2 and 3 form a complex with CUL-2 and act as an E3 ligase to ubiquitinate TRA-1. TRA-1 is then recognized by the RPN-10 subunit (Starostina *et al*, 2007) and degraded by the proteasome. These studies have shown the importance of proteasome in the sex determination pathway and how dysfunction of the proteasome could lead to a feminized germline in *C. elegans*.

### **The proteasome and cell division**

Proteasomes have a major role in mitosis, degrading proteins allowing the progression of anaphase. Sister chromatids are held together by the protein complex cohesin. For a cell to progress into anaphase, when the chromatids are separated, cohesin must be destroyed. Securin and cyclin B inhibit the cohesin protease, separase, safeguarding cohesin from cleavage, which would result in premature chromatid separation. The anaphase promoting complex (APC) is an E3 ubiquitin ligase that targets securin and cyclin B for degradation via the 26S proteasome, allowing for separase to degrade cohesin, initiating the progression into anaphase. Consequently, disruption of

proteasome activity halts cell division at metaphase (Reddy *et al*, 2007). The fact that proteasome disruption is detrimental to any cells that undergo mitosis makes organism-wide knock-down and knock-out studies difficult.

Recent studies have shown the proteasome plays a role in the unpackaging of DNA through histone interactions during mitosis, as lack of proteasome causes a buildup of excess histone upon mitosis (Wike *et al*, 2016). It is unclear if sex cells undergoing meiosis retain the exact same mechanism, especially because sperm histones are replaced with protamines in mammalian sperm. Interestingly, mammalian sperm proteasomes are not efficient at degrading ubiquitinated proteins yet are efficient at degrading histones (Qian *et al*, 2013) suggesting a ubiquitin independent role of the proteasome in sperm tissue. The differences between sperm proteasome and somatic tissue proteasome are with their subunit composition, but functions of these distinct subunits have not been classified.

In the past decade, studies of the UPS system have shed light onto some of its possible functions during meiosis. A major difference between mitosis and meiosis is that of synapsis. Synapsis is unique to prophase I of meiosis, referring to the period when two homologous chromosomes pair-up. Once the pairing of homologous chromosomes is complete, non-sister chromatids may undergo recombination. Recent studies have identified proteasome to be a required for synapsis, thereby regulating meiotic recombination. Rao *et al* (2017) showed that small ubiquitin-like modifier (SUMO) and the UPS system localize to chromosome axes and regulate meiotic prophase in mice spermatocytes. Proteasome also seems to be required for homologous crossover formation and recombination during meiosis I but does not seem to be needed for non-crossover formation (Ahuja *et al*, 2017). Whether or not these mechanisms are conserved in *C. elegans* has not been studied. Also, partial silencing of *pbs-4* and *pas-5* of the 20S core

results in impaired entry into meiosis of developing germ cells in *C. elegans* (MacDonald *et al*, 2008), strengthening the evidence of a role of proteasome in the differentiation of germ cells. Additionally, in mice, inactivation of the testis-enriched proteasomal activator PA200 leads to defective spermatocytes and high levels of apoptosis (Khor *et al*, 2006). Despite evidence regarding the requirement of the UPS for meiosis, the roles of specific proteasomal subunits (including sperm-specific proteasome components) during spermatogenesis and inside spermatids remains enigmatic.

### **Alternate proteasome subunits**

The structure of the proteasome is extremely similar between tissue types, however there are rare instances of alternate proteasome compositions. Alternate subunits of the proteasome may be switched out depending on tissue type or during an immune response. The proteasome has an important role in the immune response; mostly to help present foreign amino acids to via the major class I histocompatibility complex (MHC-1) (Tanaka *et al*, 1998). Immunoproteasomes degrade foreign proteins into small peptides that are then presented by the MHC-1 molecules. Interferon- $\gamma$  induces the switching out of proteasome catalytic subunits  $\beta 5$ ,  $\beta 6$ , and  $\beta 7$  into their immune specific counterparts. The immunoproteasomes are then able to produce more hydrophobic carboxyl termini containing peptides which better associate with MHC-1 molecules. A thymus specific immunoproteasome containing an alternate  $\beta 5$  subunit has also been identified (Murata *et al*, 2007). This unique immunoproteasome is required for CD8<sup>+</sup> T-cell development during thymic selection in mice.

There are some cases in which proteasome subunits are post-translationally modified to alter their function. The  $\alpha 6$  subunit of ascidians is post-translationally modified in sperm to form a unique subunit (Yokoto *et al*, 2011) but its biological function remains unknown. Sperm specific proteasomal subunits have been identified in multiple systems including mammals (Uechi *et al*, 2014) and *D. melanogaster* (Ma *et al*, 2002) but whether or not they have alternate functions inside spermatids and spermatozoa remain a mystery. *Drosophila melanogaster* have sperm specific proteasome subunits that localize to the nucleus during spermatogenesis and remains present in the motile sperm (Ma *et al*, 2002), but again, the biological relevance of these is not understood. Tissue-specific expression of certain proteasome subunits seems to indicate an alternate function of the proteasome. Further research is needed to understand the role of these enriched proteasomal subunits in sperm.

### **Rpn6 Function**

The regulatory non-ATPase subunit (RPN) family of proteasomal subunits on the 19S regulatory particle are characterized as non-ATPase subunits with various functions including, but not limited to, maintaining structural integrity of the proteasome (Pathare *et al*, 2012), deubiquitinase activity (Pathare *et al*, 2014), and substrate recognition (Lam *et al*, 2002). Recent discoveries have also helped to characterize functions of individual 19S non-ATPase subunits such as Rpn6 and associated homologs. Pathare *et al*, 2012 have determined the crystalized structure of Rpn6 from *Drosophila melanogaster*. They show that Rpn6 acts as a scaffolding protein helping to stabilize the lid, the ATPase base and the proteolytic 20S core. Proteasome stability seems to be paired with its efficiency, especially in somatic tissues under stress. The overexpression of *rpn-6.1* in *C. elegans* under heat

stress (34 °C) nearly doubles their lifespan compared to the control group (from 22 day lifespan in control to 44 days in treatment) (Vilchez *et al*, 2012), most likely due to the increased maintenance of proteostasis. Additionally, in *Saccharomyces cerevisiae*, Rpn6p has been shown to be required for the assembly and activity of the 26S proteasome (Santamaría *et al*, 2003) providing further evidence supporting the conserved role of Rpn6 to stabilize the proteasome. The Rpn6 unique function in stabilizing not only the 19S regulatory particle but also its interaction with the 20S core suggests a similar role in sperm specific proteasome.

### **Rpn6 Interactions**

In *C. elegans*, there are two paralogs of Rpn6; *rpn-6.1*, which is expressed in somatic cells, and *rpn-6.2*, which is primarily expressed in the germline. Predicted protein products of RPN-6.1 and RPN-6.2 share 67% protein similarity and 42% protein identity based on amino acid composition. RPN-6.2 is predicted to have two isoforms; isoforms A and B (Figure 3, 4). RNA sequencing data from the Troyanskaya Laboratory (Princeton, NJ) shows increased expression of *rpn-6.2* in *C. elegans* spermatogenic cells such as spermatids and spermatozoa. Based on protein homology, *rpn-6.2* is a sperm specific proteasomal subunit paralog of *rpn-6.1*.

Studies show Rpn6 directly interacts with Rpt6 of the base and with the  $\alpha 2$  subunit of the 20S core  $\alpha$ -ring (Pathare *et al*, 2012). The Rpn6-Rpt6 and Rpn6- $\alpha 2$  interactions most likely function to stabilize the lid to the base and the 19S cap to the 20S core, respectively. These interactions help stimulate the  $\alpha$  ring, opening the 20S core. The proteasome, COP9, initiation factor 3 (PCI) domains of Rpn6 are necessary for the

interactions with Rpt6 and  $\alpha 2$  and are conserved between humans and *C. elegans*. Interestingly, RPN-6.2 isoform B lacks the  $\alpha 2$  binding domain present in other homologs.

A unique proteasome structural subunit expressed in sperm tissue while most of the other subunits are shared with the canonical proteasome found in somatic cells is worth studying. RPN-6.2 may have a unique function specific to sperm proteasome while also preserving its structural role. We hypothesize RPN-6.2 is required for stability of the 26S sperm proteasome and defects in RPN-6.2 may be detrimental to the health of developing spermatozoa. Establishing the cellular localization of RPN-6.2 in different forms of sperm may help determine the function of sperm-specific proteasomes. Through the characterization of RPN-6.2, we hope to help elucidate the function of sperm proteasome.

Here, we report several distinct phenotypes resulting from dysfunctional RPN-6.2 and have also characterized its subcellular localization in sperm tissue of *C. elegans*. The specific function of the proteasomal subunit RPN-6.2 in spermatids remains unknown, but we hope these studies provide a foundation for further research.

RPN-6.2a ( <i>C. elegans</i> )	1	-----MSATPVTLKA-----VQS-EVSAQTAKSS	EAEVKRC	EDL	33
RPN-6.2b ( <i>C. elegans</i> )	1	MR ETS SR ED TNN I GKAP EM SGGT I MD TMT S LPH QNDQNV	---	IRHLTNLVKSPASGDDDIKKK	EDS 63
RPN-6.1 ( <i>C. elegans</i> )	1	-----MAAAAVVEFQRAQSLLSTDR	EASIDI-LHS-IVKRD	IQENDE	EAVQVKEQS 49
RPN6 (Human)	1	-----MAGATLFFERAQLSSVNR	EEQDSSLNKLVRDQ	EGAENDE	ERIRIK
Rpn6 (Fruit fly)	1	-----			EQG 49
RPN-6.2a ( <i>C. elegans</i> )	34	I L S Y S R Q L A K E K D I T G I R T L V E S I R S F Y D L V G	K A R A S K L I R	D I V E H A L T I D Q G V G P A L D H G K K E I D	100
RPN-6.2b ( <i>C. elegans</i> )	34	-----			
RPN-6.1 ( <i>C. elegans</i> )	64	I M E L G N I L A Q N K Q T E E L R N M I E Q T R P F L V S L G	K A K A A K L V R	D L V D L C L K I D D Q D G D	-----I K V G 123
RPN6 (Human)	50	I L E L G S L L A K T G Q A A E L G G L L K Y V R P F L N S I S	K A K A A R L V R	S L L D L F L D M E A A T G	-----Q E V E 108
Rpn6 (Fruit fly)	50	I L Q Q G E L Y K Q E G K A K E L A D L I K V T R P F L S S I S	K A K A A K L V R	S L V D M F L D M D A C T G	-----I E V Q 108
RPN-6.2a ( <i>C. elegans</i> )	101	L L T N C I G W A T S N K R E F L R R S L Q A R L I R L Y N D I R D F T N A Q K L A Q D L S K E L K K L E D R E L L I E V S V E E S K			167
RPN-6.2b ( <i>C. elegans</i> )	124	L V K E C I Q W A T E Q N R T F L R Q T L T A R L V R L Y N D L Q R Y T Q A L P L A A D L I R E L K K V D D K D V L V E V E L E E S K			190
RPN-6.1 ( <i>C. elegans</i> )	109	L C L E C I E W A K S E K R T F L R Q A L E A R L V S L Y F D T K R Y Q E A L H L G S Q L L R E L K K M D D K A L L V E V Q L L E S K			175
RPN6 (Human)	109	L C K D C I E W A K Q E K R T F L R Q S L E A R L I A L Y F D T A L Y T E A L A L G A Q L L R E L K K L D D K N L L V E V Q L L E S K			175
Rpn6 (Fruit fly)	109	L C K D C I E W A K Q E K R T F L R Q S L E A R L I A L Y F D T A L Y T E A L A L G A Q L L R E L K K L D D K N L L V E V Q L L E S K			175
RPN-6.2a ( <i>C. elegans</i> )	168	S S F N L N N L A K A K T A L T A K T N T N S A F A S P Q L Q A S V D M Q S G V L Y S A E E R D Y K T S F	S Y F Y E A F E G F A S I		234
RPN-6.2b ( <i>C. elegans</i> )	1	-----M Q S G V L Y S A E E R D Y K T S F	S Y F Y E A F E G F A S I		31
RPN-6.1 ( <i>C. elegans</i> )	191	A Y Y N L S N I G R A R A S L T G A R T T A N A I Y V N P R M Q A A L D L Q S G I L H A A E K D F K T A F	S Y F Y E A F E G Y D S V		257
RPN6 (Human)	176	T Y H A L S N L P K A R A A L T S A R T T A N A I Y C P P K L Q A T L D M Q S G I I H A A E E K D W K T A Y	S Y F Y E A F E G Y D S I		242
Rpn6 (Fruit fly)	176	T Y H A L S N L P K A R A A L T S A R T T A N A I Y C P P K V Q G A L D L Q S G I L H A A D E R D F K T A F	S Y F Y E A F E G F D S V		242
RPN-6.2a ( <i>C. elegans</i> )	235	G D K I N A T S A L K Y M I L C K I M L N E T E Q L A G L L A A K E I V A Y Q K S P R I I A I R S M A D A F R K R S L K D F V K A L A			301
RPN-6.2b ( <i>C. elegans</i> )	32	G D K I N A T S A L K Y M I L C K I M L N E T E Q L A G L L A A K E I V A Y Q K S P R I I A I R S M A D A F R K R S L K D F V K A L A			98
RPN-6.1 ( <i>C. elegans</i> )	258	D E K V S A L T A L K Y M L L C K V M L D L P E D V N S L L S A K A L K Y N G - S D L D A M K A I A A A Q K R S L K D F Q V A F G			323
RPN6 (Human)	243	D S - P K A I T S L K Y M L L C K I M L N T P E D V Q A L V S G K L A L R Y A G - R Q T E A L K C V A Q A S K N R S L A D F E K A L T			307
Rpn6 (Fruit fly)	243	D S - V K A L T S L K Y M L L C K I M L G Q S D D V N Q L V S G K L A I T Y S G - R D I D A M K S V A E A S H K R S L A D F Q A A L K			307
RPN-6.2a ( <i>C. elegans</i> )	302	E H K I E L V E D K V V A V H S Q N L E R N M L E K E I S R V I E P Y S E I E L S Y I A R V I G M T V P P V E R A I A R M I L D K K L			368
RPN-6.2b ( <i>C. elegans</i> )	99	E H K I E L V E D K V V A V H S Q N L E R N M L E K E I S R V I E P Y S E I E L S Y I A R V I G M T V P P V E R A I A R M I L D K K L			165
RPN-6.1 ( <i>C. elegans</i> )	324	S F P Q E L Q M D P V V R K H F H S L S E R M L E K D L C R I I E P Y S F V Q I E H V A Q Q I G I D R S K V E K K L S Q M I L D Q K L			390
RPN6 (Human)	308	D Y R A E L R D D P I I S T H L A K L Y D N L L E Q N L I R V I E P F S R V Q I E H I S S L I K L S K A D V E R K L S Q M I L D K K F			374
Rpn6 (Fruit fly)	308	E Y K K E L A E D V I V Q A H L G T L Y D T M L E Q N L C R I I E P Y S R V Q V A H V A E S I Q L P M P Q V E K K L S Q M I L D K K F			374
RPN-6.2a ( <i>C. elegans</i> )	369	M C S I D Q H G D T V V V Y P K A D A A N Q F T R S L K T I R E L T K T V D V S Y S R T K H F K			416
RPN-6.2b ( <i>C. elegans</i> )	166	M C S I D Q H G D T V V V Y P K A D A A N Q F T R S L K T I R E L T K T V D V S Y S R T K H F K			213
RPN-6.1 ( <i>C. elegans</i> )	391	S G S L D Q G E G M L I V F E I A V P D E A Y Q T A L D T I H A M G E V D A L Y S N A S K I N			438
RPN6 (Human)	375	H C I L D Q G E G V L I I F D E P P V D K T Y E A A L E T I Q N M S K V V D S L Y N K A K K L T			422
Rpn6 (Fruit fly)	375	S G I L D Q G E G V L I V F E E T P V D K T Y E R V L E T I Q S M G K V V D T L Y Q K A K K L S			422

**Figure 3: *rpn-6.2* homology**

*rpn-6.2* is a sperm specific ortholog of Rpn6. Pre8 is the  $\alpha$ -2 subunit of the 20S proteasome and Rpt6 is an ATPase subunit of the regulatory cap. Comparison of *rpn-6.2* with other homologs and the *rpn-6.1* somatic tissue paralog show the binding motifs of Pre8 and Rpt6 are conserved. Darker shades represent closer amino acid homology between the samples.

## CHAPTER II: MATERIALS AND METHODS

### Strain Maintenance

*C. elegans* were grown on nematode growth media (NGM) plates at 20 °C unless indicated otherwise. NGM was made with 17 g of agar, 3 g NaCl, and 2.5 g of peptone per liter. NGM solution was then autoclaved and then placed in a heat bath to cool for 30 minutes. After cooling to 37 °C, 1 mL 1M CaCl<sub>2</sub>, 1 mL cholesterol (5 mg/mL), 1 mL of 1M MgSO<sub>4</sub>, and 25 mL 1M KPO<sub>4</sub> were added to the NGM. Plates contained either 5 mL of NGM (35 mm, tiny plates), 15 mL of NGM (60 mm, medium plates) or 50 mL of NGM (100 mm, large plates). The plates were left to dry for 3-4 days prior to seeding with *E. coli*.

OP50 *E. coli* was streaked onto tryptic soy agar (TSA) plates. An individual colony of bacteria was then grown in a shaking incubator in tryptic soy broth (TSB) for 16 hours at 36 °C. Tiny plates were seeded with 125 µL of inoculated culture, medium plates were seeded with 750 µL of culture, and large plates were seeded with 2 mL of culture.

### RNAi by feeding

RNA interference (RNAi) is a molecular technique used to silence the expression of a gene by targeting mRNA molecules in the host cell. RNAi clones from the Ahringer library (Kamath *et al*, 2003) were used for the knock-down studies. RNAi clones refer to HT115 *E. coli* that have the pL4440 plasmid containing the *C. elegans* gene of interest and Ampicillin resistance. Controls for the RNAi experiments included a L4440 plasmid (vector) without any gene inserted in the HT115 bacterial strain, as well as L4440 with



the embryonic lethal gene (*ubc-2*) as a positive control. The RNAi clone of interest was streaked on a TSA plate containing ampicillin + tetracycline and incubated overnight. A single RNAi clone was selected and grown in TSB media containing ampicillin (100 µg/mL) and tetracycline (10 µg/mL) for 16 hours. Inoculated culture was then seeded onto RNAi plates containing 0.2% lactose.

**Table 1: *C. elegans* strains used in study**

<b>Strain Name</b>	<b>Genotype</b>
<b>N2 (Bristol)</b>	Wild type
<b>LN130</b>	<i>rcIs31 [pie-1p::GFP::Ub + unc-119(+)]; ltIs37[pie-1p::mCherry::his-58]</i>
<b>WDC1</b>	<i>rpn-7(ana1[gfp::rpn-7])</i>
<b>WDC3</b>	<i>rpn-6.1(ana3[gfp::rpn-6.1])</i>
<b>WDC4</b>	<i>rpn-7(ana4[gfp::rpn-8])</i>
<b>WDC5</b>	<i>rpn-9(ana5[gfp::rpn-9])</i>
<b>LN151</b>	<i>rcSi1[Pmex-5::rpt-1::mCh + unc-119]II; unc-119 (ed3)fx (pLB38)</i>
<b>LN167</b>	<i>rpn-6.2(rc2[rpn-6.2::GFP])</i>
<b>LN169</b>	<i>rc2[rpn-6.2::GFP]; rcSi1[Pmex::rpt-1::mCherry+ unc-119]</i>
<b>IT1187</b>	<i>unc-119(ed3) III; kpIs100 [pie-1p::Ub(G76V)::GFP::H2B::drp-1 3' UTR; unc-119(+)]</i>
<b>LN170</b>	<i>rc3[GFP+SEC::rpn-6.2b]III</i>
<b>LN171</b>	<i>rc3[GFP::rpn-6.2b]III</i>

**Confocal microscopy**

A Zeiss LSM 700 confocal microscope was used for imaging and Zen black software was used for analysis. Fluorophores were excited using 488 nm, 555 nm, 405 nm lasers and emitted fluorescence was captured using a high-resolution AxioCam microscope camera. Pinhole was set at 69  $\mu\text{m}$  in diameter.

**Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE)**

Protein lysate was produced by homogenization of *C. elegans* via Bullet Blender (Bulldog Bio, Portsmouth, NH) in phosphate buffered saline (PBS). Resolving gels were made from combining 9 mL ddi H<sub>2</sub>O, 2ml acrylamide/BIS, 3.8 mL gel buffer, 150  $\mu\text{l}$  10% SDS, 20  $\mu\text{l}$  TEMED, and 100  $\mu\text{l}$  APS. Stacking gels were composed of 7.7 mL ddi H<sub>2</sub>O, 3.3 mL acrylamide/BIS, 3.75 mL gel buffer, 150  $\mu\text{L}$  10% SDS, 10  $\mu\text{L}$  TEMED, and 150  $\mu\text{L}$  APS. Samples were diluted in a 3:1 ratio with 4xLaemli buffer (Biorad) and heated for 30 minutes at 37 °C to prevent the denaturing of the fluorophore. 20  $\mu\text{L}$  of sample was loaded into wells. Samples were electrophorized at 75 V until the ladder cleared the stacking gel, then electrophorized at 100 V for 1 hour or until the dye front reached the bottom of the gel. SDS PAGE gels were then imaged with the Typhoon Trio + Variable Mode Imager (GE Healthcare Life Sciences). SDS gels were scanned on normal sensitivity. 488 nm laser was used to detect GFP fluorophore.

**Egg count assay**

L4 LN167 and N2 hermaphrodites were grown on tiny *rpn-6.2* RNAi and vector NGM plates, respectively. L4 LN170 rolling hermaphrodites were also singled out onto tiny vector NGM plates. These worms were designated P1. The F1 generations were used for the assays. 7-10 F1 L4 hermaphrodites of LN167, LN170, and N2 from the F1 generation were singled out onto their previously designated plates. Hermaphrodite worms were transferred onto a fresh plate every 8 hours. The number of eggs laid in the 8-hour period were recorded for each worm.

**Brood size assay**

Embryonic viability was determined by comparing the number of eggs laid (from the egg count assay) and then counting how many had hatched 48 hours later. The number of successful progeny a single *C. elegans* produces while fertile is referred to as brood size. Brood size was recorded as the sum of hatched eggs from each worm after a 5-day period.

**Sperm count assay**

We used the *rpn-6.2* mutant strain to determine whether the decrease in progeny observed in *rpn-6.2* mutants was due to a decrease in sperm. *rpn-6.2* mutant and GFP::*rpn-6.2* young adults were obtained by synchronizing L4 hermaphrodites onto NGM plates and incubating for 12-14 hours. The young adults were then frozen in liquid nitrogen for 5 minutes and fixed in methanol for 20 minutes. Worms were then washed three times for 5 minutes in PBS + 0.5% tween then mounted on a glass slide with

Vectasheild (Vector Labs, Burlingame, CA). Z-stacks were taken of young adult worm spermathecas containing at most 1-5 embryos in the gonad. A Zeiss LSM 700 confocal microscope was used to detect the presence of spermatozoa indicated by blue puncta.

### ***In vitro* sperm activation assay**

L4 stage males with rounded tails were picked and placed on fresh, seeded, NGM plates and grew for 1-2 days. The males were transferred to a new plate without bacteria and allowed to crawl for few minutes to remove the bacteria surrounding the cuticle of the worms. 30  $\mu$ L of Boyd Buffer (60 mM NaCl, 32 mM KCl, 3 mM Na<sub>2</sub>HPO<sub>4</sub>, 2 mM MgCl<sub>2</sub>, 2 mM CaCl<sub>2</sub>, 5 mM HEPES, 0.2% glucose) was added to a slide and 10 males were transferred into the droplet. Worms were cut open using needles to release spermatids and incubated in a humidifier for 1 hour at 20 °C. After incubation, a coverslip was placed on the surface of dissected sample and spermatids or spermatozoa were observed using a Zeiss LSM 700 confocal microscope.

### **Mitotracker experiment**

94.1  $\mu$ L of DMSO was added to lyophilized Mitotracker Red CMXRos (Invitrogen) powder and vortexed for 5 seconds to produce a 10 mM stock. 3  $\mu$ L of the 10 mM stock was added to 297  $\mu$ L of M9 to produce a 10  $\mu$ M working solution. Male LN167 worms were soaked in the 10  $\mu$ M working solution for 1-2 hours then were left to crawl around on a seeded NGM plate for an additional hour. 18  $\mu$ L of Sperm media (50mM HEPES, 25mM KCl, 45mM NaCl, 1mM MgSO<sub>4</sub>, 5mM CaCl<sub>2</sub>, 10mM glucose) was dropped on a slide and at least 10 males were placed in the drop. Males were then cut using a needle to

expose spermatids. Spermatids were then imaged using a 63X oil immersion lens on a Zeiss LSM 700 confocal microscope.

### **Proteasome activity experiment**

IT1187 L4 roller worms, designated P1, were selected and placed on either Vector RNAi plates or *rpn-6.2* RNAi plates. F1 generation rollers were selected and imaged using a Zeiss LSM 700 confocal microscope for the presence or absence of GFP expression.

### **Antibodies**

The only primary antibody used in this study was mouse monoclonal anti-1CB4 (generous gift from Steve L'Hernault at Emory University) at 1:100 dilution. The secondary antibody used for immunofluorescence at 1:100 dilution was goat anti-mouse FITC (Jackson ImmunoResearch Laboratories).

### **Antibody stains**

Additional poly-L-lysine was added to poly-L-lysine coated slides. Male worms were washed with M9 buffer and put into a drop of egg buffer with a 1:20 dilution of 0.1 mg/ml tetramisole. Sperm was then extracted using needle. A coverslip was gently placed over worms and the slide was immediately frozen in liquid nitrogen for at least 5 minutes. Slides were removed from the liquid nitrogen and the cover slip was quickly pried off using a razor blade. Slides were then immediately fixed in methanol at -20 °C for 20 minutes. Slides were then washed in PBST for 5 minutes three times to remove residual MeOH. Slides were incubated in primary antibody overnight at 4 °C and then incubated with

secondary for 2 hours at room temperature. Primary and Secondary antibodies were diluted in PBST:30% NGS (normal goat serum) and three proceeding washes were done in PBST after each stain. Vectasheild (Vector Labs, Burlingame, CA), which contained DAPI, was used as the mounting medium. Stained samples were observed using the LSM 700 confocal microscope.

### **Generating male populations**

Medium plates (4-6) with five late L4 hermaphrodites each were set up. Worms were then heat shocked at 31°C for 5-6 hours in order to induce male progeny. Males resulting from heat-shock plates were then mated with hermaphrodites to maintain male populations. Male populations were also made by growing worms on *him-4* or *him-14* RNAi plates for 2 generations.

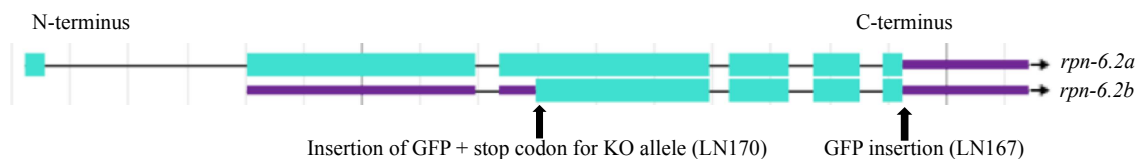
### **Statistical analysis**

The average and standard error of the mean (s.e.m) in each sample were calculated and the data was analyzed using either a Students T-test or a one-way analysis of variance ANOVA and a Tukey's post hoc test. Vassar Stats was used to conduct ANOVA and Graphpad was used to conduct Student T-test.

## CHAPTER III: RESULTS

### Characterization of *rpn-6.2* phenotypes

The proteasome is involved in a vast number of processes ranging from degradation of misfolded proteins, sex determination, pathogen resistance, regulating the cell cycle, and DNA repair. As reviewed by Tanaka *et al*, 2009 the proteasome plays a role in many different functions, yet its protein composition seems, for the most part, consistent from



**Figure 4: RPN-6.2 predicted isoforms and GFP alleles**

RPN-6.2 is predicted to have two isoforms expressed via two different promoters. The GFP CRISPR is at the 3' end of the gene. Expression of either or both isoforms will contain the GFP tag.

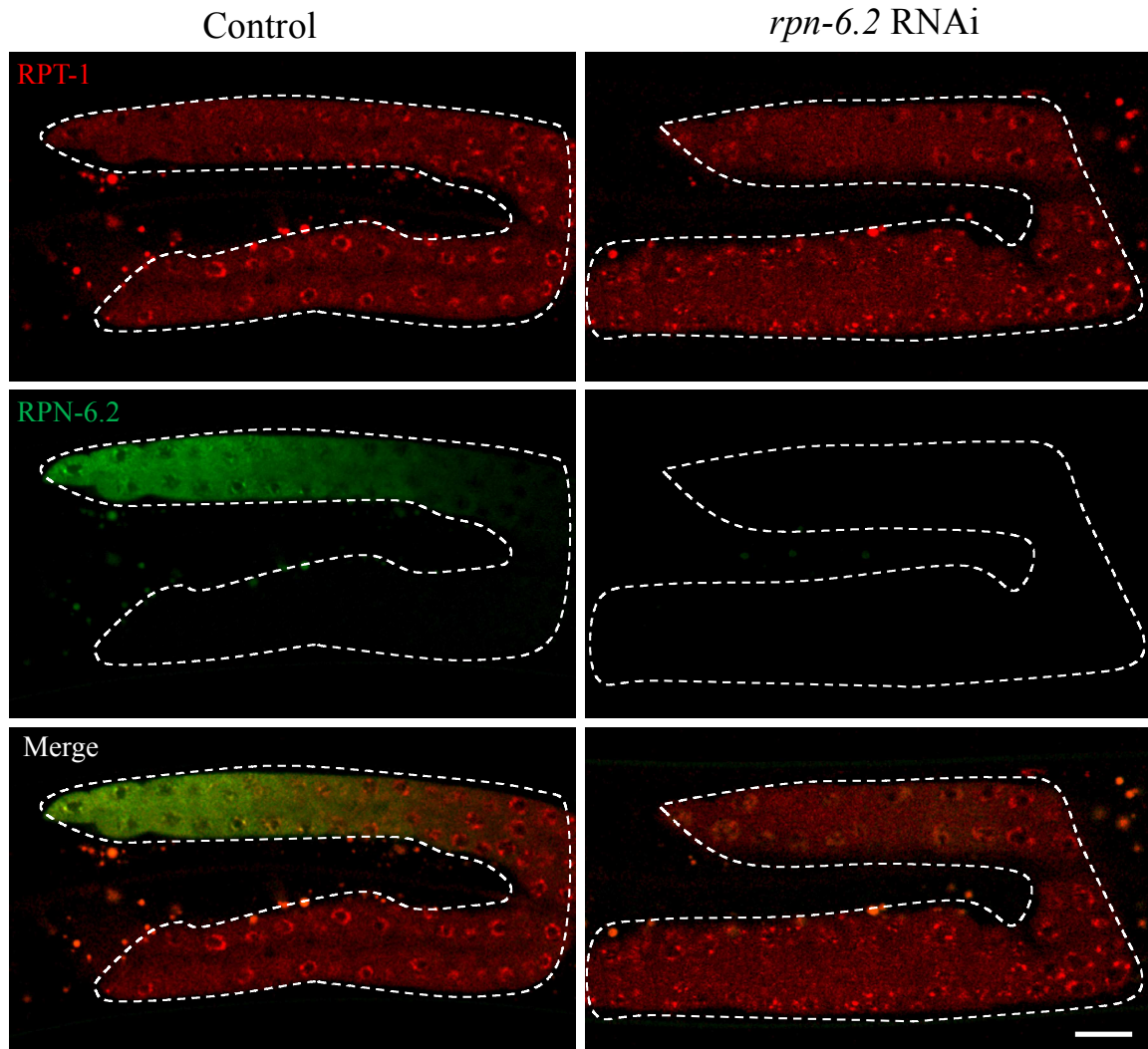
tissue to tissue. This makes studying tissue- or even cell-specific proteasome difficult in that disrupting proteasome function of one tissue will most likely disrupt proteasome activity in all tissues. RPN-6.2 is a sperm specific homolog of RPN-6.1 of the 19S regulatory particle and is thought to help stabilize the 26S proteasome. Utilizing the expression of a tissue specific proteasome subunit, we're able to observe its localization



and disrupt a tissue specific proteasome to better understand its function in *C. elegans*. RPN-6.2 is predicted to have two isoforms due to different transcriptional start sites; Isoform A, which is 45.76 kDa (416 aa), and isoform B, which is 23.43 kDa (213 aa). There is no known evidence of both isoforms existing as proteins, only as RNA transcripts (Robert H. Waterson). We made a CRISPR allele for *rpn-6.2* containing a GFP tag at its C-terminus; which should be present in both isoforms. We also generated a strain inserting a GFP and a stop codon downstream of the 1<sup>st</sup> ATG start codon in *rpn-6.2b*, producing a truncated version of RPN-6.2, which we later confirmed is non-functional.

### ***rpn-6.2* KD confirmation**

The presence of a sperm-specific proteasomal subunit may indicate an alternate function of the proteasome during spermatogenesis. As the proteasome is required in nearly every aspect of cellular function, disruption of the proteasome is often fatal. Knocking down or knocking out a tissue specific proteasome subunit should bypass any lethality phenotypes that result from the inhibition of somatic tissue proteasome. We searched RNA sequencing databases for sperm specific proteasome transcripts in *C. elegans* and identified *rpn-6.2* to be primarily expressed in the sperm. We utilized RNAi to knock-down *rpn-6.2* in GFP::*rpn-6.2*; *Pmex::rpt-1::mCherry*. Knock-down was confirmed using fluorescence imaging to show a lack of GFP expression in treated worms (Figure 5).

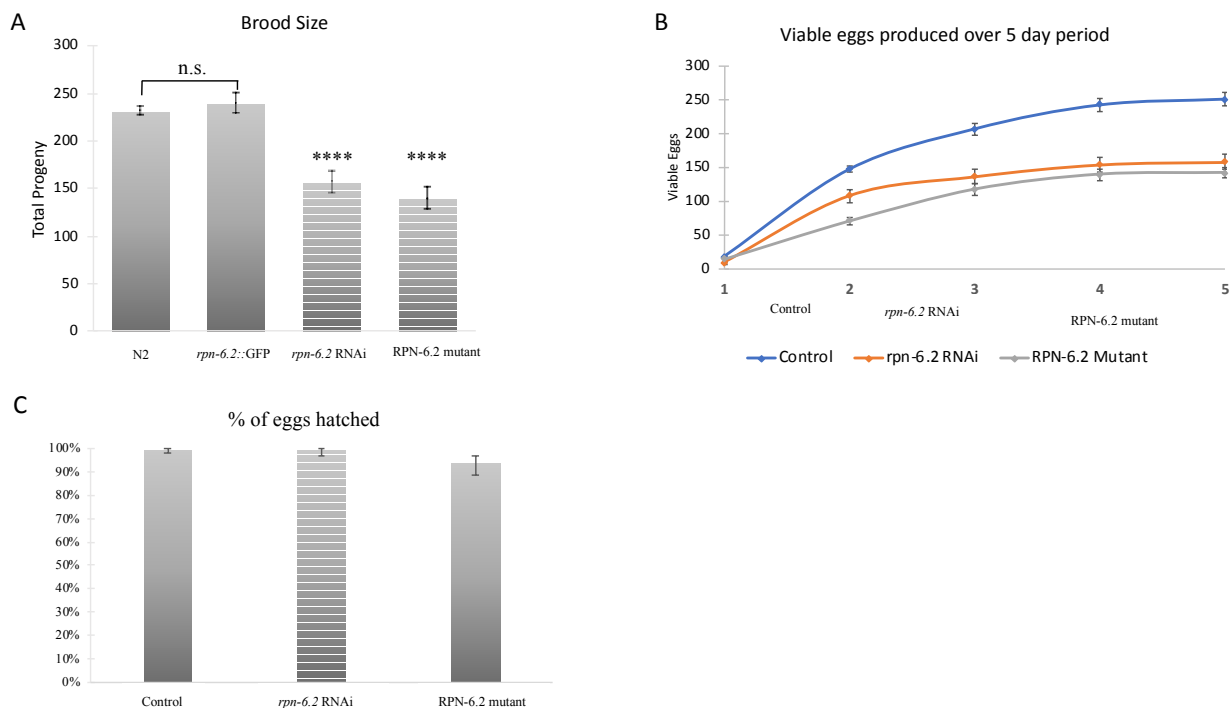


**Figure 5: Knock-down of *rpn-6.2* in LN169 worms**

Left panels depict LN169 control worm and right panels depict *rpn-6.2* knock-down in LN169. Middle right panel displays knock-down of *rpn-6.2* by the absence of GFP. Worms are at L4 stage in development. Dashed line indicates gonad of worm. Representative images from 3 biological repeats. Scale bar = 10  $\mu$ M

### **Brood size is compromised in *C. elegans* with disrupted *rpn-6.2***

Rpn6 has been characterized as a molecular clamp that helps stabilize the cap, ring, and 20S core in *D. melanogaster* (Pathare *et al*, 2012). If proteasome is involved in pre- or post-fertilization events we would expect brood size to be altered in some manner. To test this hypothesis, we looked at brood sizes of *rpn-6.2* knock-down and mutant *C. elegans*. N2 and our GFP::*rpn-6.2* strain produced average brood sizes of 232.5 (N=8) and 240 (N=14), respectively, during their reproductive span (approximately 5 days) (Figure 6a). The averages between N2 and the GFP::*rpn-6.2* strain were not statistically different. When we knocked-down *rpn-6.2* in the GFP::*rpn-6.2* strain, the average brood size was 157.6 (N=13). Similarly, our *rpn-6.2* mutants average brood size was 140 (n=14) worms (Figure 6a). The similar defect shown between the *rpn-6.2* RNAi and our *rpn-6.2* mutant confirmed the dysfunctionality of the mutant. Additionally, there was an overall decrease in the overall rate of eggs laid over a 5 day period (Figure 6b). As the number of eggs laid consistently matches the number of eggs hatched, the decrease in brood size does not seem to stem from post-fertilization issues.



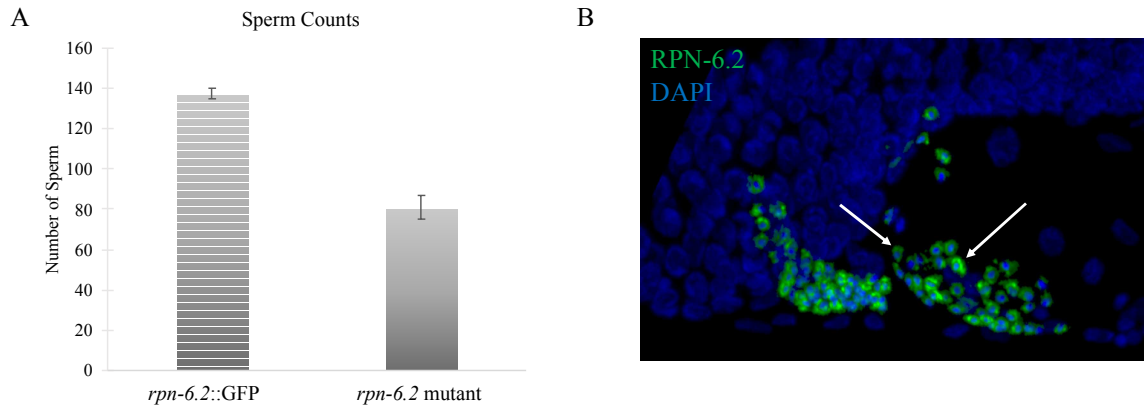
### Figure 6: Brood size is compromised in *C. elegans* with disrupted *rpn-6.2*

(A) Brood size was determined by counting the number of *C. elegans* eggs laid and hatched until sperm depletion. (B) Graphical representation of accumulative eggs laid over 5 days. There *rpn-6.2* RNAi and *rpn-6.2* mutant worms laid less viable eggs per day than control worms. (C) There was no statistical difference between eggs laid and eggs hatched of control, *rpn-6.2* knock-down worms, and *rpn-6.2* mutant worms. n=14 Error bars indicate s.e.m. Statistical significance was determined using a one-way analysis ANOVA followed by a Tukey HSD test ( $P < .01$ )

### Decrease in brood size may be contributed to low sperm count in *rpn-6.2* mutants

Next, we aimed to determine whether the lower progeny numbers were due to a sperm-egg fusion defect or a decrease in the overall number of sperm produced. We isolated young adult GFP::*rpn-6.2* and the *rpn-6.2* mutant strain worms (12 hours after L4 stage), and fixed them, and stained with DAPI to count the number of sperm in and around the spermatheca (Represented by maximum projection image in figure 7b). It's worth noting that a single *C. elegans* has two spermathecas and only one was counted in this assay, chosen at random. Average sperm counts of one arm of the gonad were markedly

lower in the *rpn-6.2* mutant strain (80.9 +/- 18.8) compared to the control (137.6 +/- 8.0) leading us to believe the brood size defect stemmed from spermatogenesis (Figure 7b).

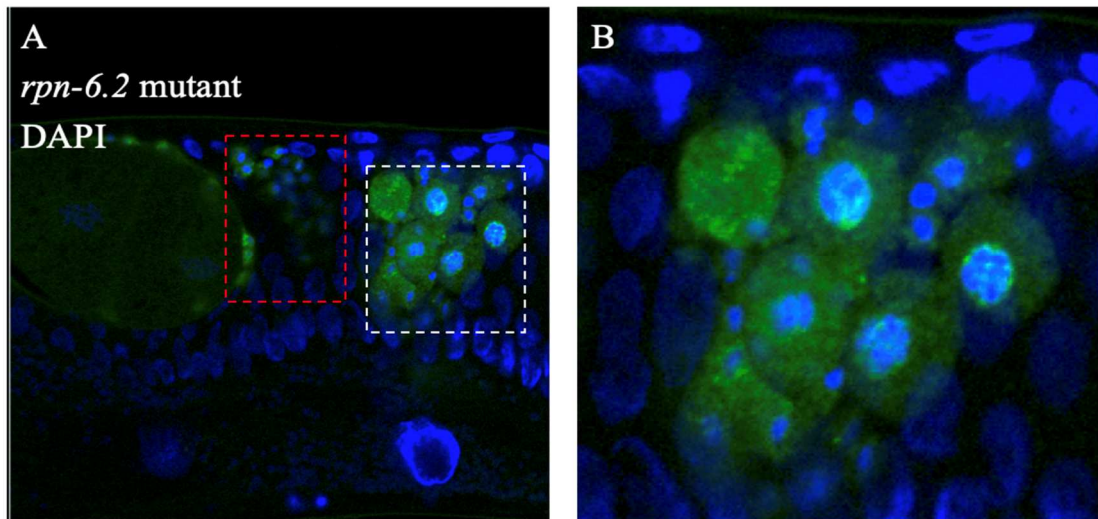


### Figure 7: Decrease in worm progeny may be due to less sperm

(A) Synchronized young adults (no more than 3 embryos in the gonad) were fixed and stained using DAPI. Z-stack images of the spermatheca were taken and the number of spermatids were quantified. Error bars indicate s.e.m. Student T test was used to determine statistical significance,  $P < 0.001$   $N = 20$   
 (B) Maximum projection of a fixed LN167 spermatheca and uterus. White arrows indicate spermatozoa.

### Decrease in sperm counts may arise from failure to undergo meiosis

While performing the sperm count assays, we observed “leftover” primary spermatocytes outside of the spermatheca in the *rpn-6.2* mutant strain but not in the control (Figure 8A and B). The primary spermatocytes contain DNA and do not seem to be undergoing meiosis. The decrease in spermatids may be due to the undivided primary spermatocytes. This observation has yet to be quantified.

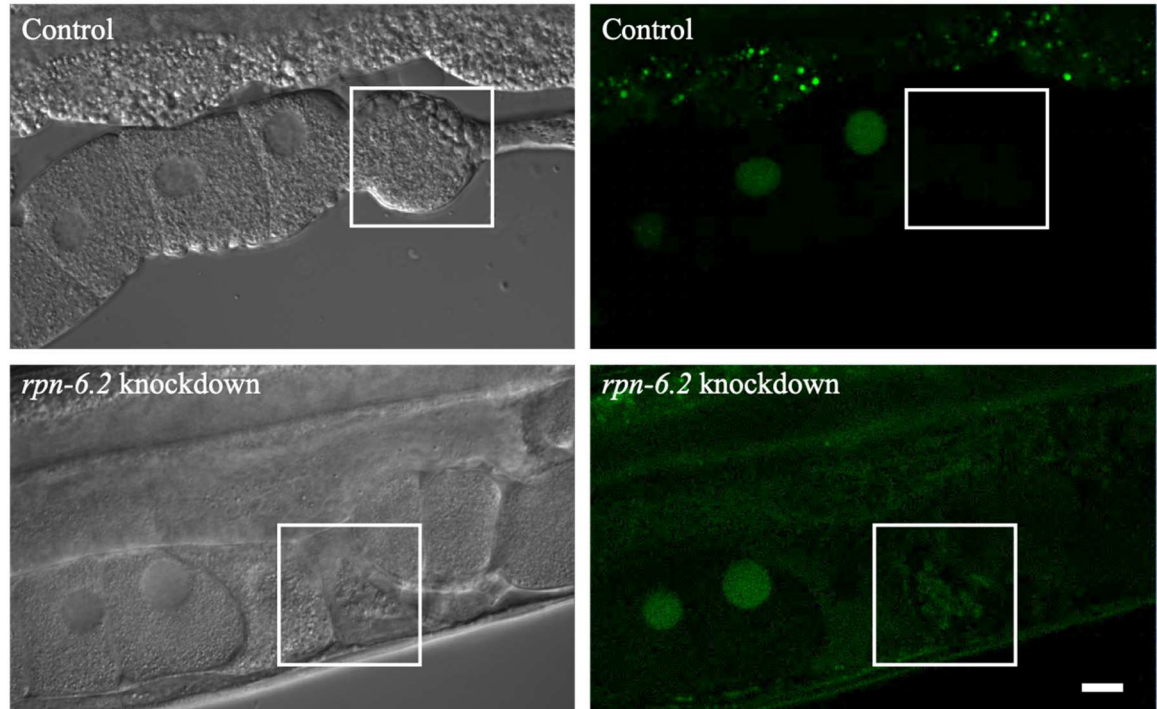


### Figure 8: Primary spermatocytes outside spermatheca

(A) Fixed gonad of a *rpn-6.2* mutant. Red box indicates location of spermatheca. White box indicates area where primary spermatocytes localize (B) “Leftover” primary spermatocytes localized outside of the spermatheca

### Sperm proteasome may be proteolytically active

The IT1187 strain expresses a Ub(G76V)::GFP::H2B tag that localizes to the nucleus and is readily polyubiquitinated and subsequently degraded by the proteasome. Presence of nuclear GFP indicates the UPS is not functional. Disrupting a part of the UPS in specific tissues will prevent the degradation of Ub(G76V)::GFP::H2B, therefore resulting in higher levels of nuclear GFP fluorescence. *rpn-6.2* was knocked-down in five IT1187 worms to see whether proteasome proteolytical activity would be affected in sperm. The presence of GFP in the sperm of *rpn-6.2* knock-down and not IT1187 control worms suggests proteolytic activity in the spermatozoa of *C. elegans* (Figure 9). Further replicates are needed to determine biological significance.



**Figure 9: Sperm proteasome may be proteolytically active**

*rpn-6.2* was knocked-down in IT1187 worms. Tissues with reduced UPS activity are indicated by GFP fluorescence. These preliminary data suggest sperm proteasome is proteolytically active and this activity requires RPN-6.2. White boxes indicate spermatheca location. Scale bar= 10  $\mu$ M

## Cellular localization of RPN-6.2

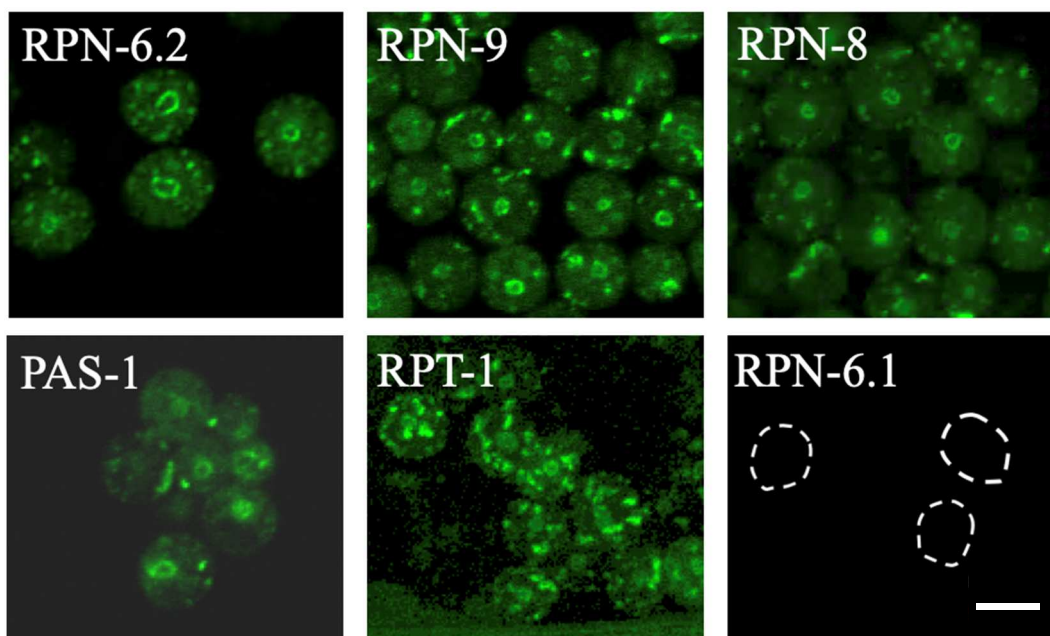
The organelles identified in spermatids of *C. elegans* include a condensed nucleoid, membranous organelles (MOs) and mitochondria; all of which are required for proper fertilization. The reliance on UPS for the homeostasis of these organelles in spermatids has yet to be studied. Degradation of pig paternal mitochondria post-fertilization has been shown to rely on proteasome activity (Sutovsky *et al*, 2004). The proteasome has also been shown to have a role in a mitochondria stress response where the UPS degrades misfolded

mitochondrial proteins (Heo *et al*, 2010) but it is unknown whether this role is conserved in sperm. Heo *et al* also showed the colocalization of proteasomes with the mitochondria during mitochondria stress response. Little is understood about the purpose of Golgi derived MOs, although their fusion to the plasma membrane of spermatids seems to be required for their progression into spermatozoa (Ward *et al*, 1981). Localization of proteasome to either of these paternal organelles may indicate a role for proteasome in spermatid homeostasis.

### **Spermatid proteasome expression**

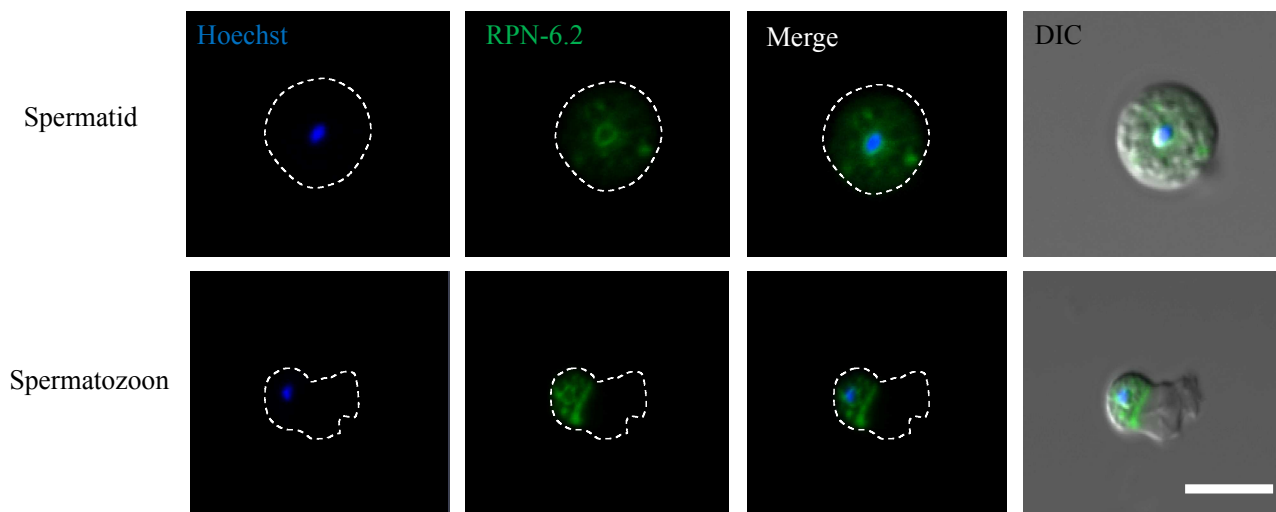
To determine the subcellular expression of specific proteasome components in spermatids, we obtained male *C. elegans* strains expressing, GFP::RPN-6.2, GFP::RPN-6.1, GFP::RPN-7 (image not shown), GFP::RPN-8, GFP::RPN-9, and GFP::PAS-1 (Kumar, G. A., & Subramaniam, K., 2018), which are all subunits of the 26S proteasome. GFP::RPN-6.1, GFP::RPN-7, GFP::RPN-8, GFP::RPN-9 strains were provided by Anna Allen at Howard University. The male *C. elegans* produces nearly 1000 sperm and the sperm themselves are much larger than the hermaphrodite equivalent making visualization of cellular structures easier. RPN-6.2, RPN-8, RPN-9, and PAS-1 all share the same expression pattern in *C. elegans* spermatids (Figure 10). The proteasome forms a spherical structure in the center of the spermatid and also appears in punctate structures in the cytoplasm.





**Figure 10: Proteasome localization in *C. elegans* spermatids**

RPN-6.2, RPN-8, RPN-9, RPT-1 and PAS-1 are all similarly localized in spermatids of *C. elegans*. RPN-6.1, the somatic tissue homolog of RPN-6.2, does not appear to be expressed in spermatids. White lines indicate area of spermatids. Green fluorescence indicates expression of proteasome subunits. n=100 spermatids per worm strain. Scale bar= 5  $\mu$ M



**Figure 11: RPN-6.2 surrounds the paternal DNA**

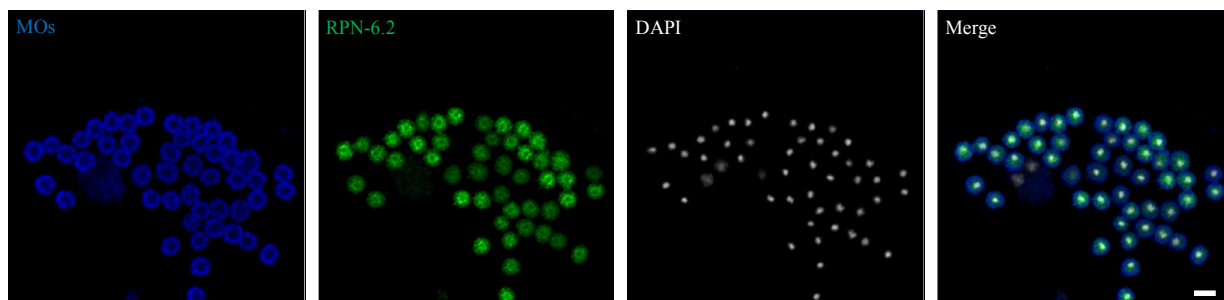
Live-cell imaging of *C. elegans* spermatids and spermatozoa expressing RPN-6.2. Male *C. elegans* were celibate for 2 days prior to sperm isolation. Hoechst was used to stain the paternal DNA. RPN-6.2 seems to surround the paternal DNA in spermatids and the motile spermatozoa. Expression pattern was seen in all spermatids and spermatozoa. Blue fluorescence indicates DNA and green fluorescence indicates presence of RPN-6.2 White lines indicate area of spermatids and spermatozoa. Scale bar= 5  $\mu$ M N=50

**RPN-6.2 surrounds the paternal DNA in spermatids and spermatozoa**

Spermatozoa are the final, motile form of sperm. When an oocyte enters and leaves the spermatheca, spermatozoa are dragged out of the spermatheca. *C. elegans* spermatozoa utilize a pseudopod tail structure to crawl back into the spermatheca. We were interested in whether the proteasome changes cellular localization upon spermatozoa activation. We activated spermatids *in-vitro* by treating spermatids with 20  $\mu$ L of Boyd Buffer and the cellular localization of proteasome resembled that of the spermatids; proteasome surrounding the paternal DNA (Figure 11). Additionally, there seems to be increased localization of proteasome to the pseudopod base.

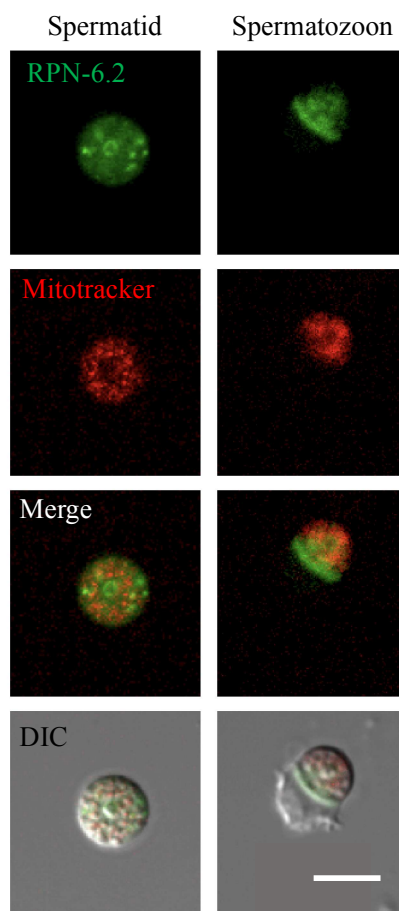
**RPN-6.2 does not localize to membranous organelles**

Previous work in the Boyd lab determined that RPT-1 to localize to MOs. To determine if RPN-6.2 also localizes to MOs, we stained MOs with anti-MO antibodies in GFP::RPN-6.2 expressing worms to compare localization (Figure 12). Interestingly, although RPT-1 colocalized with MOs, as expected, RPN-6.2 did not. RPT-1 is the only proteasomal subunit we have seen that does not share the proteasome expression pattern mentioned previously.



**Figure 12: RPN-6.2 does not localize to MOs or RPT-1**

*C. elegans* spermatids expressing mCherry::*rpt-1* and GFP::*rpn-6.2* were stained for membranous organelles with 1CB4 primary antibody. RPN-6.2 does not localize with RPT-1 of the 19S ring or membranous organelles (MO's). Localization pattern was seen in 100% of spermatids. Scale bar= 5  $\mu$ M N=50



**Figure 13: RPN-6.2 does not localize to mitochondria in spermatids or spermatozoa**

*C. elegans* males expressing GFP::RPN-6.2 were soaked in Mitotracker for 2 hours prior to spermatid extraction. Mitochondria does not seem to localize to RPN-6.2 in spermatids or spermatozoa. n=20 spermatids and spermatozoa. Scale bar= 5  $\mu$ M

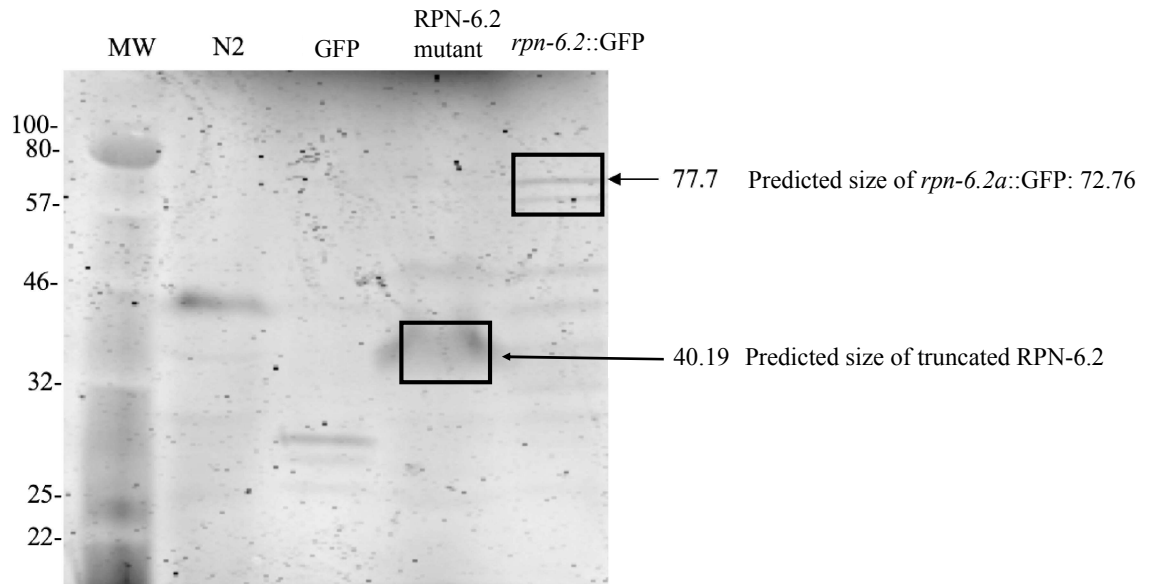
### **RPN-6.2 does not localize to mitochondria**

Localization of proteasome to the mitochondria may imply a role of proteasome in maintaining mitochondria homeostasis. We used Mitotracker to stain the spermatids and spermatozoa of GFP::*rpn-6.2* male worms to see if RPN-6.2 localized to mitochondria. RPN-6.2 does not localize to mitochondria (Figure 13).

## **RPN-6.2 isoform expression**

### **RPN-6.2 Isoform A expression**

RPN-6.2 is predicted to have two isoforms due to different transcriptional start sites. Isoform A has a predicted size of 46 kDa and isoform B has a predicted size of 23 kDa based on their composition of amino acids (416 aa and 213 aa, respectively). There is no evidence that both isoforms are translated as the current evidence of RPN-6.2 is from RNA sequencing data. DNA sequence encoding GFP has been introduced into the *rpn-6.2* gene locus in the LN167 transgenic strain, in-frame with the coding sequence of the gene at the 3' end. If the gene is expressed at the protein level, as either isoform, these proteins will have a C-terminal GFP tag. To further characterize RPN-6.2, we ran whole worm lysates of LN167 through an SDS page and then used Typhoon laser imaging to image GFP fluorescence on the gel (Figure 14). A band which is estimated to be 77 kDa was expressed which is close to the predicted size for the isoform A GFP fusion (72 kDa). The predicted size of isoform B GFP fusion is 50.33 and the truncated *rpn-6.2* mutant with a GFP fusion expressed by the isoform A promoter is 40 kDa. Our evidence suggests that isoform A, but not isoform B, is present in spermatids of *C. elegans* young adults.



**Figure 14: RPN-6.2 isoform A is expressed in *C. elegans* sperm tissue**

Whole worm lysates of N2 (wildtype), LN130 (GFP::ubiquitin), LN170 (*rpn-6.2* mutant) and LN167 (GFP::RPN-6.2) were ran through SDS PAGE. LN167 lysates produced a prominent band at 77.7 kDa and LN170 produced a band at 40.19 kDa. LN130 expressing GFP::ubiquitin was used as a control for GFP. LN130 produced a band close to 28 kDa, close to predicted size of GFP of 27 kDa.

## CHAPTER IV: DISCUSSION

Although spermatogenesis is a widely studied process, little is understood about the role played by proteasome in regulating spermatogenesis and spermatid homeostasis. The proteasome is present in the acrosome of mammalian sperm and has been implicated in the degradation of the vitelline coat of eggs, but *C. elegans* lack this mechanism and yet still have proteasome in their sperm. Although these key differences between mammalian and *C. elegans* sperm are apparent, they carry out many of the same processes during spermatogenesis (reviewed by L'Hernault *et al*, 1997). Decrease in sperm counts of men in western countries (with little to no evidence of why) highlights the importance of basic research studies to help determine the mechanisms behind functional sperm. The goal of this study was to help characterize *C. elegans* sperm proteasome. Here, we identify RPN-6.2 to be a sperm specific proteasomal subunit surrounding the sperm DNA in spermatids and spermatocytes. We also provide evidence that RPN-6.2 is required for proper spermatogenesis.

### **Disruption of RPN-6.2**

RPN-6.2 is a proteasome subunit expressed only in sperm tissue. Our knock-down confirmed we can disrupt *rpn-6.2* expression without knocking-down the closely related RPN-6.1, which would be detrimental to the somatic tissues of the worm. As RPN-6.1 is not expressed during spermatogenesis, utilizing the tissue specificity of RPN-6.2 gives us a great way to study RPN-6.2 requirements in sperm.

There are at least two possibilities regarding how the RPN-6.2 knock-down and mutant may affect proteasomal function in sperm tissue: 1) Disrupting RPN-6.2 may destabilize the proteasome, weakening the interaction between the 19S particle and the 20S

core leading to decreased proteasomal activity and 2) Disruption of RPN-6.2 may interrupt a substrate recognition role for the proteasome in the degradation of spermatogenic factors. There is stronger evidence supporting the former. RPN-6.2 and its homologs are thought to be scaffolding proteins acting as a molecular clamp; stabilizing the connection between the non-ATPase regulatory cap, ATPase ring, and the alpha-3 subunit of the 20S core (Pathare *et al*, 2012). Knock-down of the *rpn-6.2* paralog, *rpn-6.1* has shown to decrease chymotrypsin-like activity of the 20S core and decreases the lifespan of *C. elegans* (Vilchez *et al*, 2012). Vilchez *et al* also showed *rpn-6.1* over expression lead to much more resistance to oxidative and heat stress. RPN-6.1 was also shown to be responsible for the increased longevity of worms without gonads under slight heat shock (25 °C). The overall hypothesis is that RPN-6.1 stabilizing the proteasome promotes its degradative ability via opening of the  $\alpha$ -rings. Given the structure and previously mentioned role of Rpn6 homologs, we predict RPN-6.2 to have a similar function in sperm proteasome. An *in vitro* study comparing sperm proteasome and sperm proteasome lacking RPN-6.2 may help to clarify the structural role of RPN-6.2 in *C. elegans* sperm.

The decrease in brood size of *C. elegans* we observed in *rpn-6.2* mutants was not due to embryonic lethality, suggesting RPN-6.2 may either have a role during fertilization or spermatogenesis. Proteasome activity is required for the progression of meiosis and also has a key role in regulating meiotic stages by the controlled degradation of cyclins. Cyclin turnover could possibly be interrupted as a consequence of sperm proteasome lacking the RPN-6.2 subunit. Cyclins assist in the ability of cyclin dependent kinases (CDKs) to recognize target substrates, which regulate meiotic stages, for phosphorylation. Cyclin B3 is a nuclear protein expressed during the zygotene stage of meiosis I but its expression is depleted by the following pachytene stage. In mice, spermatogenesis occurs in

seminiferous tubules (SF) and may be observed for defects in spermatogenesis. Studies in mice have shown cyclin B3 expression during pachytene induces various defects in spermatogenesis including a depletion of germ cells in the SF, SFs packed with sperm at various meiotic stages, and defects in maturation of spermatids (Refik-Rogers *et al*, 2006). The presence of cyclin B3 is also required for female fertility in *Drosophila* but is not required for the completion of mitosis. (Jacobs HW *et al*, 1998). Additionally, nondegradable cyclin B3 leads to condensed DNA by the end of mitosis (Parry *et al*, 2001). These results insinuate a role for proteasome to degrade cyclin B3 during pachytene.

Recent studies have shown oogenic cyclin B3 is indeed targeted for degradation by the APC complex during meiosis I in mice (Karasu *et al*, 2018). In our study, deficient proteasome as a consequence of lacking RPN-6.2 may not be able to degrade all of cyclin B3 prior to pachytene. The increase in cyclin B3 expression during pachytene of spermatogenesis could be the reason behind decreased sperm production in *rpn-6.2* mutant and knock-down worms. An enzyme-linked immunosorbent assay may be appropriate to determine whether cyclin B3 expression is increased in *rpn-6.2* mutant worms.

The proteasome's downstream involvement in the APC mechanism must also be taken into consideration. *emb-30* is a subunit of the APC and is required for the metaphase/anaphase transition of meiosis I (Furata *et al*, 2000). Disruption of *emb-30* halts oogenic and sperm cells at metaphase, preventing any sex cells from developing. These studies exemplify the necessity of the APC during meiosis. It's easy to speculate that the decrease in sperm counts is due to a defective proteasome unable to degrade targets polyubiquitinated by the APC for degradation, but this does not explain the presence of proteasome in spermatids and spermatozoa. Additionally, studies disrupting the APC



complex in meiotic cells lead to a much more drastic phenotype as most cells cannot complete anaphase. There is also a possibility that the loss of RPN-6.2 has a slight effect on proteasome activity, producing the less extreme phenotype we see in our *rpn-6.2* knock-down and mutant worms.

Disruption of RPN-6.2 may also affect a non-proteolytic function of the proteasome. There is little information on how kinases dissociate from their respective cyclin while escaping proteasomal degradation. Interestingly, dissociation of cyclin B from Cdc2 kinase during meiosis II is dependent on the 26S proteasome but independent of proteolytic activity (Nishiyama *et al*, 2000). Disruption of RPN-6.2 may interfere with the proteasomes ability to assist the dissociation of cyclins from the cyclin-CDK complex prior to their degradation.

### **Proteasome localization in spermatids and spermatozoa**

RPN-6.2 is expressed in the nucleus of primary spermatocytes and in the spermatids of *C. elegans*. However, our imaging suggests a different subcellular expression pattern in spermatids. RPN-6.2 along with RPN-8, RPN-9, PAS-1, and RPT-1 surround the paternal DNA in spermatids and spermatozoa. This is an interesting finding, as it is commonly believed that the sperm lack a nuclear membrane. Instead, SEM imaging has identified what is referred to as a “nuclear halo” that surrounds the paternal DNA but its function is not understood (Ward *et al*, 1981). The composition of the nuclear halo described by Ward *et al* in 1981 is still an enigma. The only other sperm protein resembling the proteasome localization in spermatids is SPE-11 (Shakes *et al*, 2000). SPE-11 has a sperm specific expression pattern similar to sperm proteasome and is required for embryogenesis, but not fertilization (L’Hernault *et al*, 1988). Interestingly, SPE-11 is the only known male derived

factor required for post-fertilization events. It appears proteasome and SPE-11 are tethered to the nuclear halo, or perhaps even to the paternal DNA but the implications of this relationship require further study. The localization around the paternal DNA seems to be the only similarity between RPN-6.2 and SPE-11, as they have different phenotypes when disrupted.

The mechanism behind the formation and function of the nuclear halo is not understood. Recently, *mib-1*, a conserved E3 ubiquitin ligase was identified to be required for the formation of the nuclear halo (Herrera *et al*, 2018). *eri-3*, a member of the Dicer-associated complex, is also required for the formation of the nuclear halo, but its role is unknown (Herrera *et al*, 2018). There is currently no direct relationship between *eri-3*, *mib-1* and *spe-11* besides their relationship to the nuclear halo.

Not only does proteasome appear to be encapsulating the paternal DNA, but it is also present in puncta scattered around the cell. RPN-6.2 expression does not co-localize with MOs nor mitochondria. Interestingly, RPT-1 has been seen to co-localize with MOs. RPT-1 does not share the same localization pattern that we have seen in the other proteasomal subunits. This may be because the mCherry::*rpt-1* is transgenically expressed under the germline promoter *mex-5* and the other proteasome subunit strains have GFP tags expressed through the endogenous promoter. The abnormal expression of *rpt-1* may account for the alternate expression pattern seen in spermatids.

Collectively, we have shown proteasome is expressed in sperm, appears to be surrounding the paternal DNA, and that RPN-6.2 is required for normal brood sizes in *C. elegans*. Our novel findings will not only contribute to elucidating the role of proteasome during spermatogenesis, but also produces a new way to study sperm proteasome using RPN-6.2 as a tissue specific marker. Finally, our data showing proteasome encapsulating

the paternal DNA in the sperm of *C. elegans* suggests the proteasome is tethered to the nuclear halo.

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