Phenotypic Characterization of PNPase Mutation and Overexpression in C. elegans

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PHENOTYPIC CHARACTERIZATION OF PNPASE MUTATION AND
OVEREXPRESSION IN C. ELEGANS

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science
at Virginia Commonwealth University

By

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Creation of overexpression models with microinjections of the plasmids and constructs were carried out by Dr. Laura Mathies.

Mutant strains (G58E (VC40327), G74R (VC20261), and G74E (VC20284) from the Caenorhabditis Genetics Center were ordered by Dr. Laura Mathies and Dr. Jill Bettinger

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**List of Abbreviations**

CDC 25.1 – Cell division cycle 25.1

CDKI p27KIP1 – Cyclin-dependent kinases 1 (tumor) protein 27 kinase inhibitory protein 1

cDNA – Complementary DNA

CGC – *Caenorhabditis* Genetics Center

c-myc – Cellular- MYC protooncogene

COIII – Cytochrome c oxidase subunit 3

CRISPR – Clustered regularly interspaced short palindromic repeats

CTB-1 – Cytochrome B – 1

DIC – Differential interference contrast microscopy

DNA – Deoxyribonucleic acid

dsRNA – Double stranded RNA

EMS – Ethyl methanesulfonate

ENU – N-ethyl-N-nitrosourea

FBF-1/2 – *fem-3* Binding factor 1 / 2

FSN PCR – Fusion PCR

FZR-1 – Fizzy and cell division cycle 20 related 1

GLD-1 – Germ line development 1

GLD-2 – Germline development 2

hsp – Heat shock protein

IFN – Interferon

IPTG – Isopropyl beta-d-1-thiogalactopyranoside

KH – K homology
LB – Liquid broth
LIN-35 – Retinoblastoma-like protein homolog lin-35
MAPK – Mitogen activated protein kinase
MEX-3 – Muscle EXcess 3
miR – MicroRNA
MMP1 – Metal metallic protease 1
MPP – Mitochondrial processing peptidase
mTP – Mitochondrial targeting peptide
mtRNA – Mitochondrial RNA
MTS – Mitochondrial targeting sequence
NGM – Nematode growth media
NOS-3 – Nanos-type domain-containing protein 3
PCR – Polymerase chain reaction
PNPT1 – Polyribonucleotide nucleotidyltransferase 1
PNPase – Polynucleotide phosphorylase
qRT-PCR – Quantitative reverse transcription-PCR
RNA – Ribonucleic acid
RNAi – RNA interference
ROS – Reactive oxygen species
RPH – RNase PH
rRNA – Ribosomal RNA
SOD2 – Superoxide dismutase 2
ssRNA – Single stranded RNA
SW-PCR – Single worm-PCR
TIM – Translocase of the inner membrane
TOM – Translocase of the outer membrane
UNC-54 – Un-coordinated 54
UTR – Untranslated region
WLB – Worm lysis buffer
Yme-1 – Yeast mitochondrial escape 1
Abstract

Phenotypic Characterization of PNPase Mutation and Overexpression in *C. elegans*

By Brian Hur, B.S.

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University

Virginia Commonwealth University, 2019

Major Director: Rita Shiang
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PNPase, polynucleotide phosphorylase, is a multifunctional exoribonuclease protein with 3’ terminal oligonucleotide polymerase activity. Coded by the *PNPT1* gene, the protein is associated with mitochondrial homeostasis and functions as a possible target for cancer therapy. In this study, *C. elegans* was used to investigate the effect of mutation and overexpression of *pnpt-1*, the gene that encodes PNPase. It was determined that two specific mutations in *pnpt-1* did not affect PNPase expression nor did they produce deleterious phenotypes that affected polycistronic transcript accumulation or ROS production. Creation of a stable overexpression model was achieved through Fusion PCR. However, different transgenic strains overexpressing PNPase produced opposite results for polycistronic transcript accumulation while ROS production saw no significant change, suggesting a mosaic overexpression model.

In a cancer model, exogenous PNPase was present in the pachytene region of the germline and where expressed the cells were in non-germline cells suggesting differentiation mechanisms associated with overexpression of PNPase. However, further analysis of different mutations in *pnpt-1* or optimizations to the overexpression model are necessary to provide a better understanding of PNPase function with mitochondria homeostasis and in a cancer model setting.
Chapter 1: Introduction

I. PNPase

Encoded by the *PNPT1* (polyribonucleotide nucleotidyltransferase 1), PNPase is an evolutionarily conserved 3’-5’ phosphate dependent exoribonuclease and 5’-3’ RNA polymerase that adds poly(A) tails to RNA molecules (Sarkar et. al., 2005; Das et. al., 2010). The protein localizes either in the inter membrane space of the mitochondria or the cytosol (Piwowarski et. al., 2003). In humans, the *PNPT1* gene is located within 2p15-2p16.1 and spans 60 kb, consisting of 28 exons (Leszczyniecka et. al., 2003). During a screen between terminally differentiated HO-1 melanoma cells and senescent progeroid fibroblasts to identify co-regulated genes, 75 genes were classified and labeled as old-1 through old-75. Among the 75 genes, old-35 presented significant sequence similarity to PNPase from other species, thus cataloging human PNPase as hPNPase old-35 (Leszczyniecka et. al., 2002).

Functioning as an exoribonuclease, PNPase selectively degrades specific targets such as c-myc mRNA, miR-221, miR-222, miR-106b, and other miRNAs involved in oncogenesis in the cytosol. (Sarkar et. al., 2003; Das et. al., 2010). PNPase also regulates translocation of small RNA molecules into the mitochondria such as MRP (mitochondria RNA processing) RNA, RNase P, and 5S rRNA (Wang et. al., 2010). Additionally, PNPase has been found to be induced by type I interferons, specifically IFN-alpha and beta. Studies identified an ISRE, IFN-stimulated response element, in the promoter of hPNPase old-35. Mutations in this site eliminated induction of gene expression (Leszczyniecka et. al., 2003).
II. PNPase Structure

PNPase is conserved across most organisms from simple species of bacteria to complex and higher mammals, with the exception of yeast (Leszczyniecka et. al., 2004). The protein is comprised of four major domains that include two RNase PH domains, one KH domain, and one S1 domain. Spatially, the two catalytic RNase PH domains are located towards the N-terminal end while the RNA-binding KH and S1 domains are located closer to the C-terminus (Figure 1A) (Leszczyniecka et. al., 2004). In humans, PNPase is a large molecule that consists of 738 amino acids with an alpha helix to separate the two RNase PH domains (Leszczyniecka et. al., 2002). Despite the presence of conserved domains of PNPase, there are variations of the protein structure within different organisms. PNPase in plants contains an N-terminal target peptide for translocation of the protein to the chloroplast while the animal variant has a N-terminal mitochondrial localization signal to direct the protein to the intermembrane space of the mitochondria (Piwowarski et. al., 2003; Sarkar et al., 2005).

On a structural level, PNPase forms a homotrimeric structure or a dimer of homotrimeric resulting in a doughnut-like shape (Figure 1B) (Carpousis et. al., 2002). The RNase PH domains create a channel for catalytic activity for single-stranded RNA molecules (Symmons et. al., 2000: Symmons et. al., 2002). Superior to the RNase PH domains are the KH and S1 domains that also contribute to central channel structure but primarily function as RNA-binding domains (Golzarroshan et. al.,2018). Catalytic activity from the RNase PH domains varies in different organisms. In a review, the catalytic activity of PNPase in bacteria is primarily carried out by the second RNase PH domain while the RNase PH domains in plants contribute equally (Sarkar and Fisher, 2006). For human PNPase, mutational analysis has determined that both RNase PH domains have equal catalytic activity and the presence of at least one RNase PH domain is
adequate for complete enzymatic activity (Sarkar et. al., 2005). The KH and S1 domains are also vital to protein function. Deletion of the S1 or KH domains results in a decrease in enzymatic activity by 50 fold and 19 fold, respectively, while deletion of both domains results in 1% of enzymatic activity (Stickney et. al., 2005)

Figure 1A: Protein motifs of PNPase. Both RNase PH, KH, and S1 domains are conserved across several organisms. PNPase in different organisms contain variations in the N-terminal targeting sequence/peptide or no targeting sequence/peptide at all. (Sohki et. al., 2013)

Figure 1B: Structure of PNPase. PNPase assembles into a homotrimer revealing a donut-like structure with a channel in the middle where catalytic activity takes place. (Shi et. al., 2008)
III.  PNPase Localization

Human PNPase is mainly localized within the intermembrane space of the mitochondria (Piwowarski et al., 2003). The N-terminal mitochondrial targeting sequence (MTS) of the protein directs it to the organelle (Chen et al., 2006). PNPase is then imported through the TOM and TIM23 pores of the mitochondria where it is cleaved by MPP (mitochondrial processing peptidase) at the region following the N-terminal targeting sequence. Yme1 serves as a translocation motor that pulls PNPase into the intermembrane space where PNPase assembles into the final multimeric complex (Figure 2) (Rainey et al., 2006). Cleavage of the N-terminal targeting sequence by MPP exposes a hydrophobic region on PNPase that is hypothesized to serve as a stop-transfer domain to stop translocation through TIM23 (Rainey et al., 2006).

Depending on the organism, PNPase localizes in different regions. In plants, PNPase localizes in the chloroplast stroma and functions as a poly(A) polymerase and exonuclease (Chen et al., 2006). Outside of plants and in multiple cell types, PNPase is found in the intermembrane space of the mitochondria, regulating mtRNA levels (Chen et al., 2006; Piwowarski et al., 2003). Other studies have also noted that hPNPase can localize in the cytoplasm where it degrades specific mRNA and miRNA molecules. (Leszczyniecka et al., 2002; Sarker et al., 2003; Das et al., 2010).
IV. Functional Studies – Mouse Knockout and Cellular Knockdown

Previous *in vivo* mouse studies had attempted knocking out PNPase but were unsuccessful since it was determined that embryos without PNPase were nonviable and embryonic lethal (Wang et. al., 2010). Instead, a conditional liver knockout of PNPase was produced in a mouse model. Knockout of PNPase in liver cells resulted in disrupted respiration and mitochondrial morphology with mitochondria cristae appearing disordered, circular, and smooth (Wang et. al., 2010). Inhibition of mitochondrial RNA transport which regulate the transcription and translation of electron transport chain proteins partially contributed to the disrupted respiration mechanisms (Wang et. al., 2010).

PNPase knockdown was also performed in melanoma cell line HEK-293T (Chen et. al., 2006). Results included impairment of the respiratory chain complex with a decrease in
enzymatic activity, mitochondria appearing filamentous and granular, and decrease in complex 3 and 4 potential of the respiratory chain complex (Chen et. al., 2006). All of this contributed to a decrease in cell growth with increased cell death (Chen et. al, 2006).

V. Functional Studies – Cellular Overexpression

Overexpression of hPNPase old-35 was studied in HO-1 cells with two different approaches to analyze growth suppression mechanisms: slow and sustained overexpression with low multiplicity of adenoviral vector and rapid overexpression with a high multiplicity of an adenoviral vector (Sarkar et. al., 2003). Each overexpression mechanism produced different results. Slow and sustained overexpression resulted in an induction of a senescent-like phenotype and growth inhibition (Sarkar et. al., 2003). Infected HO-1 cells arrested in the G1 phase of the cell cycle with a reduction of cells in the S phase that eventually lead to apoptosis (Sarkar et. al., 2003; van Maerken et. al., 2009). Rapid overexpression of PNPase resulted in induced growth arrest and promotion of apoptosis without any changes to the cell cycle (Sarkar et. al., 2003).

Overexpression of PNPase also decreased expression of c-myc mRNA and Myc protein, a transcription factor that regulates cell growth (Sarkar et. al., 2003). hPNPase old-35 selectively degraded c-myc RNA molecules through recognition of the 3’ UTR, which led to blocked mitogenic signals and cells driven to terminal differentiation (Sarkar et. al., 2003; Sarkar et al 2006). Other effects of overexpressing hPNPase included inhibition of DNA synthesis and telomerase activity, and up regulation of CDKI p27KIP1 (Sarkar et. al., 2005). All of these factors contributed to the senescent-like phenotype associated with overexpression of hPNPase old-35 (Sarkar et. al., 2003; Sarkar et. al., 2005).
Overexpressed hPNPase old-35 also targets and degrades other RNA molecules such as miR-221, miR-222, and miR-106b (Das et al., 2009). miR-221 is often upregulated in various human cancers including glioblastoma, liver, bladder, thyroid, pancreatic, gastric and prostate carcinomas (Lupini et al., 2013). miR-222 is also upregulated in several cancer types, targeting MMP1 (metal metallic protease 1) and SOD2 (superoxide dismutase 2) (Liu et al., 2009). miR-106b targets several tumor suppressor genes (Liu et al., 2014). In a comparison with normal cells, tumorigenic cells presented down-regulated expression of hPNPase old-35, allowing upregulation of oncogenic miRNA molecules to facilitate tumorigenesis (Das et al., 2009). The correlation between miRNA targets of hPNPase and cancer progression makes overexpression of hPNPase old-35 an appealing anti-cancer approach.

VI. PNPas in the Mitochondria

Due to the localization of hPNPase old-35 in the intermembrane space of the mitochondria, several studies investigated if hPNPase-old 35 has a role in mitochondrial homeostasis. Overexpressing hPNPase old-35 resulted in an increase in reactive oxygen specie (ROS) production (Sarkar et al., 2004). Increase in ROS production led to an increased activation of the NF-kB pathway and the downstream genes associated with proinflammatory cytokine synthesis (Sarkar et al., 2004). Ultimately, overexpression of hPNPase old-35 induced a senescent-like growth arrest phenotype with increased production of ROS contributing to irreversible growth arrest of the cell (Sarkar et al., 2004). Knockdown studies in HEK293T cells that decreased expression of hPNPase old-35 resulted in mitochondrial dysfunction (Chen et al., 2006). Mitochondria appeared to be fragmented, filamentous, and granular shaped with decreased membrane potential (Chen et al., 2006). At the enzymatic level, coupled respiratory complexes I/III, and II/III presented reduced enzymatic activity with dysfunction in complex I of
the electron transport chain, resulting in elevated lactate and decreased ATP levels (Chen et. al., 2006).

hPNPase old-35 also functions as an importer trafficking small RNA molecules into the mitochondria, specifically RNase P RNA, MRP RNA, and 5S RNA (Wang et. al., 2012). RNase P is an endoribonuclease that processes mitochondrial tRNAs. MRP RNA is a site-specific endonuclease that processes mtRNA transcripts to form primers to initiate replication of mtDNA. 5S rRNA is a ribosomal component that provides regulatory interactions among the functional sites of the translating protein (Wang et. al., 2012). The figure below displays the various roles PNPase plays within the cell at different locations (Figure 3).

**Figure 3: Roles and subcellular localization of PNPase.** PNPase in the cytoplasm functions in miRNA and c-myc degradation. Upon entry into the mitochondria through the TOM/TIM complex, PNPase mechanisms associate with ROS production, mtRNA processing, RNA polymerization, and RNA import into the organelle. (Sohki et. al., 2013)
VII. Human Diseases

In a clinical setting, mutations in PNPase have been associated with myopathy, encephalopathy, and neuropathy (Vedrenne et. al., 2012) and human hereditary hearing loss (von Ameln et. al., 2012). These studies highlight PNPase’s connection with mitochondrial function and homeostasis. In the first study, two siblings of consanguineous parents presented with a homozygous missense mutation in the second RNase PH catalytic domain of PNPT1 (c. 1160 A>G)(Q387R). In the first child, there was a significant decrease in 5S rRNA and MRP RNA import into the mitochondria and decreased rate of mitochondrial translation. The child had slow voluntary movements, dystonia, dyskinesia, choreoathetosis, global hypotonia, severe muscle weakness, and no head control. Due to muscle atrophy, deep tendon reflexes were barely detected. The second child developed motor regression with trunk hypotonia, choreoathetotic movements, major dystonia in the limbs, and global hypotonia. Overall, the two children had fixed but nonprogressive encephalopathy with mildly elevated levels of lactate in the plasma and cerebrospinal fluid. On a molecular level, there was decreased activity of respiratory chain complexes III and IV in the liver in the first child. The second child presented normal enzymatic activity in both skeletal muscle samples and cultured skin fibroblasts (Vedrenne et. al., 2012). The results above provided strong evidence of the role of PNPase in RNA import into the mitochondria and maintenance of a functional respiratory chain complex (Vedrenne et. al., 2012).

The second study consisted of 3 siblings from a consanguineous family that presented with severe hearing impairment. Sequence analysis identified a missense mutation in the second RNase PH catalytic PNPT1 (c. 1424 A>G)(E475G) in the second RNase-PH domain. The missense mutation interfered with oligomerization and prevented PNPase from properly forming
a homotrimer, but the protein was able to behave as a hypomorph. Trimerization was reduced with increased monomer formation that had decreased protein function. As a result, a decrease of RNase P RNA import into the mitochondrion was observed. Due to the high energy demand of the inner ear, it was hypothesized that the slower rate of import of small RNA molecules of the PNPase hypomorph negatively affected the inner ear tissue and not others. Variation of phenotypes observed between the two families led researchers to hypothesize that the different functional deficits of PNPase was dependent on the severity of the mutation (von Ameln et. al., 2012).

A third human study identified two siblings from a non-consanguineous family that presented disease phenotypes which included severe axonal neuropathy, optic atrophy, intellectual disability, auditory neuropathy and chronic respiratory and gut disturbances (Alodaib et. al., 2016). Unlike the previous studies, whole exome sequencing on all family members identified compound heterozygous missense variants in the PNPT1 gene in the two affected siblings with mutations Q254K in the first catalytic RNase PH domain and A510P in the second RNase PH domain that were predicted to be damaging (Alodaib et. al., 2016). In patient fibroblasts, the missense mutations affected quaternary formation of PNPase protein and presented a reduction in protein and mRNA expression of PNPT1 when compared to samples from unaffected family members. However, mRNA expression of PNPT1 did not vary in blood samples from unaffected and affected family members. Analysis of the oxidative phosphorylation complexes identified significant reduction of expression with complex I, III, and IV and decreased enzymatic activity of complex I and IV in the patient fibroblasts. The study also confirmed impairment of mitochondrial translation with a 33% reduction in total mitochondrial protein synthesis in comparison to the unaffected control (Alodaib et. al., 2016).
Another study observed the effects of the disease-causing missense mutations, Q387R and E475G, in the second RNase PH catalytic domain of PNPase on a proteomic and molecular level using *Escherichia coli*. As a result of the mutations, PNPase formed dimers rather than trimers and had significantly lower RNA binding and degradation activities compared to wild-type trimeric PNPase (Golzarroshan et. al., 2018). Specifically, human PNPase mutants formed a dimeric structure with a disrupted KH pore that impeded ssRNA binding. Analysis of the KH pore in trimeric PNPase identified exposed GXXG motifs within the KH domain oriented inward toward the pore. This positioning of GXXG motifs facilitated interactions with and binding to ssRNA (Golzarroshan et. al., 2018). However, the dimeric mutant PNPase presented a disrupted KH pore with GXXG motifs located far apart, making them less available for RNA binding and interaction (Figure 4). These findings explained the decreased ssRNA binding and degrading activity in mutant dimeric PNPase compared to trimeric PNPase (Golzarroshan et. al., 2018).
Figure 4: Wildtype vs Mutant PNPase. (A) Wildtype PNPase trimer forms a donut-like shape with GXXG motifs of KH domains located close to each other at the KH pore. (B) Mutant PNPase that resulted in a dimeric protein with KH domains distant from each other. (Golzarroshan et al., 2018)

VIII. ROS

ROS, products of cellular respiration, are synthesized when molecular oxygen (O2) is reduced. Such species include hydrogen peroxide, hydroxyl radical, and superoxide. Within the mitochondrial electron transport chain, Complex I and Complex III are responsible for the synthesis and release of superoxides into the intermembrane space of the mitochondria. Sizeable accumulations of ROS within the cell damages proteins, lipids, and DNA. However, low amounts of ROS molecules can serve as activators for signaling pathways associated with
proliferation and transcription (Trachootham et. al., 2008; Droge et. al., 2002; Thannickal et. al., 2000). Studies have proven that slight increases in ROS activate beneficial stress responses that lead to lifespan extension rather than producing harmful effects (Zarse et. al., 2012; Schulz et. al., 2007). To offset the accumulation and effect of superoxides, superoxide dismutase converts superoxide into hydrogen peroxide. The hydrogen peroxide is then eliminated by other peroxiredoxins and peroxidases (Sena and Chandel, 2012).

IX. C. elegans

*Caenorhabditis elegans* is a free living nematode that is used as a popular model organism for studies of developmental biology and basic functions and mechanisms of eukaryotic cells (Corsi et. al., 2015). Championed by Syndey Brenner, this specific nematode species proves to be a valuable model to study mutations that contribute to human diseases. The animal exists mostly as self-fertilizing hermaphrodites with a genotype of XX for sex chromosomes. Male *C. elegans* are present but arise at a low frequency of <0.2% with a XO genotype (Corsi et. al., 2015). Their self-fertilizing nature allows stocks to be maintained from a single animal, giving rise to an entire population.

*C. elegans* is an attractive model due to a short three day life cycle to develop from an egg to a 1 millimeter long egg-laying adult, small size, complete sequenced genome, and ease of growth and maintenance. Their transparent body throughout their life cycle allows easy examination of cells with differential interference contrast (DIC) microscopy (Corsi et. al., 2015). Due to their simple nature, the nematode is an optimal system to investigate the genetics of basic behavior mechanisms such as foraging, feeding, defecation, movement, egg laying, sensory responses to touch, smell, and other simple forms of behavior (Rankin et. al., 2002). However, there are limitations that prevent *C. elegans* from being the perfect model organism.
Such as lacking specialized tissues found in higher organisms (Van Raamsdonk and Hekimi, 2010).

![Figure 5: Anatomy of C. elegans hermaphrodite body](https://en.wikipedia.org/wiki/Caenorhabditis_elegans#/media/File:Caenorhabditis_elegans_hermaphrodite_adult-en.svg)

**X. PNPase in C. elegans**

In *C. elegans*, the ortholog for PNPT1 is *pnpt-1* and consists of 10 exons located on chromosome III. The *pnpt-1* transcript was initially identified during an RNA interference (RNAi) profiling of embryogenesis (Sonnichsen et. al., 2005). *tm1909*, a deletion mutant, resulted in lethal and sterile phenotypes. However, this deletion mutant was not limited to *pnpt-1* and included a neighboring upstream gene, *chin-1*.

*C. elegans* proved to be the ideal model for this project due to ease of gene expression manipulation through RNAi, a molecular process where dsRNA inhibits gene expression or translation, for knockdown analysis, ectopic expression through transgenic overexpression...
analysis, the availability of mutant strains from the *Caenorhabditis* Genetics Center (CGC) for mutant analysis, and the ease and varied approaches at producing a cancer model. Since previous attempts to produce a knockout model in other organisms were met with little success due to embryonic lethality (Wang et. al., 2010), and with no documentation of an overexpression animal model present, using *C. elegans* will provide a foundational understanding of the phenotypic effects of varied PNPase expression and observation of the senescent induced phenotype associated with overexpression of PNPase in a cancer model setting.

**XI. *C. elegans* Germline**

Germline development of *C. elegans* can be organized into three distinct phases: specification, growth, and maintenance (Figure 5A). During early embryogenesis, P blastomeres are distinguished from somatic cells with the germline founder cell, P4, giving rise to only germ cells with no contribution to the soma. P granules are segregated into the P blastomeres, contributing to germline development. During the hatching stages of the nematode, the gonad consists to two primordial germ cells, Z2 and Z3, flanked by two somatic gonad precursors, Z1 and Z4. During the first two larval stages, the somatic gonad precursors divide to initially produce 12 cells, ten of which rearrange into somatic gonad primordium while the other two form the distal tip cells. During the L3 stage, both gonad arms extend concurrently with proliferation of the germ line. The L4 stage is identified by four-fold growth of germ cell number with germ cell proliferation in the distal mitotic zone (Hubbard et. al., 2005). Entry from the mitotic zone into meiotic division at the proximal end of the gonad requires coordination of GLD-1, GLD-2, FBF-1/2, and NOS-3 signaling (Figure 5B) (Nayak et. al., 2004). Within the transition zone between the mitotic and meiotic zones, chromosomes pair and undergo meiotic recombination until the pachytene stage. Upon entry into the meiotic zone, homologous
recombination and formation of synaptonemal complexes occur with germ cell chromosomes. Mitogen activated protein kinase (MAPK) signaling in the germline allows progression from pachytene to diplotene. From diplotene to diakineses, meiotic chromosomes become highly condensed and form six discrete oocyte bivalents (Hubbard et al., 2005).

Gametogenesis varies depending on the age of the nematode. The late L4 stage is marked by spermatogenesis while the adult stage is marked by oogenesis (Hubbard et al., 2005). During L4, GLD-1 and FOG-2 work together to inhibit tra-2 activity allowing fem-3 to be active and promote spermatogenesis. When entering the adult stage, gld-1 and fog-2 are inactive, increasing tra-2 activity that causes repression of fem-3 function and switching the germline to oogenesis (Figure 5B) (Nayak et al., 2004). Ultimately, gonads of hermaphroditic C. elegans consist of a linear sequence of germ cells in different developmental stages (Ciosk et al., 2006).
Figure 6A: Development of the C. elegans germline. (A). Development of the germline founder cell (P4) and its associated primordial germ cells, Z2 and Z3, from the zygote stage of the nematode. (B). Gonad formation and growth at different stages of nematode development. Red cells represent the distal tip cells. Yellow represents the mitotic region. Light green represents the transition phase consisting of early prophase of meiosis I. Dark green represents pachytene. Pink represents oogenesis. Blue sheath represents the spermatheca precursor cells. Light blue sheath represents nuclei. Grey represents the spermatheca. White represents the uterus. (Hubbard et. al., 2005)
Figure 6B: Genes involved in gametogenesis. (a) Interactions between several factors that regulate the switch between spermatogenesis and oogenesis. (b) Schematic of fog-2 and gld-1 inhibition on tra-2 mRNA, allowing spermatogenesis. (Nayak et. al., 2004)

XII. gld-1 Cancer Model in C. elegans

Several factors allow C. elegans to be useful for cancer research. The model is completely characterized with an invariant somatic cell lineage allowing ease of identification of phenotypes that disrupt normal proliferation and patterning (Sulston and Horvitz, 1977; Sulston et. al., 1983). The animal’s transparent body throughout its entire lifespan allows cells to be visualized during their division and movement. Finally, many human genes and pathways associated with cancer are conserved in C. elegans (Kirienko et. al., 2014). While the pathways and mechanisms are complex in a human setting, regulatory networks related to cancer in C. elegans are simplified with fewer genes involved (Hunter and Pines, 1994).
For our cancer model, *gld-1* knockdown in the nematode germline was used to create an over proliferation and tumor phenotype. Under normal conditions, GLD-1, an RNA binding protein, functions as a germline specific tumor suppressor that is responsible for the transition from spermatogenesis to oogenesis (Nayak et. al., 2004). The protein regulates the transition between mitotic division in the distal end to meiotic division at the proximal end of the gonad (Ciosk et. al., 2006). GLD-1 expression is inhibited in the distal mitotic zone (Crittenden et. al., 2002) but is observed to have higher levels of expression in the central region of the gonad (Jones et. al., 1996). With loss of function mutations of *gld-1*, tumors develop from germ cells that initiate meiosis but return to mitosis prior to completion of meiotic prophase (Figure 6A and 6B) (Francis et al., 1995). In addition to mass mitotic division, one study has identified nuclei in the germline resembling nuclei found in somatic tissues upon knockdown of *gld-1* but saw higher incidence of these nuclei in *gld-1* and *mex-3* knockdown (Ciosk et. al., 2006). Further analysis has determined the non-germline cells were differentiated somatic cells such as muscle cells, neurons, and intestinal cells (Ciosk et. al., 2006). Using *gld-1* knockdown as a cancer model in the germline will allow observation of the effects of overexpression of ectopic PNPase during tumor development in a whole animal model setting.
Figure 7: Effects of gld-1 mutants. Mutation in gld-1 produces a tumor-like phenotype with mass proliferation of mitotically dividing cells present in the proximal region of the gonad where meiotic division normally takes place. (Kirienko et. al., 2014)

XIII. Preliminary Studies of PNPase Knockdown in C. elegans

Previous work has studied the phenotypic effect of PNPase knockdown in C. elegans. Knockdown was achieved through feeding RNAi using an RNAi clone that produced dsRNA corresponding to pnpt-1. The clone consisted of a 558 base pair region 5’ of exon 1 to exon 3 of wPNPase. The study determined that decrease in PNPase expression caused an increase in lifespan due to increased ROS production. Additionally, decreased PNPase expression contributed to an altered fission/fusion ratio of the mitochondria but with no presentation of
disordered cristae. The lack of disordered cristae could be contributed to actions of other pathways and recovery mechanisms (Lambert 2015).

XIV. Project Outline

The main goal of this project is to generate animal models for *pnpt-1* (mutant and overexpression) in *C. elegans* to analyze their effect on mitochondrial homeostasis and induction of cellular senescence in a cancer model. To carry out this plan, mutant and overexpression models were produced and tested for downstream phenotypes including testing the effects of *pnpt-1* overexpression in a *gld-1* nematode cancer model to determine if it ameliorates aberrant proliferating cells. Strains that contain mutations in *wpnpt-1* will be obtained from the CGC and backcrossed to generate a background removed of unlinked mutations. Production of transgenic lines for an overexpression model of PNPase will first require synthesis of the ectopic construct. Fusion PCR will be used to create the construct under a heat-inducible promoter and it will be introduced into *C. elegans* by microinjection. Protocols to induce expression will be optimized to activate exogenous PNPase expression. Knockdown of PNPase will be achieved by RNAi mechanisms, following a similar protocol adopted by previous work (Lambert 2015). Once all three models have been created and optimized, polycistronic transcripts and total mitochondrial transcripts will be quantified, and ROS levels will be measured. A tumorous phenotype will be achieved using the *gld-1* cancer model to produce a proliferating tumor phenotype. Immunofluorescence will be used to analyze germline cells in the nematode gonad and will determine the senescent-inducing and differentiating role of PNPase overexpression.
Chapter 2: Materials and Methods

OP50 Liquid Cultures/Seeding NGM plates

Nematodes were fed OP50 bacterial cultures for regular maintenance. Bacteria from glycerol stocks of OP50 was streaked for isolation on LB plates. Individual bacterial colonies were picked with a P200 pipette tip and placed in 3 mLs of LB liquid media. Liquid cultures were incubated overnight at 37°C and 225 rpm. Approximately 150 uL of OP50 liquid culture was used to seed each medium (60mm x 15 mm) NGM (nematode growth media) plate. The 150 uL solution was distributed among 5 individual dots on the plate surface. Seeded plates were incubated overnight at 37°C and then placed in 4°C for long term storage.

C. elegans Maintenance

C. elegans were grown on NGM and fed OP50 bacteria and maintained at 20°C. Plates were individually parafilmed to prevent contamination. All worm strains were regularly maintained by either chunk transferring once a week or transferring 5-10 adult hermaphrodites every 4 days.

C. elegans Strains

For RNAi experiments, mutant strain CF3152 [rrf-3 (pk1426)] was obtained from Dr. Malene Hanson. Mutant studies consisted of strains containing point mutations in pnp-t-1:G58E (VC40327), G74R (VC20261), and G74E (VC20284). VC2010, a N2 wildtype derivative, was used as a control for mutant studies. These strain were obtained from the CGC and were generated as part of the million mutation project (Thompson et. al., 2013).
Frozen Stocks of Worms

Plates of recently starved L1/L2 hermaphrodites were washed with 0.6 mLs S Buffer (64.5 mM K2HPO4, 43.55 mM KH2PO4, 0.1 M NaCl) and transferred to cryotubes. Equal volumes of S Buffer + 30% glycerin (sterile) were added to a cryotube and vortexed to mix contents well. Samples were stored in -80°C freezer.

To reconstitute a worm line, cryotubes were thawed at room temperature and gently vortexed to mix contents. Half a milliliter of solution was pipetted onto OP50 seeded NGM plate. Plates were parafilmed and stored at 20°C for worms to develop.

RNAi Bacterial Strains

RNAi was induced through feeding the worms bacteria expressing dsRNA. Liquid bacterial cultures were initially grown overnight in LB with ampicillin (100 ng/uL) at 225 rpm at 37°C. Overnight cultures were diluted 1:100 in fresh LB with ampicillin (100 ng/uL). Cultures were then incubated in a shaker at 225 rpm and 37°C for 3 hours. IPTG was added (0.4 mM) with additional ampicillin (100 ng/uL). Samples were returned to the 37°C shaker for 2 hours. Ampicillin (100 ng/uL) and IPTG (0.4 mM) were added one last time. Plates were seeded with 200 uL of culture per 6 cm NGM + carbenicillin (0.1 mg/mL) plate (Lambert 2015).

Bacterial strains for RNAi were Exon 3 for knockdown of *wpnpt-1* (Lambert 2015), L4440 for empty vector control, T74 B4-7 for *dpy10*, a positive control that produced the “dumpy” phenotype (Dr. Jill Bettinger), and 1-F308 for *gld-1* (Dr. Malene Hansen) knockdown to produce the overgrowth phenotype in the cancer model studies. Strain Exon 3 was synthesized in our lab by Laura Lambert.
RNAi induced strains were plated with seven L4 hermaphroditic CF3152 worms per plate. L4 hermaphrodites were allowed to lay eggs overnight and removed to produced age matched progeny after 4 days of development. For RNA extraction, L4 CF3152 hermaphrodites were not removed after overnight egg laying but were allowed to continuously lay eggs.

**Extracting DNA from C. elegans**

Ten to twenty adult worms were picked and dropped into 20 uL of worm lysis buffer (WLB) (10mM Tris (pH 8.0), 50 mM KCl, 2.5 mM MgCl2, 0.45% Tween 20, 0.45% NP-40, and 0.05% Gelatin) with freshly added Proteinase K at a final concentration of 60 ng/uL. The worm and buffer mixture was frozen at -80°C for at least 30 min. Samples were then incubated in a 65°C water bath for 60 minutes. Samples were then incubated at 95°C for 15 min to inactivate the Proteinase K. DNA was stored at -20°C. For single worm PCR (SW-PCR), only one adult worm was picked and dropped into 20 uL of WLB with Proteinase K. All subsequent steps remained the same.

**Extracting RNA from C. elegans**

For mutant and transgenic lines, ten L4 hermaphrodites were transferred to fresh OP50 seeded NGM plates. Five 60 mm x 15 mm plates were used for each line. For RNAi samples, seven L4 CF3152 hermaphrodites were transferred to RNAi carbenicillin plates. Seven 60mm x 15mm plates were used for each RNAi induced sample. Progeny were allowed to develop for four days at 20°C prior to extracting RNA. Worms were washed off with 1 mL of M9 twice and transferred to a 15 mL conical tube. Worms were pelleted at 1000 rpm for 5 min and the supernatant was discarded. Worms were washed 3 more times with 5 mLs of M9. After the
supernatant was removed after the final wash, 200 uL Trizol reagent (Ambion 15596026) was added. Worms in Trizol were frozen at -80°C for at least 30 minutes. Worms were then freeze-cracked: alternating 20 seconds in liquid nitrogen followed by 1 minute thaw in 37°C water bath and repeated 10 times. The trizol/worm mixture was transferred to 1.5 mL tube and 200 uL of chloroform was added. The sample was spun at 13,000 rpm at 4°C for 15 min. The aqueous fraction was transferred to a new 1.5 mL tube. An equal volume of 70% isopropanol was added to the aqueous fraction. The Qiagen RNeasy Mini Kit was used for subsequent steps of RNA isolation following manufacturer’s instructions. RNA samples were stored at -80°C.

RNA samples were DNase treated using the RQ1 RNase-Free DNase Kit (Promega) to remove DNA contamination. One Unit per ug RNA of RQ1 RNase-Free DNase, and RQ1 RNase-Free DNase 10X buffer to bring the final concentration of buffer to 1X was added to the RNA. The sample was incubated at 37°C for 30 minutes. RQ1 DNase Stop Solution was added (same volume as 10X buffer) and samples were incubated at 65°C for 10 minutes to inactivate the DNase. DNase treated RNA samples were stored in -80°C.

**Generating cDNA**

The following reagents were added in a 0.65 mL tube: 0.5 ug RNA, 100 ng oligo dT primer, 100 ng random primer, and DEPC H₂O to 12 uL. Samples were incubated at 70°C for 10 minutes. A mix of 1X MMLV RT buffer, 10 mM DTT, 1 mM dNTPs, 10 units RNasin (Promega), and 100 units of MMLV RT (Promega) was added. Samples were incubated at 37°C for 1 hour followed by incubation at 95°C for 5 minutes. cDNA was used for downstream applications or stored at -20°C.
PCR

PCR was performed to obtain annealing conditions for qPCR, optimizing cloning primers, to genotype worms for backcrosses, to create the transgenic *pnpt-1* clone by traditional cloning methods and Gibson assembly, and to produce fragments for sequencing analysis. Phire Hot Start II DNA Polymerase Kit (Thermo Fisher Scientific F122S) and its corresponding 5X buffer was used for all PCR reactions. The list of primers used in PCR reactions are listed below along with their annealing conditions (Table 18 and 19). PCR reaction components are found in Table 1 and cycling parameters were:

1.5’ 94°C – [30” 94°C – 30” annealing temperature – 30” 72°C] x30 – 7’ 72°C.

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Final Concentration</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA</td>
<td>X uL</td>
<td>N/A</td>
</tr>
<tr>
<td>5X Phire II Buffer</td>
<td>1X</td>
<td>Thermo Fisher Scientific - F122S</td>
</tr>
<tr>
<td>Phire Hot Start II DNA</td>
<td>0.05 – 0.08 uL</td>
<td>Thermo Fisher Scientific - F122S</td>
</tr>
<tr>
<td>Polymerase</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primers</td>
<td>0.5 uM</td>
<td>IDT</td>
</tr>
<tr>
<td>dNTPs</td>
<td>2mM</td>
<td>Bioline</td>
</tr>
<tr>
<td>Water (Hyclone Water)</td>
<td>Bring to final reaction volume</td>
<td>GE</td>
</tr>
</tbody>
</table>

**Table 1**: General PCR Protocol Outline: List of reagents and their final concentrations for a PCR protocol * For backcross genotyping PCR protocol, see Table 4 ** For Fusion PCR experiments, see Tables 8, 10, 12, and 14.

qPCR

qPCRs were all performed at 60°C annealing temperatures under standard reaction conditions (Table 2). iTaq Universal SYBR Green Supermix (Bio-Rad) was used with primers at a final concentration of 0.94 uM and diluted cDNA in a 15 uL single reaction. Reactions were run in triplicate in a 96 well plate. Control cDNA was used to determine ideal cDNA dilutions for each experiment (Table 3). Fold changes were determined from cT, dCT, and ddCT values.
when comparing cT values between control and sample of interest. dCT values were determine by: 
\[ dCT = cT_{GOI} - cT_{actin} \] with GOI = gene of interest. ddCT values were determined by:
\[ ddCT = dCT - dCT_{control} \]. Fold change was determined by: 
\[ \text{Fold Change} = 2^{-ddCT} \].

Controls for heat shocked transgenic line strains were non-heat shocked transgenic lines. Control for mutant lines was VC2010. Control for Exon 3 RNAi knockdown was L4440 RNAi knockdown.

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Concentration (Volume Used)</th>
</tr>
</thead>
<tbody>
<tr>
<td>cDNA</td>
<td>See dilutions Table 3 (1 uL)</td>
</tr>
<tr>
<td>Forward/Reverse Primers (6.25 uM stock)</td>
<td>0.933 uM (2.24 uL)</td>
</tr>
<tr>
<td>iTaq Universal SYBR Green Supermix (2X)</td>
<td>1X (7.5 uL)</td>
</tr>
<tr>
<td>ddH20</td>
<td>(4.26 uL)</td>
</tr>
</tbody>
</table>

**Table 2:** Standard qPCR Reaction (15 uL) and reagent components

The qPCR protocol carried out is listed as follows: 
10’ 95°C – [1’ 95°C – 1’ 60°C] x40 – 15” 95°C – 1’ 60°C – 15” 95°C.

<table>
<thead>
<tr>
<th>qPCR Experiments (primers)</th>
<th>cDNA Dilutions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Determining cDNA dilution</td>
<td>1:8</td>
</tr>
<tr>
<td>Measuring exogenous PNPase expression via FLAG Tag (qPCR PNPT1 FWD FLAGTAG / qPCR PNPT1 REV FLAGTAG)</td>
<td>1:16</td>
</tr>
<tr>
<td>Measuring endogenous PNPase expression (wPNPase ex 9 F / wPNPase ex 10 R)</td>
<td>1:16</td>
</tr>
<tr>
<td>Measuring actin expression (w act-2 F / w act-2 R)</td>
<td>1:16</td>
</tr>
<tr>
<td>Measuring polycistronic transcript accumulation (ctb-1 F / COIII R)</td>
<td>1:4</td>
</tr>
<tr>
<td>Measuring total mitochondrial transcripts (COIII Set 7 F/R)</td>
<td>1:16</td>
</tr>
</tbody>
</table>

**Table 3:** qPCR Experiments and cDNA Dilutions
Analysis of *C. elegans* *pnpt-1* Point Mutations

SIFT (Sorting Intolerant from Tolerant) and Polyphen-2 (Polymorphism Phenotyping v2) were used to determine the predicted deleteriousness or benign-ness of missense mutations located on *pnpt-1*. SIFT predicts if amino acid substitutions affect protein function. Both WT and mutant sequences were inputted into the software to determine the predicted degree of deleteriousness of the missense mutation. Polyphen-2 determines the possible impact the amino acid substitution has on protein structure and function. Both WT protein sequence and amino acid change were inputted into the algorithm.

SIFT: [https://sift.bii.a-star.edu.sg/](https://sift.bii.a-star.edu.sg/)

Polyphen-2: [http://genetics.bwh.harvard.edu/pph2/](http://genetics.bwh.harvard.edu/pph2/)

**Determination of Mitochondrial Targeting Peptide (mTP)**

Peptide sequence was analyzed using TargetP V1.0 software to determine if worm PNPase contains an N-terminal mitochondrial targeting sequence or peptide motif. Peptide sequence was inputted into the software and a predicted score determined if a mitochondrial targeting sequence was present or not.

TargetP V1.0: [http://www.cbs.dtu.dk/services/TargetP-1.0/](http://www.cbs.dtu.dk/services/TargetP-1.0/)

**Male *C. elegans* generation**

Ten L4 hermaphrodites were transferred on OP50 seeded 60 mm x 15 mm NGM plate. Worms were heat shocked in 30°C incubator for four hours without parafilm. After heat shock
treatment, the plates were parafilmed and stored at 20°C. F1 male progeny were isolated after 3-4 days post heat shock.

To maintain male nematode stocks, males and L4 hermaphrodites were grouped onto a 30 mm x 15 mm plate in a 1:1 ratio and allowed to mate overnight at 20°C. After 24 hours of mating, hermaphrodites were isolated and singled onto separate OP50 seeded 30 mm x 15 mm NGM plates. Progeny were allowed to grow and develop for four days. Male progeny were isolated and used to maintain male stocks or used for backcrossing.

**Backcrossing**

Mutant hermaphrodites (VC20261, VC40327, VC20284) were backcrossed eight times to wildtype VC2010 N2 variant strain to produce a predicted 99.61% wildtype background. Each backcross event was grouped into 2X backcrossing cycles (Figure 8).

Male VC2010 worms and mutant L4 hermaphrodites were transferred onto a 30 mm x 15 mm seeded plate in a 2:1 ratio of males:hermaphrodites for mating. Worms were allowed to mate overnight at 20°C. Following overnight mating, mated mutant hermaphrodites were singled onto 30mm x 15mm OP50 seeded plates and allowed to lay progeny for 3 days at 20°C. Heterozygous male F1 progeny were isolated and mated with VC2010 L4 hermaphrodites in a 2:1 ratio of males:hermaphrodites on a 30mm x 15mm OP50 seeded plate. Worms were allowed to mate overnight at 20°C. After mating, VC2010 hermaphrodites were singled onto 30mm x 15 mm OP50 seeded plates and allowed to lay progeny for 3 days at 20°C. Adult hermaphrodites of F1 progeny were singled onto 30 mm x 15 mm OP50 seeded NGM plates and allowed to lay eggs overnight. Then, the adult hermaphrodites were genotyped using DNA extracted using the single worm PCR protocol.
Progeny of heterozygous hermaphrodites were allowed to develop for 3 days to adulthood. Adult F2 progeny of F1 heterozygous hermaphrodites were singled on 30 mm x 15 mm OP50 seeded NGM plates and allowed to lay eggs overnight. Adult F2 hermaphrodites were genotyped using the single worm PCR protocol. Extracted DNA was used for genotyping through PCR and digest protocols to determine the genotype of the adult hermaphrodite. Progeny of homozygous mutant F2 adult hermaphrodites were maintained, concluding the 2X Backcross cycle. Four 2X backcross cycles were carried out for each mutant strain to produce the predicted 99.61% wildtype background. Genotyping consisting of a PCR and Digest protocol for each mutant strain are listed below in Table 4.
**Figure 8: 2X Backcrossing Mechanism for Mutant Strains**

<table>
<thead>
<tr>
<th>Step</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>D1</td>
<td>Mutant Hermaphrodite L4 ( \text{mut} / \text{mut} ) (\times) VC2010 Adult Male (+ / +) (\text{Cross 1})</td>
</tr>
<tr>
<td>D2</td>
<td>Singled mated mutant hermaphrodite adult</td>
</tr>
<tr>
<td>D5</td>
<td>F1 Progeny (50% hermaphrodites: 50% males) (\text{mut} / +)</td>
</tr>
<tr>
<td>D6</td>
<td>Singled mated VC2010 hermaphrodite adults</td>
</tr>
<tr>
<td>D10</td>
<td>F1 Progeny of Cross II (50% hermaphrodites: 50% males) (\text{mut} / +) (+ / +) (1:1) Ratio</td>
</tr>
<tr>
<td>D11</td>
<td>Genotyped F1 hermaphrodite adults that were allowed to lay eggs via SWPCR</td>
</tr>
<tr>
<td>D15</td>
<td>F2 progeny from heterozygous F1 hermaphrodite adults (+ / +) (\text{mut} / +) (\text{mut} / \text{mut}) (1:2:1) Ratio</td>
</tr>
<tr>
<td>D16</td>
<td>SWPCR of singled F2 hermaphrodite adults from D15 for genotyping. Singled 8-10 F2 hermaphrodite adults from mutant hermaphrodite adults from SWPCR of D11 (allowed to self-fertilize and lay embryos before genotyping)</td>
</tr>
<tr>
<td>D17</td>
<td>SWPCR of singled F2 hermaphrodite adults from d16. Picked worms from homozygous mutant F2 hermaphrodite adult plates to maintain 2x backcrossed mutant strain</td>
</tr>
</tbody>
</table>

Tirumala 2012
<table>
<thead>
<tr>
<th>Sample</th>
<th>Primers</th>
<th>PCR Product Size</th>
<th>Digest of Mutation Allele</th>
</tr>
</thead>
<tbody>
<tr>
<td>VC40327 (G58E)</td>
<td>PNPT1_Genomic_F / PNPT1_Genomic_R</td>
<td>399 bp</td>
<td>218 bp / 181 bp (BstBI: NEB)</td>
</tr>
<tr>
<td>VC20261 (G74R)</td>
<td>VC20286/61_StyI_F / Reverse 3</td>
<td>152 bp</td>
<td>115 bp / 27 bp (StyI_HF: NEB)</td>
</tr>
<tr>
<td>VC20284 (G74E)</td>
<td>VC20286/61_StyI_F / Reverse 3</td>
<td>152 bp</td>
<td>115 bp / 27 bp (StyI_HF: NEB)</td>
</tr>
</tbody>
</table>

Table 4: PCR and Digest Protocol for Mutation Genotyping

**PCR Purification and Concentration**

PCR products were purified and concentrated prior to sequence submissions, TA Cloning, Gibson Assembly, and Fusion PCR. DNA Clean and Concentrator-5 (D4003), by Zymo Technologies was used to carry out this protocol. PCR products were mixed with DNA Binding Buffer in a 1:5 ratio. The mixture was transferred to the Zymo-Spin Column and spun down for 30 seconds at 13,300 rpm. Flow through was discarded. To the spin column, 200 uL of DNA Wash Buffer was added and was spun down for 30 seconds at 13,300 rpm. The spin column was transferred to a 1.7 mL tube and 6-10 uL of DNA Elution Buffer was added to the column matrix. Column matrix was allowed to incubate at room temperature for one min. The spin column was then spun down at 13,300 rpm for 30 seconds. DNA sample was stored at -20°C for downstream applications.

**Sequencing**

DNA samples were Sanger sequenced using Eurofins Genomics sequencing services. Samples were submitted per company guidelines.
Gel Electrophoresis

Depending on DNA size or application, gel electrophoresis was carried out on either 1% Agarose (Seakem LE) in TAE buffer or 6% acrylamide with 0.5X TBE buffer. One percent agarose gels were used for gel extractions and PCR and digested products greater than 200 bp used. Genotyping of SW-PCR and allele specific PCR, or PCR and digested products less than 200 bp used 6% acrylamide gels.

Gel Extraction

Desired bands of digest products or PCR products were cut out after performing gel electrophoresis in agarose gels. Excised sections were placed in Freeze ‘N Squeeze DNA Gel Extraction spin columns (Bio-Rad) and placed at -20°C for 5 minutes. DNA was eluted at 13,000 x g for 3 minutes at room temperature. Purified DNA samples were stored at -20°C.

TA Cloning

Sections of the wPNPT1 cDNA were PCR amplified and ligated into pBluescript SK+ that had been prepared by cutting with EcoRV and t-tailed. Prior to ligation, PCR samples were purified and concentrated. The amount of PCR product used for ligation followed the formula:

\[ X \text{ ng PCR product} = \frac{(Y \text{ bp PCR product})(50 \text{ ng Vector})}{\text{size in bp of vector}} \]

The ligation reaction used 3x the amount of PCR product for a 3:1 molar ratio between insert to vector respectively. The reaction consisted of PCR product, 50 ng of vector DNA, 400 U of T4 DNA Ligase (NEB M0202S), 1x ligation buffer, and ddH20 to bring the final volume to 10 uL. Ligations were incubated at 14°C overnight.
Transformations were performed using 1 µL of the ligation mix added to 50 µL of thawed supercompetent cells (DH5alpha, DH10b, Able C, Able K, or BL21-DE3) and incubated on ice for 30 minutes. The transformation was heat shocked in a 42°C water bath for 30 seconds followed with a 2 minute incubation on ice. LB media (950 µL) was added to the mixture and incubated in a 37°C shaker for 1 hour. Cells were plated onto LB ampicillin plates (0.1 mg/mL ampicillin) prepped with 20 µL of X-Gal and 0.09 M IPTG. Plates were incubated overnight at 37°C.

**Gibson Assembly**

Individual cloned fragments of *pnpt-1* were assembled using NEBuilder HiFi DNA Assembly (NEB E2621G). Transformed bacteria containing cloned gene fragments were miniprepped and digested to isolate *pnpt-1* fragments. Digest products were extracted after gel electrophoresis through Freeze ‘N Squeeze DNA Gel Extraction Spin Columns (Bio-Rad). Protocol for ligating 2-3 fragments in a single reaction (2-3 Fragment assembly) was followed per manufacturer guidelines.
Individual segments of \textit{pnpt-1}, \textit{hsp-16.41} promoter, and \textit{unc-54} 3′-UTR with overlapping segments were assembled using a series of PCR reactions. The \textit{hsp-16.41} promoter, and \textit{unc-54} 3′-UTR fragments were obtained from PCR reactions using vector pPD48.93 as a template (Table 5). Fragments were assembled either 2 or 3 at a time using Q5 Hot Start High Fidelity DNA Polymerase (NEB M0493S). Reaction mix contained equimolar ratio of individual fragments, Q5 Hot Start High-Fidelity DNA Polymerase, 1X buffer, and ddH2O to volume. Forward and reverse primers were added unless otherwise stated. Thermocycler protocols are listed below. Outline of the multistep PCR Fusion Protocol is displayed above in Figure 9.
**Fusion PCR – Step 1: Amplification of the Individual Fragments**

The primers, templates, annealing temperatures, fragment sizes to produce the five fragments for the PCR fusion are described in Table 5. PCR reactions are show in Table 6. PCR cycles used were 30” 98°C – [10” 98°C – 30” annealing temperature – 40” 72°C] x30 – 2’ 72°C.

<table>
<thead>
<tr>
<th>Fragment Produced</th>
<th>Primers</th>
<th>Template</th>
<th>Annealing Temperature</th>
<th>Product Size</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>hsp-16.41</strong></td>
<td>PNPT1_Foward_A / PNPT1_Reverse_B</td>
<td>vector pPD 49.83</td>
<td>54.7°C</td>
<td>541 bp</td>
</tr>
<tr>
<td><strong>wPNPT1 Fragment 1+2</strong></td>
<td>PNPT1_Foward_C / PNPT1_Reverse_D</td>
<td>wPNPT1 Fragment 1+2 in vector pBSSK</td>
<td>56.8°C</td>
<td>1357 bp</td>
</tr>
<tr>
<td><strong>wPNPT1 Fragment 3 pt 1</strong></td>
<td>wPNPT1_Frag3F / wPNPT1_Frag3_pt1_R</td>
<td>wPNPT1 Fragment 3 part 1 in vector pBSSK</td>
<td>58.4°C</td>
<td>558 bp</td>
</tr>
<tr>
<td><strong>wPNPT1 Fragment 3 pt 2</strong></td>
<td>wPNPT1_Frag3_pt2_F / PNPT1_Reverse_H</td>
<td>wPNPT1 Fragment 3 part 2 in vector pBSSK</td>
<td>58.4°C</td>
<td>511 bp</td>
</tr>
<tr>
<td><strong>unc-54 3` UTR</strong></td>
<td>PNPT1_Foward_I / PNPT1_Reverse_J</td>
<td>vector pPD 49.83</td>
<td>54.7°C</td>
<td>737 bp</td>
</tr>
</tbody>
</table>

*Table 5: Primer, Template, Annealing Temperature, and Product Size of Step 1*

**PCR Protocol**

<table>
<thead>
<tr>
<th>Reagents (stock concentration)</th>
<th>Concentration/Amount Used</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA (2pg/uL stock)</td>
<td>5 uL</td>
<td></td>
</tr>
<tr>
<td>5X Q5 Reaction Buffer</td>
<td>5 uL</td>
<td>NEB M0495S</td>
</tr>
<tr>
<td>Q5 HiFi</td>
<td>0.25 uL</td>
<td>NEB M0493S</td>
</tr>
<tr>
<td>dNTPs (2.5 mM stock)</td>
<td>2 uL (0.2 mM)</td>
<td>Bioline</td>
</tr>
<tr>
<td>Primers (6.25 uM stock)</td>
<td>2 uL each (1 uM)</td>
<td>IDT</td>
</tr>
<tr>
<td>Water (Hyclone)</td>
<td>8.75 uL</td>
<td>GE</td>
</tr>
<tr>
<td>Total Volume</td>
<td>25 ul</td>
<td></td>
</tr>
</tbody>
</table>

*Table 6: Step 1 PCR Protocol for PCR Fusion*
Fusion PCR – Step 2A Combining Fragments

To combine the overlapping fragments, the fragments generated above are denatured and anneal with one another and therefore become both the template and primer for the amplification reaction. In this step the promoter and \( \text{pnpt-1 fragment 1 + 2} \) are combined (fragment 5’) and the three most 3’ fragments are annealed (fragment 3’) (Table 7). PCR Reaction are shown in Table 8. The cycling parameters used for these reactions were 30” 98°C – [10” 98°C – 30” 58°C – 1’ 72°C] x15 – 2’ 72°C.

<table>
<thead>
<tr>
<th>Fragment Produced</th>
<th>Templates</th>
<th>Annealing Temperature</th>
<th>Product Size</th>
<th>Equimolar Concentrations of Fragments used</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fragment 5’</td>
<td>( hsp-16.41 / wPNPT1 ) Fragment 1+2</td>
<td>58°C</td>
<td>1898 bp</td>
<td>0.22 pmols</td>
</tr>
<tr>
<td>Fragment 3’</td>
<td>( wPNPT1 ) Fragment 3 pt1 / ( wPNPT1 ) Fragment 3 pt2 / ( unc-54 3' UTR )</td>
<td>58°C</td>
<td>1809 bp</td>
<td>0.41 pmols</td>
</tr>
</tbody>
</table>

**Table 7**: Template, Annealing Temperature, and Product Size of Step 2A

<table>
<thead>
<tr>
<th>Reagents (Stock concentration)</th>
<th>Concentration/Amount Used</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA</td>
<td>X uL (see table 7)</td>
<td></td>
</tr>
<tr>
<td>5X Q5 Reaction Buffer</td>
<td>20 uL</td>
<td>NEB M0495S</td>
</tr>
<tr>
<td>Q5 HiFi</td>
<td>1 uL</td>
<td>NEB M0493S</td>
</tr>
<tr>
<td>dNTPs (2.5 mM stock)</td>
<td>8 uL (0.2 mM)</td>
<td>Bioline</td>
</tr>
<tr>
<td>Water (Hyclone)</td>
<td>X uL to bring to final volume</td>
<td>GE</td>
</tr>
<tr>
<td>Total reaction volume</td>
<td>100 ul</td>
<td></td>
</tr>
</tbody>
</table>

**Table 8**: Step 2A PCR Protocol for PCR Fusion
Fusion PCR – Step 2B Generating Additional Copies of Fragments

Step 2B consisted of amplification of fused fragments 5’ and 3’ from Step 2A for use for the next fusion step (Tables 9 and 10). The fragments were run on a 1% agarose gel and the fragments were excised and extracted from the gel but not purified cycling parameters were: 30” 98°C – [10” 98°C – 30” annealing temperature – 1’ 72°C] x35 – 2’ 72°C.

<table>
<thead>
<tr>
<th>Fragments Produced</th>
<th>Primers</th>
<th>Template</th>
<th>Annealing Temperature</th>
<th>Product Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fragment 5’</td>
<td>PNPT1_Forward_A / PNPT1_Reverse_D</td>
<td>PCR fusion product Fragments 5’</td>
<td>59.1 / 60.1°C</td>
<td>1898 bp</td>
</tr>
<tr>
<td>Fragment 3’</td>
<td>wPNPT1/Frag3F / PNPT1_Reverse_J</td>
<td>PCR fusion product Fragments 3’</td>
<td>58 / 59.1°C</td>
<td>1809 bp</td>
</tr>
</tbody>
</table>

**Table 9:** Primer, Template, Annealing Temperature, and Product Size of Step 2B

**PCR Protocol**

<table>
<thead>
<tr>
<th>Reagents (Stock concentration)</th>
<th>Concentration/Amount Used</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gel extracted DNA</td>
<td>2 uL</td>
<td></td>
</tr>
<tr>
<td>5X Q5 Reaction Buffer</td>
<td>10 uL</td>
<td>NEB M0495S</td>
</tr>
<tr>
<td>Q5 HiFi</td>
<td>0.5 uL</td>
<td>NEB M0493S</td>
</tr>
<tr>
<td>dNTPs (2.5 mM stock)</td>
<td>4 uL (0.2 mM)</td>
<td>Bioline</td>
</tr>
<tr>
<td>Primers (6.25 uM stock)</td>
<td>4 uL each (1 uM)</td>
<td>IDT</td>
</tr>
<tr>
<td>Water (Hyclone)</td>
<td>25.5 uL</td>
<td>GE</td>
</tr>
<tr>
<td>Total reaction volume</td>
<td>50 ul</td>
<td></td>
</tr>
</tbody>
</table>

**Table 10:** Step 2B PCR Protocol for PCR Fusion
Fusion PCR – Step 3A Combining of Fragments 5’ and 3’

Step 3A consisted of fusing the two large fragments together at the overlapping region again with the annealed fragments serving as primer and template. (Tables 11 and 12). This fragment was called FSN PCR. The PCR cycling parameters were: 30” 98°C – [10” 98°C – 30” 58°C – 2’ 72°C] x15 – 2’ 72°C.

<table>
<thead>
<tr>
<th>Fragment Produced</th>
<th>Templates</th>
<th>Annealing Temperature</th>
<th>Product Size</th>
<th>Equimolar Concentrations of Fragments used</th>
</tr>
</thead>
<tbody>
<tr>
<td>FSN PCR</td>
<td>Fragment 5’/ Fragment 3’</td>
<td>58°C</td>
<td>3707 bp</td>
<td>0.23 pmols</td>
</tr>
</tbody>
</table>

Table 11: Template, Annealing Temperature, and Product Size of Step 3A

PCR Protocol

<table>
<thead>
<tr>
<th>Reagents (Stock concentration)</th>
<th>Concentration/Amount Used</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA</td>
<td>X uL (see table 11)</td>
<td></td>
</tr>
<tr>
<td>5X Q5 Reaction Buffer</td>
<td>20 uL</td>
<td>NEB M0495S</td>
</tr>
<tr>
<td>Q5 HiFi</td>
<td>1 uL</td>
<td>NEB M0493S</td>
</tr>
<tr>
<td>dNTPs (2.5 mM stock)</td>
<td>8 uL (0.2 mM)</td>
<td>Bioline</td>
</tr>
<tr>
<td>Water (Hyclone)</td>
<td>X uL to bring to final volume</td>
<td></td>
</tr>
<tr>
<td>Total reaction volume</td>
<td>100 uL</td>
<td>GE</td>
</tr>
</tbody>
</table>

Table 12: Step 3A PCR Protocol for PCR Fusion

Fusion PCR – Step 3B Generating Additional Copies of the Fused FSN PCR Product

Step 3B consisted of amplification of the FSN PCR product created from Step 3A with nested primers to generate material for worm injections (Tables 13 and 14). PCR protocol ran as follows: 30” 98°C – [10” 98°C – 30” 58°C – 2’ 72°C] x35 – 2’ 72°C.
Table 13: Primer, Template, Annealing Temperature, and Product Size of Step 3B

<table>
<thead>
<tr>
<th>Fragment Produced</th>
<th>Primers</th>
<th>Template</th>
<th>Annealing Temperature</th>
<th>Product Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>FSN PCR</td>
<td>PNPT1_Nested_Forward / PNPT1_Reverse_J</td>
<td>FSN PCR</td>
<td>58°C</td>
<td>3707 bp</td>
</tr>
</tbody>
</table>

Table 14: Step 3B PCR Protocol for PCR Fusion

### PCR Protocol

<table>
<thead>
<tr>
<th>Reagents (Stock concentration)</th>
<th>Concentration/Amount Used</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gel Extracted DNA</td>
<td>3 uL</td>
<td></td>
</tr>
<tr>
<td>5X Q5 Reaction Buffer</td>
<td>10 uL</td>
<td>NEB M0495S</td>
</tr>
<tr>
<td>Q5 HiFi</td>
<td>0.5 uL</td>
<td>NEB M0493S</td>
</tr>
<tr>
<td>dNTPs (2.5 mM stock)</td>
<td>4 uL(0.2 mM)</td>
<td>Bioline</td>
</tr>
<tr>
<td>Primers (6.25 uM stock)</td>
<td>4 uL each (1 uM)</td>
<td>IDT</td>
</tr>
<tr>
<td>Water (Hyclone)</td>
<td>24.5 uL</td>
<td>GE</td>
</tr>
<tr>
<td>Total Reaction Volume</td>
<td>50 ul</td>
<td></td>
</tr>
</tbody>
</table>

pnpt-1 Overexpression Transgenic Line Generation

Microinjections were carried out in young adult hermaphroditic worms into VC2010 or CF3152 strains by Dr. Laura Mathies. Equal amounts (100 ng) of pPD49.83 heat shock vector or FSN PCR (PCR Fusion construct) with pTG96 suf-5::GFP reporter vector were added in a 40 uL reaction mix. Reaction mixes were injected into the gonad arm of young adult hermaphrodites. suf-5::GFP is expressed in the nucleus of somatic cells (Gonzalez-Serricchio et al., 2006) and serves as a co-injection marker.

Transgenic Line Stabilization

Stable transgenic pnpt-1 overexpression VC2010 and CF3152 lines were selected over three generations. For each generation, five to ten GFP expressing hermaphrodites were singled, allowed to mature, and produce eggs and the progeny screened for GFP-expression. Regular
nematode maintenance followed after stabilization of the line and propagated with GFP-expressing animals.

**Overexpression of pnpt-1**

**Method 1**

Ten adult transgenic hermaphrodites expressing GFP were transferred onto medium OP50 seeded plates. Plates were parafilmed and placed in 20°C for 4 days until F1 progeny developed into adults. GFP-expressing nematodes were heat shocked for 2 intervals of 2 hours at 30°C without parafilm with a 30 min break at 20°C between the two heat shock incubations.

**Method 2 (Cancer Models)**

Five adult transgenic hermaphrodites expressing GFP were transferred onto medium *gld-1* RNAi seeded plates. Plates were parafilmed and placed in 20°C for 3 days until F1 progeny developed into L4’s. Plates were heat shocked at 30°C for 2 hours at L4 stage (day 3) and adult stage (day 4) without parafilm. Worm plates were parafilmed and stored at 20°C in between when worms were heat shocked.

**Western Protocol**

**Protein Extractions**

Ten L4 hermaphrodites were transferred to newly seeded NGM plates. Each strain was staged on 5 plates. Progeny were allowed to develop for 4 days at 20°C prior to sample preparation for protein extraction. Plates of nematodes were washed with 1mL of M9 2X and collected in a 15 mL tube. Worms were pelleted at 1,000 rpm for 5 minutes, and the supernatant
was discarded. They were then washed with 5 mLs of fresh M9 buffer 3X. Protease inhibitor cocktail (Sigma 8340) was added in a 1:100 dilution to the 300 uL worm pellet in M9 prior to storage in -80°C. When ready to use worm pellets were thawed and transferred to a 1.7 mL tube and 200 uL of 1X RIPA Buffer (Cell Signaling Technologies #9806) was added to the pellet. Samples were homogenized with a pellet pestle for 2 minutes. They were then placed on ice and gently rocked on a platform shaker for 20 minutes at 4°C. Samples were spun down at 13,300 rpm for 1 minute at 4°C. Supernatant consisting of protein lysates was transferred to a new 1.7 mL tube and kept on ice to measure the concentration using the Lowry Assay.

**Lowry Protein Assay**

The DC Protein Assay (Bio-Rad) was used to measure concentrations of protein lysates. A standard curve was generated using 25 uL BSA standards (0.125 mg/mL, 0.25 mg/mL, 0.5 mg/mL, 1 mg/mL, and 1.4 mg/mL). Protein lysates were diluted with ddH2O to a 1:5 ratio respectively. Final version of Reagent A was prepared by mixing a ratio of 20 uL of Reagent S and 1 mL of Reagent A. Then, final version of reagent A (125 uL) mix and Reagent B (1mL) were added to 25 uL BSA standards and protein samples and samples were inverted 5 times to mix the reagents. Samples were allowed to incubate at room temperature for 15 minutes. BSA standards were read at 750 nm with Beckman Coulter DU 530 DNA/Protein Analyzer Spectrophotometer to produce the standard curve. Protein lysates were read and plotted against the standard curve to determine concentration. Protein lysate samples were aliquoted into 50 ug aliquots and stored at -80°C.
Western Analysis

4X Laemmli Sample Buffer (BioRad – 161-0747) was added to the 50 ug aliquots of protein lysates to bring the final concentration of Sample Buffer to 1X. Samples were boiled at 100°C for 10 minutes followed by pelleting in a 4°C centrifuge at 13,300 rpm for 30 seconds. Supernatents were loaded onto a 7.5% acrylamide resolving gel and a 3.9% acrylamide stacking gel with 10 uL protein standard (Bio-Rad 161-0374). Gel electrophoresis of the protein lysates was carried out at 100 volts for ~2 hours in 1X Tris glycine running buffer. After gel electrophoresis, the stacking gel was discarded and the resolving gel was prepped for transfer. The gel was soaked in 1X transfer buffer for >15 minutes and the protein lysates were transferred onto an Immun-Blot PVDF Membrane for Protein Blotting (Bio-Rad 162-0177). The membrane was prepped prior to the transfer step by soaking in cold methanol for 15 seconds, ddH2O for 2 minutes, then 1X transfer buffer for > 5 minutes.

The transfer step was carried out in 1X transfer buffer with methanol for ~2 hours at 100 volts with an ice block in the gel box. During the 2 hour transfer, the ice block was replaced with a new ice block every 30-40 minutes to keep the gel box cold. After the transfer step, the membrane was removed from the cassette and rinsed in TBS for 10 minutes on a platform shaker at room temperature.

Block / Primary Ab / Secondary Ab

After blocking for 4 hours at room temperature, membranes were rinsed in TBST prior to incubation in primary Ab overnight at 4°C. After primary Ab incubation, membranes were washed in TBST for 3 x 10 minutes on platform shaker prior to secondary Ab application. After secondary Ab incubation, membrane was rinsed 2 x 10 minutes in 5% milk/TBST, rinsed in
TBST, and then washed in TBST for 10 minutes on platform shaker. Parameters for protein
detection of specific antibodies are found in Table 15.

<table>
<thead>
<tr>
<th>Antibody (Experiment)</th>
<th>Block</th>
<th>Primary Ab</th>
<th>Secondary Ab</th>
</tr>
</thead>
<tbody>
<tr>
<td>FLAG Tag (Determining exogenous PNPase protein synthesis)</td>
<td>5% milk/TBST</td>
<td>Monoclonal Anti-FLAG M2 Produced in Mouse (Sigma F1804)</td>
<td>ECL Anti Mouse IgG Horseradish Peroxidase-linked Whole Antibody from Sheep (GE Healthcare NA931V)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1:1000 dilution in 3% milk/TBS</td>
<td>1:10,000 dilution in 5% milk/TBST</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Incubate covered at 4°C overnight</td>
<td>Incubate covered for ~45 min at room temperature on platform shaker</td>
</tr>
<tr>
<td>alpha-Tubulin (loading control)</td>
<td>5% milk/TBST</td>
<td>Anti-alpha-Tubulin (Mouse Monoclonal) (Sigma T6199)</td>
<td>ECL Anti Mouse IgG Horseradish Peroxidase-linked Whole Antibody from Sheep (GE Healthcare NA931V)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1:5000 dilution in 5% milk/TBST</td>
<td>1:10,000 dilution in 5% milk/TBST</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Incubate covered at 4°C overnight</td>
<td>Incubate covered for ~45 min at room temperature on platform shaker</td>
</tr>
</tbody>
</table>

**Table 15: Parameters and Conditions for Antibodies**

Detection

Antibodies were detected through chemiluminescence using Super Signal West Femto
Maximum Sensitivity Substrate Kit (ThermoFisher 34096). Stable Peroxide Solution and
Luminol/Enhancer Solution were mixed in a 1:1 ratio. Membranes were placed in the detection
solution protein-side down and incubated at room temperature for 5 minutes and wrapped in
plastic wrap. Membranes were exposed to X-ray film in a dark room for 5-10 seconds and then
15-30 seconds. Film was developed in a Kodak film developer. Membrane was stored in TBS at 4°C.

**Stripping**

BlotFresh Western Blot Stripping Reagent Ver II (SignaGen Laboratories SL100324) reagent was used to strip membranes prior to reapplication of antibodies. Membranes were placed in 30 mLs of stripping reagent on a platform shaker for 10 minutes at room temperature. Membranes were then washed in TBST for 2X 10 minutes on a platform shaker.

**Immunofluorescence**

**Poly-L-Lysine Slide Preparation**

ColorFrost Plus Slides (Fisher Scientific) were coated with poly-L-Lysine solution (Sigma Aldrich P8920). Poly-L-Lysine solution was diluted 1:1 with ddH2O. Slides were briefly heated at “setting 4” on a hot plate. Ten microliters of solution was added to one heated slide and sandwiched to another heated slide to spread solution. Slides were separated and residual Poly-L-Lysine solution were left to dry at room temperature.

**Edgar Buffer Dissecting Media**

Worms were dissected in 20 uL of Edgar’s Buffer. Buffer consisted of 60 mM NaCl, 32 mM KCl, 3 mM Na₂HPO₄, 2 mM MgCl₂, 2 mM CaCl₂, 5 mM HEPEs, 0.2% glucose pH 7.2. The buffer was filter sterilized and stored in 4°C. Levamisole hydrochloride (Sigma L-9756) at 25 mM concentration was added to Edgars Buffer in a 1:100 ratio prior to dissection. Levamisole served as a paralytic aide for gonad isolation and dissection.
Gonad Isolation

Worms were placed in 20 µL of Edgar’s Buffer with 0.25 mM levamisole on a poly-L-Lysine coated charged slide. A 30 gauge needle was used to open the nematode at the pharynx. Gonad arms and the other internal organs were separated using an eyebrow hair attached to a toothpick. Four dots of vacuum jelly were placed around the dissection media and a 22 mm x 50 mm coverslip (Fisherbrand 12-543-C) was placed on top and slightly depressed until touching the gonad arms and dissected worms. Slide was then placed in liquid nitrogen until all dissections were completed. Upon completion, slides were taken out of liquid nitrogen and freeze-cracked by flicking off the coverslip. Slides were immediately placed in fixation media.

Fixation

Samples were fixed in cold 100% methanol for 4 minutes followed by 4 minutes in cold acetone. Coplin jars contained Type 3A molecular sieves at the bottom of the jar for static dehydration in the fixation media. Fixation media was chilled in wet ice for >15 minutes prior to fixation of dissected samples after freeze cracking.

Following fixation, slides were washed in 1X PBS for 3X for 5 minutes on a platform shaker. Slides were soaked in 50 mL blocking solution in a coplin jar and incubated for ~20 minutes on platform shaker at room temperature. Blocking media consisted of 0.5% BSA, 0.04% azide, and 0.01% Tween-20 in 1X PBS. Reagent was filter sterilized with Steriflip Vacuum-driven Filtration System with 0.22 um membrane (Millipore Sigma – SE1M179M6) and stored at 4°C.
A hydrophobic ring was placed around the sample on the slide with vacuum grease (Dow Corning High Vacuum Grease) and 30 uL primary antibody solution was pipetted on top of the samples.

Samples were incubated with primary antibody for 2 hours in a humid chamber. Humid chamber consisted of a Tupperware container with soaked paper towels lining the bottom and pipette tip wafers serving as an elevated platform for the slides. Antibody buffer solution consisted of 3% BSA, and 1% azide in sterile ddH2O. The reagent was filter sterilized with Steriflip Vacuum-driven Filtration System with 0.22 um membrane (Millipore Sigma – SE1M179M6) and stored at 4°C. Following primary antibody incubation, slides were washed in 1X PBS 3X for 10 min on a platform shaker. Excess 1X PBS was wiped off and 30 uL of secondary antibody solution was pipetted on top of the sample. Samples were incubated in the dark for 2 hours in a humid chamber. Slides were then washed in 1X PBS for 3 X five minutes on a platform shaker and dip washed in ddH2O. Excess solution and hydrophobic grease ring was wiped off using a kin wipe. Five to ten microliters of Fluoro-Gel II Mounting Medium (Electron Microscopy Sciences 17985-50) solution was added to an 18 mm x 18 mm coverslip and coverslip was placed on top of the slide covering the sample. Samples were incubated overnight at 4°C in a slide folder. Antibody information for primary and secondary antibodies and the double staining protocol for FLAG-Tag and K76 are listed in the tables below (Table 16 and 17).
<table>
<thead>
<tr>
<th>Antibody</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>K76 (Developmental Studies Hybridoma Bank)</td>
<td>Anti-PGL-1 for p granules detection</td>
</tr>
<tr>
<td>Rabbit ANTI-FLAG antibody (Sigma F7425)</td>
<td>Polyclonal Flag tag antibody produced in rabbit</td>
</tr>
<tr>
<td>Donkey anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 488 (Thermo Fisher A21202)</td>
<td>Alexa Fluor 488 Donkey anti Mouse</td>
</tr>
<tr>
<td>Donkey anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 568 (Thermo Fisher A10042)</td>
<td>Alexa Fluor 568 Donkey anti Rabbit</td>
</tr>
</tbody>
</table>

**Table 16:** Primary and Secondary antibody list and associated description

<table>
<thead>
<tr>
<th>Protocol</th>
<th>Primary Ab</th>
<th>Secondary Ab</th>
</tr>
</thead>
<tbody>
<tr>
<td>FLAG-Tag and K76</td>
<td>Anti-Flag (Rabbit): 1:3000 dilution</td>
<td>Alexa Fluor 488 Donkey anti Mouse: 1:4000</td>
</tr>
<tr>
<td></td>
<td>K76: Final concentration of 5 ug/mL</td>
<td>Alexa Fluor 568 Donkey anti Rabbit: 1:4000</td>
</tr>
</tbody>
</table>

**Table 17:** Double Staining Protocol for FLAG-Tag and K76

**ROS Assay**

Seven L4 hermaphroditic worms were picked onto RNAi or NGM plates and allowed to mature and lay eggs overnight (up to 24 hours). Adults were removed from the plates the next day and eggs were allowed to mature into adults over the next three days. At least 150 adult hermaphrodites were picked and transferred to a new seeded RNAi or NGM plate. For transgenic lines, adult GFP-expressing hermaphrodites were picked and transferred to a seeded NGM plate. Fifty worms were individually picked and dropped into 0.65 mL tubes containing 250 uL of 1X Sample Buffer from AmplexRed (Life Technologies) kit in triplicate. Worms were spun down at 15,000 rpm for 2 min and 150 uL of supernatant was removed. Worms were washed for a total of 4X. One hundred microliters of 1X Sample Buffer was added to the tube and samples were pulsed on a vortex at maximum speed. Tubes were spun down at 1,000 rpm
for 2 mins and 100 uL of supernatant was removed. Fifty uL of worms/1X Sample Buffer mixture were aliquoted into a 96 well plate.

DMSO and 1 vial of Amplex Red Reagent were allowed to thaw to room temperature while covered from light. Sixty uL of thawed DMSO was added to the Amplex Red Reagent vial to dissolve the contents. This step was done right before preparing the working solution. 1X Sample Buffer was prepared by diluting 4 mL of 5X Sample Buffer with 16 mL of sterile deionized water. Next, 22.7 uL of 3% H₂O₂ was mixed with 977 uL of 1X Sample Buffer to produce 20 mM H₂O₂. The 20 mM H₂O₂ diluted stock was further diluted to 0.01 mM in 1X Sample Buffer to produce the positive control. 1X Sample Buffer served as the negative control. A vial of Horseradish Peroxidase (HRP) was mixed with 1 mL of 1X Sample Buffer. The solution was divided into single use 100 uL aliquots and stored at -20C.

After transferring worm samples and the positive and negative controls to the 96 well plate, a working solution were prepared with 100 uM Amplex Red Reagent and 0.2 U/mL HRP. Fifty microliters of working solution was added to each sample well. The 96 well plate was loosely covered with parafilm and incubated at room temperature and covered from light. Absorbance readings were taken with a BioTek SYNERGY|HTX multimode reader at 540 nm and 620 nm after 30 minute incubation and 2.5 hour incubation. Measured values were analyzed with Gen5 2.06 software.

Final amounts of H₂O₂ was determined by subtracting the 540 nm absorbance values from the 620 nm absorbance values. Triplicates were averaged and significance of differences were determined with a t-test.
<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence</th>
<th>Notes/Desc</th>
</tr>
</thead>
<tbody>
<tr>
<td>wPNPT1_Genomic_F</td>
<td>AGT GGA ATC GAG CTG AAA GC</td>
<td>For sequencing mutant strains</td>
</tr>
<tr>
<td>wPNPT1_Genomic_R</td>
<td>TTG CTG ACG CTG ACG GTC T</td>
<td>For sequencing mutant strains and reverse primer for allele specific PCR for VC20284 and VC20261</td>
</tr>
<tr>
<td>VC40327_G_F</td>
<td>GTA AGA CCG TAG TTG CTT CAT TCC G</td>
<td>Allele specific PCR for genotyping for VC40327 (Mutant allele)</td>
</tr>
<tr>
<td>VC40327_A_F</td>
<td>ACT CTA GTA GAC ATA ACC GTA GTT GCT TCA TCC GA</td>
<td>Allele specific PCR for genotyping for VC40327 (wildtype allele)</td>
</tr>
<tr>
<td>Reverse 2</td>
<td>CCG ATA TTC AAC TTG TAG CGG</td>
<td>Allele specific PCR for genotyping for mutant line VC40327</td>
</tr>
<tr>
<td>VC20284_G_F</td>
<td>GTA AGC AAC GGA AAT CCA AGC AAC G</td>
<td>Allele specific PCR for genotyping for VC20284 (mutant allele)</td>
</tr>
<tr>
<td>VC20284_A_F</td>
<td>AAG CAT CTC AAG CAT CAA CGG AAA TCC AAG CAG GA</td>
<td>Allele specific PCR for genotyping for VC20284 (wildtype allele)</td>
</tr>
<tr>
<td>Reverse 3</td>
<td>GCT GAC GGT CTG GAA TTT TCA G</td>
<td>Allele specific PCR for genotyping for mutant lines VC20284 and VC20261</td>
</tr>
<tr>
<td>VC20261_G_F</td>
<td>CAG AGC AAC GGA AAT CCA AGC ATG</td>
<td>Allele specific PCR for genotyping for VC20261 (mutant allele)</td>
</tr>
<tr>
<td>VC20261_A_F</td>
<td>GAA GCA TCT CAA GCA CAA CGG AAA TCC AAG CGA A</td>
<td>Allele specific PCR for genotyping for VC20261 (wildtype allele)</td>
</tr>
<tr>
<td>VC20284/61_StyI_F</td>
<td>CCG CAG GTA GTA CAA CGG AAA TCC AAC CAA</td>
<td>Allele specific PCR for genotyping for VC20261 and VC20284</td>
</tr>
<tr>
<td>PNPT1_Nested_Forward</td>
<td>GCA GGT CGA CTC TAG AGG ATC AC</td>
<td>Primer for amplifying final Fusion PCR construct and sequencing Fusion PCR construct</td>
</tr>
<tr>
<td>PNPT1_Foward_A</td>
<td>ATG ACC ATG ATT ACG CCA AGC</td>
<td>Fusion PCR: Forward primer for hsp-16.41 promoter</td>
</tr>
<tr>
<td>PNPT1_Reverse_B</td>
<td>GGC AAC CGG GAA AGT TTC ATA GAT ATC AAT ACC ATG GTA CCG TCG</td>
<td>Fusion PCR: Reverse primer for hsp-16.41 promoter</td>
</tr>
<tr>
<td>Primer Name</td>
<td>Sequence</td>
<td>Function</td>
</tr>
<tr>
<td>---------------------------</td>
<td>------------------------</td>
<td>--------------------------------------------------------------------------</td>
</tr>
<tr>
<td>PNPT1_Forward_C</td>
<td>ATG AAA CTT TCC CGG TTG CC</td>
<td>Fusion PCR: Forward primer for \textit{wPNPT1} Fragment 1+2</td>
</tr>
<tr>
<td>PNPT1_Reverse_D</td>
<td>AAA CCT GGC ATG CGA GCC GT</td>
<td>Fusion PCR: Reverse primer for \textit{wPNPT1} Fragment 1+2</td>
</tr>
<tr>
<td>wPNPT1Frag3F</td>
<td>GCC GAT TTC CCA TAC GCC AC</td>
<td>Fusion PCR: Forward primer for \textit{wPNPT1} Fragment 3 part 1</td>
</tr>
<tr>
<td>wPNPT1_Frag3_pt1_R</td>
<td>CTG AGC CAA CAG TGA GAT GTG AGC</td>
<td>Fusion PCR: Reverse primer for \textit{wPNPT1} Fragment 3 part 1</td>
</tr>
<tr>
<td>wPNPT1_Frag3_pt2_F</td>
<td>GCA AGT TGA TCG AGG CGG AG</td>
<td>Fusion PCR: Forward primer for \textit{wPNPT1} Fragment 3 part 2</td>
</tr>
<tr>
<td>PNPT1_Reverse_H</td>
<td>GGA CTT AGA CAG AGG CAC GGT CAC TTG TCG TCA TCG TCT TTG TAG TCC</td>
<td>Fusion PCR: Reverse primer for \textit{wPNPT1} Fragment 3 part 2</td>
</tr>
<tr>
<td>PNPT1_Forward_I</td>
<td>CCG TGC CTC TGA CTT CTA AGT CC</td>
<td>Fusion PCR: Forward primer for \textit{unc-54} 3` UTR</td>
</tr>
<tr>
<td>PNPT1_Reverse_J</td>
<td>CGT ACG GCC GAC TAG TAG GAA</td>
<td>Fusion PCR: Reverse primer for \textit{unc-54} 3` UTR</td>
</tr>
<tr>
<td>FSNPCR_Seq_Check_F</td>
<td>TCA GGA GGA CCC TTG GCT AGC GTC</td>
<td>Sequencing Fusion PCR construct in transgenic strains</td>
</tr>
<tr>
<td>FSNPCR_Seq_Check_R</td>
<td>GAG CAT GTA GGG ATG TTG AAG AGT AAT TGG</td>
<td>Sequencing Fusion PCR construct in transgenic strains</td>
</tr>
<tr>
<td>FSN_Seq_CheckV2_F</td>
<td>CGG TAC CAT GGT ATT GAT ATC TAT GAA AC</td>
<td>Did not use</td>
</tr>
<tr>
<td>FSN_Seq_CheckV2_R</td>
<td>CAG AGG CAC GGT CAC TTG TCG</td>
<td>Did not use</td>
</tr>
<tr>
<td>PNPT1_Missing_F</td>
<td>AGT GCT CCG CGA CAC CAC</td>
<td>Sequencing section of \textit{FSN} PCR construct of \textit{wPNPT1}</td>
</tr>
<tr>
<td>PNPT1_Missing_R</td>
<td>GAC TGA AGC CAT TGA TGA TGA GCC</td>
<td>Sequencing section of \textit{FSN} PCR construct of \textit{wPNPT1}</td>
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<tr>
<td>qPCR PNPT1 FWD FLAGTAG</td>
<td>GCG ATG AGC ACA CTG GAA ATA</td>
<td>qPCR for exogenous \textit{wPNPT1} expression</td>
</tr>
<tr>
<td>qPCR PNPT1 REV FLAGTAG</td>
<td>CGT CAT CGT CTT TGT AGT CCT TT</td>
<td>qPCR for exogenous \textit{wPNPT1} expression</td>
</tr>
<tr>
<td>wPNPT1Frag3 FLAG_R</td>
<td>TCA CTT GTC GTC ATC GTC TTT GTA GTC CTT</td>
<td>Amplification of Fragment 3 of \textit{wPNPT1} for cloning</td>
</tr>
<tr>
<td>Forward</td>
<td>Reverse</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>TTT TTT CTG TGA CGT GGC AGG TG</td>
<td>ACA GTA ACT TGA GCA CAT CAC A</td>
<td>Measuring total mitochondrial transcripts with qPCR</td>
</tr>
<tr>
<td>COIII FWD Set 7</td>
<td>COIII REV Set 7</td>
<td></td>
</tr>
<tr>
<td>AAA TGC TAA GAA CAA ACC ACC AC</td>
<td>GCC TCA GC GGA ATG TTA</td>
<td>Measuring total mitochondrial transcripts with qPCR</td>
</tr>
<tr>
<td>COIII FWD Set 3</td>
<td>COIII REV Set 3</td>
<td></td>
</tr>
<tr>
<td>GCC TCA GC GGA ATG TTA</td>
<td>GTG ATC AAG TCT CTC CCA ACT C</td>
<td>Measuring total mitochondrial transcripts with qPCR</td>
</tr>
<tr>
<td>COIII FWD Set 7</td>
<td>COIII REV Set 7</td>
<td></td>
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<tr>
<td>tbt-1 F</td>
<td>tbt-1 R</td>
<td></td>
</tr>
<tr>
<td>AAG ATG ACT AGG TCA ATG CA</td>
<td>TAT GCA TAC CTT GAA AGT CT</td>
<td>qPCR testing for polycistronic transcripts</td>
</tr>
<tr>
<td>COIII FWD Set 3</td>
<td>COIII REV Set 3</td>
<td></td>
</tr>
<tr>
<td>ATG ATG AAT GAT GTG CTC GA</td>
<td>ATC GTC CTC GAC TCT GGA GAT G</td>
<td>Worm actin</td>
</tr>
<tr>
<td>w act-2 F</td>
<td>w act-2 R</td>
<td></td>
</tr>
<tr>
<td>TCA CGT CCA GCC AAG TCA AG</td>
<td>TCA CGT CCA GCC AAG TCA AG</td>
<td>Worm actin</td>
</tr>
<tr>
<td>wPNPase ex 9 F</td>
<td>wPNPase ex 10 R</td>
<td></td>
</tr>
<tr>
<td>ATG ATG AAT GAT GTG CTC GA</td>
<td>GGA TTC AGG CTT AGG TGG TT</td>
<td>qPCR for total PNPase expression</td>
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Table 19 – Primer Annealing Temperature

<table>
<thead>
<tr>
<th>Primer Pairs</th>
<th>Annealing Temperature</th>
<th>Product Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>wPNPT1 Frag 3 F / wPNPT1 Frag 3 FLAGR</td>
<td>60°C</td>
<td>919 bp</td>
</tr>
<tr>
<td>wPNPT1 Frag 3 F / wPNPT1 Frag 3 pt1 R</td>
<td>60°C</td>
<td>554 bp</td>
</tr>
<tr>
<td>wPNPT1 Frag 3 pt2 F / wPNPT1 Frag 3 FLAG R</td>
<td>60°C</td>
<td>443 bpP</td>
</tr>
<tr>
<td>wPNPT1_Genomic_F / wPNPT1_Genomic_R</td>
<td>58°C</td>
<td>399 bp</td>
</tr>
<tr>
<td>VC40327_G F / Reverse 2</td>
<td>55 / 60 / 63 / 65.1 / 66.6°C</td>
<td>110 bp</td>
</tr>
<tr>
<td>VC40327_A F / Reverse 2</td>
<td>60 / 65.1 / 66.6°C</td>
<td>120 bp</td>
</tr>
<tr>
<td>VC20284 G F / PNPT1 Genomic R</td>
<td>58 / 60°C</td>
<td>154 bp</td>
</tr>
<tr>
<td>VC20284 G F / Reverse 3</td>
<td>58 / 60 / 63 / 66.6 / 68.7°C</td>
<td>146 bp</td>
</tr>
<tr>
<td>VC20284_A F / PNPT1 Genomic R</td>
<td>58 / 60°C</td>
<td>164 bp</td>
</tr>
<tr>
<td>VC20284_A F / Reverse 3</td>
<td>58 / 60 / 63 / 65.1 / 66.6 / 68.7 / 70.3°C</td>
<td>156 bp</td>
</tr>
<tr>
<td>VC20261_G_F / Reverse 3</td>
<td>55 / 63 / 65.1 / 66.6 / 68.7 / 70.3°C</td>
<td>147 bp</td>
</tr>
<tr>
<td>VC20261_A F / Reverse 3</td>
<td>55 / 58 / 60 / 63 / 65.1 / 66.6 / 68.7 / 70.3°C</td>
<td>157 bp</td>
</tr>
<tr>
<td>VC20284/61_StyI_F / Reverse 3</td>
<td>63°C</td>
<td>152 bp</td>
</tr>
<tr>
<td>PNPT1_Forward_A / PNPT1_Reverse_B</td>
<td>54.7°C</td>
<td>541 bp</td>
</tr>
<tr>
<td>PNPT1_Forward_C / PNPT1_Reverse_D</td>
<td>56.8°C</td>
<td>1357 bp</td>
</tr>
<tr>
<td>wPNPT1Frag3F / wPNPT1_Frag3_pt1_R</td>
<td>58.4°C</td>
<td>558 bp</td>
</tr>
<tr>
<td>wPNPT1_Frag3_pt2_F / PNPT1_Reverse_H</td>
<td>58.4 / 59 / 59.7 / 60 / 60.5 / 61°C (3 temperatures used)</td>
<td>511 bp</td>
</tr>
<tr>
<td>PNPT1_Forward_I / PNPT1_Reverse_J</td>
<td>54.7°C</td>
<td>737 bp</td>
</tr>
<tr>
<td>PNPT1_Forward_A / PNPT1_Reverse_D</td>
<td>59.1 / 60.1°C</td>
<td>1898 bp</td>
</tr>
<tr>
<td>Forward E / PNPT1_Reverse_J</td>
<td>58 / 59.1°C</td>
<td>1898 bp</td>
</tr>
<tr>
<td>PNPT1_Nested_Foward / PNPT1_Reverse_J</td>
<td>58°C</td>
<td>3707 bp</td>
</tr>
<tr>
<td>FSNPCR_Seq_Check_F / FSNPCR_Seq_Check_R</td>
<td>60°C</td>
<td>3074 bp</td>
</tr>
<tr>
<td>PNPT1_Missing_F / PNPT1_Missing_R</td>
<td>58.2 / 59 / 60.1 / 61.1 / 61.7°C</td>
<td>427 bp</td>
</tr>
<tr>
<td>qPCR_PNPT1_FWD_FLAGTAG / qPCR_PNPT1_REV_FLAGTAG</td>
<td>60°C</td>
<td>102 bp</td>
</tr>
<tr>
<td>wPNPase ex9 F / wPNPase ex10 R</td>
<td>60°C</td>
<td>161 bp</td>
</tr>
<tr>
<td>COIII Set 3 FWD / COIII Set 3 REV</td>
<td>60°C</td>
<td>283 bp</td>
</tr>
<tr>
<td>COIII set 7 FWD / COIII Set 7 REV</td>
<td>60°C</td>
<td>219 bp</td>
</tr>
<tr>
<td>----------------------------------</td>
<td>--------</td>
<td>--------</td>
</tr>
<tr>
<td>w act-2 F / w act-2 R</td>
<td>60°C</td>
<td>101 bp</td>
</tr>
<tr>
<td>ctb-1 F/COIII R</td>
<td>60°C</td>
<td>231 bp</td>
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</table>

**Bold temperatures used and finalized after optimizing conditions**
Chapter 3: Results

Mutant Studies of PNPase in C. elegans

The goal of the mutant studies was to identify strains that contained mutations located in *pnpt-1* and to determine how their phenotypes compared to the knockdown and overexpression studies.

Missense Mutation Analysis

Mutant strains with homozygous mutations within *pnpt-1* were identified and selected from the *Caenorhabditis* Genetics Center. Mutations were induced through EMS and ENU mutagens on VC2010 (N2 wildtype derivative). Independent clonal lines were allowed to self proliferate, driving individual mutations to homozygosity (Thompson et. al., 2013). Of the 24 strains available, none of them presented nonsense mutations while all of them presented homozygous missense mutations in exons of the gene. All missense mutations were analyzed through SIFT and Polyphen-2 software to determine the predicted effect of the mutation on gene function. A score of 1 or close to 1 in Polyphen-2 predicted the mutation to be deleterious while a score of <0.05 in SIFT predicted the mutations to be deleterious. Among the 24, G58E (VC40327), G74R (VC20261), and G74E (VC20284) were selected (Table 20). G58E and G74R mutations were predicted to be deleterious or damaging while G74E was predicted to be benign. G74E and G74R were selected due to differences in their predicted deleteriousness/benigness while having a missense mutation affecting the same amino acid. G58E was selected as another predicted deleterious mutant strain to serve as a comparison with G74R, also a predicted deleterious mutant strain. All three mutations were found to be located in the first catalytic domain of *pnpt-1*. These specific strains were selected to determine if
predicted disruption of the protein within the first catalytic RNase PH domain would affect normal protein function. One study identified compound heterozygous missense mutations, one in the first RNase PH domain and one in the second RNase PH domain, in the *PNPT1* gene that produced a disease phenotype (Alodaib et al., 2016). The RNase PH domains provide the exoribonuclease function of PNPase. Although there are two RNase PH domains, selecting strains with mutations in the first catalytic domain would determine if the mutation affects total protein function, if the mutation results in reduced PNPase function as a result of disrupting the first RNase PH domain and not the second, and if both RNase PH domains are required for normal protein function.

Since mutant lines were generated through random mutagenesis with EMS and ENU at the *Caenorhabditis* Genetics Center, each line was backcrossed with VC2010, N2 wildtype derivative, for a total of eight times to generate a predicted 99.61% wildtype background to remove unlinked mutations located outside of *pnpt-1*. 
<table>
<thead>
<tr>
<th>Mutant Strains Available for ( \text{pnp1} )</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Wildtype</strong></td>
</tr>
<tr>
<td><strong>Polyphen-2</strong></td>
</tr>
<tr>
<td><strong>AA1</strong></td>
</tr>
<tr>
<td><strong>Prediction</strong></td>
</tr>
<tr>
<td><strong>Score</strong></td>
</tr>
<tr>
<td><strong>SIFT Results</strong></td>
</tr>
<tr>
<td>Median Sequence Conservation</td>
</tr>
<tr>
<td>Sequences Present at this Position</td>
</tr>
<tr>
<td>VCA0327</td>
</tr>
<tr>
<td>Amino acids with probabilities &lt;0.05 are predicted to be deleterious</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Polyphen-2</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>AA1</strong></td>
</tr>
<tr>
<td><strong>Prediction</strong></td>
</tr>
<tr>
<td><strong>Score</strong></td>
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<td><strong>Sensitivity/Specificity</strong></td>
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**Table 20: Mutant strains available for \( \text{pnp1} \).** SIFT predicted the effects of amino acid substitution on protein function. Polyphen-2 determined the impact amino acid substitution has on protein function and structure. The mutants highlighted in green were used for this study. A score of 1 from Polyphen-2 and a score of 0 or close to 0 identified the missense mutation as predicted to be deleterious. Strains with mutations located in the first catalytic RNase PH domain include gk74448, gk576099, gk327843, gk165911, gk165912, gk165915, and gk388087. Strains with mutations located in the second catalytic RNase PH domain include gk165917, gk347759, gk360199, gk364277, gk379909, gk715189, gk779183, and gk803047. Strains with mutations located in the S1 domain include otn17845, and otn7416.
Endogenous PNPase Expression of Mutants

In both laboratory models and human studies, mutations located in the gene coding (exon) regions of *pnpt-1* could reduce normal protein function and or prevent trimeric formation of the protein (Vedrenne et al., 2012; Golzarroshan et al., 2018). Specifically, mutations in the second catalytic RNase PH domain resulted in lower RNA binding and degradation activities (Golzarroshan et al., 2018). Depending on the severity of the mutation, missense mutations in *pnpt-1* have been shown to contribute to a disease phenotype in a human study setting. Although the mutations were not located in the regulatory regions of the gene, PNPase expression was still quantified using qRT-PCR with primers that spanned the exon 9 and exon 10 junction to determine if PNPase expression was altered with the selected mutations. In a comparison to VC2010, the N2 wildtype derivative, strains G74R, G58E, and G74E showed no significant difference in PNPase expression (p=0.07) (Figure 10).

![PNPase Expression in Mutant Strains](image)

**Figure 10: PNPase Expression in Mutant Strains.** PNPase expression in mutant lines were not significantly different than wildtype (p=0.07) (n=3)
**Polycistronic Transcript Accumulation**

The genome of the mitochondria is a circular piece of DNA that is transcribed as a single polycistronic transcript where a single mRNA can code for several proteins upon RNA processing into individual RNA molecules (Figure 11). One of the enzymes responsible for processing polycistronic transcripts in the mitochondrion and excising intervening tRNAs is RNase P. Since RNase P is one of the RNA molecules transported by PNPase, the goal of this experiment was to determine if mutations in PNPase affected accumulation and processing of polycistronic transcripts as a result of disrupted transport of RNase P RNA into the mitochondria. Previous knockdown studies determined that a decrease in PNPase expression resulted in an increase in polycistronic transcript accumulation (Laura 2015). To determine the degree of polycistronic transcripts accumulation, total mitochondrial and polycistronic transcripts were quantified. This data would allow determination of percentage of polycistronic transcripts among total mitochondrial transcripts produced. The amount of polycistronic transcripts were quantified using qRT-PCR with primers at the region between *ctb-1* and *COIII*. When compared to VC2010 wildtype worms, polycistronic transcript accumulation was not significantly different in the mutants (p=0.31). Samples G58E and G74E each produced one outlier among the three trials that contributed to the high error bars and high variance. G74R presented a 0.59x fold change, G58E presented an 8.72x fold change, and G74E presented a 10.21x fold change (Figure 12).
Figure 11: Mitochondrial DNA Gene Map of C. Elegans. Locations of the protein coding genes (gray arrows), tRNAs (circles), and rRNAs (black arrows) coded by the mitochondrial genome. Genes are transcribed as a polycistronic transcript and later processed to single RNA species. (From wormbook.com)

Figure 12: Polycistronic Transcript Accumulation. Polycistronic transcript accumulation were not significantly different in the mutants when compared to VC2010 wildtype(p = 0.31)(n=3)
Total mitochondrial transcripts, both processed and polycistronic, were quantified using qRT-PCR by measuring the expression of COIII. When comparing to VC2010 wildtype, G58E show a trend of a large increase while G74E presented a trend of a modest increase for total mitochondrial transcripts (Figure 13). Due to technical errors with the qRT-PCR protocol, the experiment was only attempted once and no statistical analysis was carried out.

![Total Mitochondrial Transcripts](image)

**Figure 13: Total Mitochondrial Transcripts measured by qRT-PCR.** Mutant strains presented variations in amount of total mitochondrial transcripts compared to VC2010. No statistical analysis could be carried since this was a single run (n=1) and all results are presented as trends.
ROS Assay

ROS are produced as a by-product of cellular respiration from the respiratory chain complex, specifically at Complex I and III. Large accumulation of ROS can damage proteins, lipids, and DNA while low accumulation of ROS can serve as activators for signaling pathways. Previous work on knockdown of PNPase expression through RNAi concluded that decreased PNPase expression correlated with an increase in ROS production (Lambert 2015). The purpose of this experiment was to determine if mutations in PNPase altered ROS production and when compared to the VC2010 wildtype.

ROS production was quantified using the Amplex Red kit that served as an assay for quantifying peroxidase activity when $H_2O_2$, a reactive oxygen species, was present. Specifically, in the presence of a peroxidase, the Amplex Red Reagent reacts with $H_2O_2$ in a 1:1 ratio to produce resorufin, a red fluorescent oxidation product. Resorufin production could be quantified by absorbance since the product had an excitation and emission spectrum of 571 nm to 585 nm. As result, the absorbance values produced are listed as followed: 0.018 for VC2010, 0.021 for G74R, 0.022 for G58E, and 0.025 for G74E. ROS production in the 3 mutants was not significantly different than wildtype (p=0.61) (Figure 14).
Figure 14: ROS production in Mutant Strains. ROS production analysis in mutant strains showed no difference when compared to the VC2010 wildtype (p = 0.61)(n=3).

Overexpression Studies

The goals of the overexpression studies were to generate an overexpression animal model in *C. elegans*, observe the phenotypic effects PNPase overexpression, and compare it to the results from the knockdown and mutant studies. Initial attempts to clone the *pnpt-1* gene into the pPD49.83 vector failed. Multiple variations and protocol optimizations to clone *pnpt-1* included using different competent cells and adjusting transformed bacteria growth conditions, but none worked. It was concluded that a 3’ fragment of *pnpt-1* was toxic to the bacterial competent cells. As a result, Fusion PCR allowed for ligation of the individual fragments of *pnpt-1*, and the *hsp-16.41* promoter and *unc-54* 3’ UTR from the pPD49.83 vector without propagation in *E. coli*. Ligation of the individual components occurred at overlapping regions of each fragment to produce a linear construct for heat shocked induced expression of exogenous PNPase.
Generation of Transgenic Lines

Transgenic strains of *C. elegans* were generated to create an overexpression model of PNPase. Upon completion of the PCR Fusion construct of *pnpt-1*, equimolar amounts of construct and pTG96 plasmid or empty vector control pPD49.83 and pTG96 plasmid were microinjected in the gonad arms of VC2010 and CF3152 strains. Transgenic VC2010 lines would serve as a comparison to both wildtype and mutants lines. CF3152 strains were microinjected to be later used for the cancer model studies through knockdown of *gld-1* with RNAi. pTG96 is a *sur-5*:GFP injection marker that allows GFP expression in somatic cells, serving as a tool to determine the success of transgenic strain synthesis. Microinjected worms were first selected for GFP expression and singled to generate individual lines. Individualized lines were allowed to propagate for at least three generations to obtain stable lines. Lines that maintained GFP expression were selected for maintenance of transgenic lines and for subsequent experiments (Figure 15, Table 21). As a result, progeny of the microinjected worms were successfully transgenic and stable with consistent expression of GFP.
**Figure 15:** Adult and young adult transgenic *C. elegans*. GFP expression is visible throughout the body length of the nematode due to the *sur*-5 promoter in pTG96 in VC2010 and CF3152 animals.

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<th>FSN PCR/TG96</th>
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<td>VC2010</td>
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<td>CF3152 FSN 3B5</td>
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**Table 21: pnpt-1 Transgenic lines.** FSN represented transgenic lines with the Fusion PCR construct for exogenous PNPase expression. pPD represented transgenic lines with the pPD 49.83 empty vector control. Strains in bold were used in subsequent assays.

**Determination of Heat Shock Protocols**

The Fusion PCR construct contains a *hsp-16.41* promoter that can activate transcription upon heat stress (Stringham et. al., 1991). Previous studies have identified the optimal temperature range to activate transcription with adequate response was 29°C to 31°C. Heat shock temperatures could be as low as 25°C but with only ~1% of cells showing activation of transgene
expression (Stringham et al., 1991). For the protocol, researchers have determined that two two-hour exposures with a 30 minute recovery period at 20°C was ideal.

Our goal was to identify a temperature and protocol that would activate the \textit{hsp-16.41} promoter for ectopic expression of \textit{pnpt-1} without killing the nematode. The experiment compared two protocols: heat shock at 30°C for two 2 hour intervals with a 30 minute recovery period at 20°C and heat shock at 30°C for one 2 hour interval (Stringham et al., 1992). We used qRT-PCR to measure FLAG TAG expression for exogenous PNPase to compare the two protocols. Both treatment conditions resulted in induction of exogenous PNPase expression. In both VC2010 FSN 9C3 and CF3152 FSN 3B3 transgenic lines with the Fusion PCR construct, there was a larger increasing trend for exogenous expression of PNPase with the two 2 hour interval at 30°C compared to one 2 hour interval at 30°C. When compared to the values from one 2 hour heat shock interval, VC2010 FSN 9C3 resulted in a 318.6% increase and CF3152 FSN 3B3 resulted in a 90.6% increase with 2 intervals of heat shock (Figure 16). This was attempted once for protocol determination only, thus no statistical analysis can be provided. Moving forward, the heat shock protocol for subsequent overexpression experiments followed the two 2 hour interval at 30°C.
Figure 16: Exogenous PNPase Expression Under Two Heat Shock Protocols. Transgenic lines VC2010 FSN 9C3 and CF3152 FSN 3B3 produced higher expression of exogenous PNPase with two two-hour intervals at 30°C when compared to one two-hour heat shock interval at 30°C. With two two-hour intervals, VC2010 resulted in a 166x fold change and CF3152 resulted in a 112x fold change compared to their non-heat shocked counterparts (n=1).

Exogenous *pnpt-1* Expression

While the transgenic strains visibly expressed the co-injection GFP maker under a fluorescent microscope, qRT-PCR experiments measuring FLAG TAG expression for ectopic PNPase would allow confirmation of successful creation of an overexpression model. Among the three heat shocked VC2010 FSN PCR transgenic lines and one heat shocked CF3152 FSN PCR transgenic line, all four presented an increase for FLAG Tag expression for exogenous PNPase in comparison to their non-heat shocked transgenic controls. Heat shocked VC2010 FSN 5A1 had 130x higher expression, heat shocked VC2010 FSN 9C3 had 130x higher
expression, heat shocked VC2010 FSN 3D1 had a 348x higher expression, and heat shocked CF3152 FSN 3B3 had 161x higher expression. Statistical analysis concluded that VC2010 FSN 5A1 (p = 0.045), VC2010 FSN 9C3 (p = 0.006), and CF3152 FSN 3B3 (p = 0.012) strains produced a significant increase in expression for exogenous PNPase while VC2010 FSN 3D1 (p = 0.192) was not significant (Figure 17). In subsequent experiments and assays, VC2010 FSN 9C3 and CF3152 FSN 3B3 were selected as the two transgenic lines to use for overexpression studies.

Transgenic lines for the empty vector control (pPD49.83) showed no fold change in FLAG Tag expression when heat shocked lines were compared with their non-heat shocked controls (data not shown). Transgenic lines VC2010 pPD 1D3 and CF3152 pPD 3A1 were used as controls for future experiments and assays.

**Figure 17: FLAG Tag Expression in Transgenic Lines.** Significant increase in fold change was determined for exogenous PNPase expression in heat shocked VC2010 FSN 5A1 (p = 0.045), VC2010 FSN 9C3 (p = 0.006), and CF3152 FSN 3B3 (0.012) when compared to their non-heat shocked counterparts. Increase in FLAG Tag expression in heat shocked VC2010 FSN 3D1 was not significant (p = 0.192) (n=3)
Total (Endogenous and Exogenous) \textit{pnpt-1} Expression

The next step was to determine the difference in total PNPase expression between FSN and pPD transgenic lines that were heat shocked and not heat shocked. The goal was to identify how much exogenous PNPase contributed to total PNPase expression. qRT-PCR was used to measure total PNPase, both endogenous and exogenous, using primers spanning the junction of exon 9 and exon 10 of \textit{pnpt-1}. All heat shocked lines that contained the Fusion PCR construct produced significantly more total \textit{pnpt-1} expression than non-heat shocked controls. Heat shocked VC2010 FSN 5A1 had 57x increase, heat shocked VC2010 FSN 9C3 had 100x increase, heat shocked VC2010 FSN 3D1 had 128x increase, and heat shocked CF3152 FSN 3B3 had 127x increase in total PNPase expression. Heat shocked FSN transgenic lines produced a significant increase in fold change for total PNPase expression compared to non-heat shocked FSN transgenic lines (p = 0.0004). Heat shocked CF2010 pPD 4C2 had 1.1x increase, heat shocked VC2010 pPD 1D3 had 1.61x increase, heat shocked VC2010 pPD 13B3 had 1.11x increase, heat shocked CF3152 pPD 3A1 had 1.27x increase, and heat shocked CF3152 pPD 5A1 had 1.01x increase in total PNPase expression. Heat shocked pPD transgenic lines fold changes were not statistically significant when compared to their non-heat shocked pPD transgenic lines (p = 0.79) (n=3) (Figure 18).
Figure 18: Total \textit{pnpt-1} Expression in Heat Shocked Transgenic lines. All four transgenic lines with FSN PCR constructs showed significant increase in total PNPase expression when heat shocked (p = 0.0004). pPD transgenic lines showed no significant difference in \textit{pnpt-1} expression when heat shocked (p = 0.79) (n=3)

Exogenous PNPase Expression

Following qRT-PCR analysis for FLAG Tag expression in transgenic lines, the next step was to determine if heat shock activation for exogenous PNPase expression resulted in exogenous PNPase protein formation. Western blot analysis was carried out using FLAG TAG M2 antibody to detect exogenous PNPase since the FSN construct contained a triple FLAG Tag at the C-terminus. Results indicated that only FSN transgenic lines that were heat shocked produced flag-tagged PNPase (Figure 19).
Figure 19: Exogenous PNPase Expression. Heat shocked FSN Transgenic lines resulted in exogenous PNPase protein production that was detected through a FLAG Tag antibody. The vector control pPD did not show any exogenous PNPase expression (n=3).

Polycistronic Transcript Accumulation

To determine if increased expression of PNPase influenced polycistronic transcript accumulation in the mitochondria, qRT-PCR was carried out measuring polycistronic transcript accumulation using primers at the junction between the *ctb-1* and COIII genes. Heat shocked VC2010 FSN 9C3 produced an 165.5% (2.66x fold change) increase in polycistronic transcript accumulation while heat shocked CF3152 FSN 3B3 resulted in a 75.6% (0.24x fold change) decrease in polycistronic transcript accumulation when compared to their non-heat shocked counterparts. Statistical analysis determined the results for heat shocked VC2010 FSN 9C3 (p = 0.0006) and CF3152 FSN 3B3 (P < 0.0001) were significant. Heat shocked VC2010 pPD 1D3 resulted in an 8% increase in polycistronic transcript accumulation (1.08x fold change) and heat shocked CF3152 pPD 3A1 resulted in a 192% increase (2.92x fold change) but was not significantly different than its non-heat shocked counterpart. Heat shocked empty vector pPD transgenic polycistronic transcript accumulation were not significantly different than their non-
heat shock counterparts for VC2010 pPD 1D3 (p = 0.7511) and CF3152 pPD 3A1 (p = 0.08) (n=3) (Figure 20).

Figure 20: Polycistronic transcript accumulation in Heat Shocked Transgenic Lines. Heat shocked VC2010 FSN 9C3 presented significant increase in polycistronic transcript accumulation (p = 0.0006). Heat shocked CF3152 FSN 3B3 presented significant decrease in polycistronic transcript accumulation (p < 0.0001). Heat shocked empty vector controls resulted in no significant change for polycistronic transcript accumulation. (n=3)

Total Mitochondrial Transcript

Total mitochondrial transcripts were quantified using qRT-PCR by measuring the expression of COIII. Heat shocked transgenic lines were compared with their non-heat shocked counterparts. Heat shocked VC2010 FSN 9C3 produced a large trend for increased total mitochondrial transcript while CF3152 FSN 3B3, VC2010 pPD 1D3, and CF3152 pPD 3A1
resulted in small trends for increased transcripts (Figure 21). Due to technical errors with this protocol, the experiment was attempted only once so no statistical analysis can be provided.

**Figure 21: Total Mitochondrial Transcripts in Heat Shocked Transgenic Lines.** Increased trends in total mitochondrial transcripts was present in heat shocked transgenic strains. (n=1)

**ROS Assay**

Previous work in cell lines determined that overexpression of PNPase resulted in an increase of ROS production as a result of induced senescence (Sarkar et al., 2004). The purpose of this experiment was to determine if overexpression of PNPase as a result of exogenous PNPase activation could change ROS production in heat shocked transgenic nematodes. ROS production was quantified using the Amplex Red kit that served as an assay for quantifying peroxidase activity when $H_2O_2$, a reactive oxygen specie, was present. In a comparison between heat shocked and non-heat shocked strains, most of the strains saw no varying trends in
absorbance changes when strains were heat shocked with the exception of VC2010 FSN 9C3. Heat shocked VC2010 FSN 9C3 saw a 50.4% decrease in ROS production, heat shocked CF3152 FSN 3B3 saw a 2.6% increase in ROS production, heat shocked VC2010 pPD 1D3 resulted in an 8.77% decrease in ROS production, and heat shocked CF3152 pPD 3A1 resulted in a 13.89% increase in ROS production. Statistical analysis for VC2010 FSN 9C3 (p = 0.19), CF3152 FSN 3B3 (p = 0.95), VC2010 pPD 1D3 (p = 0.55), and CF3152 pPD 3A1 (p = 0.82) determined the results to be not significant (n=3) (Figure 22).

![ROS Assay](image)

**Figure 22: ROS Production in Heat Shocked Transgenic Lines.** Results between heat shocked and non-heat shocked transgenic lines varied little across different transgenic strains. Statistical analysis revealed no significant differences between treatment groups (VC2010 FSN 9C3 (p = 0.19), CF3152 FSN 3B3 (p = 0.95), VC2010 pPD 1D3 (p = 0.55), CF3152 pPD 3A1 (p = 0.82) (n=3).
Cancer Model Studies

The goal of the cancer model studies was to combine the PNPase overexpression model in a *gld-1* knockdown cancer model nematode and to determine if PNPase overexpression differentiates cells into non-germline cells in the nematode germline.

Heat Shock Protocol Optimization for Cancer Models

Protocol to heat shock transgenic worms was optimized for both temperature and duration in order to produce exogenous PNPase expression over a 2-day period during knockdown of *gld-1* without killing the nematode. qRT-PCR measuring FLAG Tag expression for exogenous PNPase in the CF3152 FSN 3B3 transgenic strain identified a suitable heat shock protocol produced the most exogenous PNPase. Adult nematodes were heat shocked at 26.5°C or 27.2°C for a 24-hour period or heat shocked at 30°C for two 2 hour intervals with a 30 minute break at 20°C in between. Heat shock protocols at 26.5°C or 27.2°C resulted in an increased fold change by 339X and 423X, respectively, but when the nematodes were heat shocked at 30°C produced the highest fold change trend at 4564X (Figure 23). Statistical analysis could not be conducted since this was attempted only once for protocol optimization. The final heat shock protocol for subsequent *gld-1* cancer experiments consisted of two 2 hour intervals at 30°C separated by a 24 hour period between each interval.
Figure 23: Exogenous PNPase Expression in Heat Shocked CF3152 FSN 3B3 strain. When animals were heat shocked at 30°C there was an 4564X increase compared to their non-heat shocked counterpart. Heat shocked the animals at 26.5°C or 27.2°C produced a smaller increase. (n=1)

Oocyte Formation

*gld-1* knockdown in *C. elegans* produces a cancer phenotype that results in massive mitotic proliferation throughout the germline and ablation of oocyte production (Kirienko et al., 2014). Overexpression of PNPase induces a senescent-like phenotype that arrest cells in G1 phase of the cell cycle and inhibits growth in HO-1 cell lines (Sarkar et al., 2003). The goal was to determine if overexpression of PNPase prevents the *gld-1* overproliferation phenotype through senescent inducing mechanisms. *gld-1* knockdown nematode strains were heat shocked, oocyte production quantified and compared to their non-heat shocked control. There was no difference in oocyte production with PNPase overexpression. Though heat shocked CF3152 FSN 3B3 resulted in a 8.47 increase in oocyte producing worms (p = 0.16), heat shocked CF3152 pPD
3A1 resulted in a 25.19% increase (p = 0.16), and heat shocked CF3152 resulted in a 7.96% decrease (p = 0.76), though there was no statistical difference (Figure 24).

**Figure 24: Oocyte Production in Heat Shocked Animals.** There was no difference in oocyte production in both FSN, pPD transgenic lines or CF3152 controls when. Results were not statistically significant (CF3152: p = 0.76, CF3152 pPD 3A1: p = 0.16, CF3152 FSN 3B3: p = 0.16) (n=3)

**P-Granule Immunohistochemistry**

The regulatory regions of the Fusion PCR construct consisted of the *hsp-16.41* promoter and the *unc-54* 3’ UTR that allow expression in most somatic cells and within late pachytene of the nematode germline. Knockdown of *gld-1* not only creates a cancer model with mass mitotic proliferation throughout the germline but also development of somatic cells such as muscle, neuronal, and intestinal cells within the gonad (Ciosk et. al., 2006). K76, antibody to PGL-1,
would detect p-granules through immunofluorescence, serving as a marker specific for germline cells in the nematode gonad. Immunofluorescence of the germline would determine if overexpression of exogenous PNPase would inhibit or reduce severity of *gld-1* cancer phenotype and increase the number of non-germline cells within the gonad through induction of senescence or differentiation.

Strains used for immunofluorescence consisted of CF3152 with and without heat shock, CF3152 pPD 3A1, and CF3152 FSN 3B3 with the same treatment. All gonads were co-stained with K76 and FLAG Tag antibodies to detect germline specific cells and exogenous PNPase, respectively. All six strains presented staining for K76, a marker that is targeting guanyl-specific ribonuclease *pgl-1* that is found exclusively in germline cells, from the distal to proximal end of the germline (Figure 25-30), and no oocyte production with the exception of one case in CF3152 FSN 3B3 no heat shock (Figure 29). Exogenous PNPase expression was only observed in CF3152 FSN 3B3 when it was heat shocked (Figure 30). Gonads in this heat shocked strain displayed cells that presented FLAG Tag expression with no K76 colocalization. No staining for K76 in those cells determined that those are non-germline cells. It was also determined that heat shock treatment and empty vector pPD49.83 had no effect on the tumorous phenotype of the *gld-1* cancer model.
Figure 25: Oocyte Development of gld-1 KD CF3152 Control Worms. Gonad of CF3152 no heat shock at 20x magnification shows no oocyte formation in the proximal gonad (blue and purple boxes_ with K76 staining present from distal to proximal in the germline (red through purple boxes). No FLAG Tag staining indicates no exogenous PNPase expression (red through purple boxes). Blue is DAPI staining, green is K76 and red is FLAG-tag PNPase (20x mag) (n=1).
Figure 26: Oocyte Development of *gld-1* KD CF3152 with Heat Shock. Gonad of CF3152 with heat shock shows no oocyte formation in the proximal gonad (blue and purple boxes) with K76 staining present from distal to proximal ends of the germline (red through purple boxes). FLAG Tag PNPase staining was not detected indicating no exogenous PNPase expression (red through purple boxes). Blue is DAPI staining, green is K76 and red is FLAG-tag PNPase (40x mag) (n=3)
Figure 27: Oocyte Development of *gld-1 KD CF3152 pPD 3A1 no Heat Shock*. Gonad of CF3152 pPD 3A1 with no heat shock shows no oocyte formation (blue and purple boxes) in the proximal gonad with K76 staining present from distal to proximal ends of the germline (red through purple boxes). No FLAG Tag detection indicates no exogenous PNPase expression (red through purple boxes). Blue is DAPI staining, green is K76 and red is FLAG-tag PNPase (40x mag) (n=1)
Figure 28: Oocyte Development gld-1 KD CF3152 pPD 3A1 with Heat Shock. Gonad of CF3152 pPD 3A1 with heat shock shows a large cellular body with chromatin morphology suggesting oocyte formation or meiotic division in the middle of the germline (light blue box). K76 staining is present from distal to proximal ends of the germline with the exception of the large body singular cell (red, yellow, green, purple boxes). No FLAG Tag detected indicating no exogenous PNPase expression (red through purple boxes). Blue is DAPI staining, green is K76 and red is FLAG-tag PNPase (40x mag)(n=2)
Figure 29: Gonad of gld-1 KD CF3152 FSN 3B3 Without Heat Shock. While oocyte development appears to be replaced with mitotic divisions in the proximal region of gonad due to gld-1 knockdown (red box), the proximal gonad contained a large mononuclear structure with no K76 staining (blue and purple boxes). No FLAG Tag staining present throughout the germline (red through purple boxes). Blue is DAPI staining, green is K76 and red is FLAG-tag PNPass (20x mag) (n=4)
Figure 30: Gonad of gld-1 KD CF3152 FSN 3B3 with Heat Shock. FLAG Tag staining (red) was observed to be present in the germline (yellow and purple boxes). Cells within the region of FLAG Tag staining did not present K76 staining (green) (yellow and purple boxes). At the proximal end of the gonad (purple box), FLAG Tag staining appeared to localize around DAPI stained DNA (blue). No oocyte formation was present in the germline (red through purple boxes). K76 staining was present from the distal to proximal ends (orange through purple boxes) with the exception of cells at the very distal tip of the germline (red box) and cells colocalized with FLAG Tag expression for ectopic PNPase expression (yellow and purple boxes) (whole germline = 20x mag | cut outs = 100x mag) (n=1)
Chapter 4: Discussion

Phenotypic characterization of overexpression and knockout of PNPase has been restricted to models such as cell lines or organ specific models, limiting the conclusions that can be drawn with regard to the organism as a whole. Additionally, no whole animal model for PNPase overexpression has been produced. Formation of an animal model for overexpression and characterization of PNPase mutants, with the already synthesized knockdown model in *C. elegans* (Lambert 2015), would allow direct comparison of the phenotypic effects of reduced and increased PNPase levels on both a gross and molecular scale. Analysis of PNPase mutants would determine if the selected missense mutations impair protein function and contribute to a disease phenotype. Having these models would allow correlation of previous PNPase findings in ROS, and mitochondrial homeostasis mechanisms and how they present in an animal model setting. A model for PNPase overexpression would also allow investigation into associated senescent inducing and differentiation mechanisms in a whole animal cancer model.

In this study, the first step was to create an overexpression and mutant models for PNPase in *C. elegans*. Then, ROS production and mitochondrial homeostasis through polycistronic and total mitochondrial transcript accumulation were measured in PNPase knockdown, mutants, and overexpression. Qualitative analysis through immunofluorescence was utilized to observe the influence of ectopic PNPase overexpression on germline tumor cells in a cancer model setting.

Knockdown Studies

RNAi mechanisms to knockdown gene expression in *C. elegans* produced a decrease in PNPase expression but were inconsistent with each experiment when attempting to confirm previous studies (see Appendix). When RNAi mechanisms did work, the results matched
previous reports that noted a 63% reduction in mRNA expression (Lambert 2015). Inconsistencies in achieving knockdown expression could have been attributed to not using the freshest reagents, plates, and bacteria. However, even when using the freshest reagents and stocks, knockdown of PNPase was only achieved 3 out of 6 times. Although inconsistent with PNPase expression, knockdown of dpy-10 to produce the dumpy phenotype as a control was consistently seen with each RNAi attempt. This suggests that the RNAi protocol is capable of knocking down gene expression for dpy-10. Issues for Exon 3 knockdown could be inefficiency of the IPTG inducer for activated transcription and production of dsRNA of Exon 3 or growth of antibiotic resistance bacteria in the same RNAi culture, reducing the knockdown effect. A previous report had indicated that despite using the most optimal RNAi technique for knockdown expression (consumption of bacteria expressing dsRNA with 1mM IPTG inducer in the plate media instead of bacteria culture), phenotypic variability of RNAi penetrance was present for different gene targets (Kamath et. al., 2001). This could explain why dumpy phenotype with knockdown of dpy-10 was consistent while knockdown of PNPase was not.

ROS were quantified as a means to determine PNPase effects on mitochondrial homeostasis. In addition to ROS being produced as a byproduct of cellular respiration, aging cells are associated with production of high levels of ROS from the mitochondria that cause damage to protein, lipids, and DNA (Davali et. al., 2016). ROS mediates senescence through accumulation of damage and contributes to the induction of replicative senescence, limiting the number of divisions a cell can undertake (Chen et. al., 1998; Passos et. al., 2010). In addition to onset of senescence, ROS also functions in a positive feedback loop mechanism to maintain the senescent phenotype (Takahashi et. al., 2006). These findings suggest ROS plays a large role in senescence.
Knockdown of PNPase resulted in an increased trend in ROS production (appendix) in agreement with previous knockdown studies (Lambert 2015). Lack of significance of the results could be attributed to inconsistencies in producing knockdown of PNPase through RNAi mechanisms. Previous studies identified a near 50% increase in ROS production in knockdown of PNPase compared to the control (Lambert 2015). While a large increase in ROS production induced and maintained senescence, our findings presented a slight increase in ROS production that could be attributed to activation of beneficial stress responses that influence extension of lifespan. Additionally, a low increase of ROS can activate signaling pathways leading to proliferation and transcription (Trachootham et. al., 2008; Thannickal and Fanburg, 2000).

Previous lifespan studies have reported that knockdown of PNPase resulted in an extension of lifespan. It was hypothesized that the slight elevation of ROS production could have activated beneficial stress responses to influence extension of lifespan (Lambert 2015). However, to determine if this level of increased ROS imparted beneficial responses such as lifespan extension, a lifespan assay comparing knockdown, mutants, and overexpression profiles of \textit{pnpt-1} would be required.

Polycistronic and total mitochondrial transcripts were measured to assess the role of PNPase in transporting RNase P RNA, an exoribonuclease responsible for processing mitochondrial tRNAs, in the mitochondria. The mitochondrial genome is a circular piece of DNA that is transcribed as a single polycistronic transcript where a single mRNA can code for several proteins upon RNA processing. Amounts of both polycistronic and total mitochondrial transcripts were determined through qRT-PCR. It was expected that our knockdown studies would produce similar results as the previous knockdown studies with an increase in polycistronic transcript accumulation. Knockdown of PNPase saw a decrease trend in both
polycistronic transcripts and total mitochondrial transcripts but results were not significant.
Previous studies concluded that PNPass knockdown through RNAi mechanisms resulted in an increase in polycistronic transcript by 66 fold. It was suggested that reduced expression of PNPass would decrease the rate of transport of RNase P RNA to the mitochondria, leading to increased accumulation of polycistronic transcripts due to improper splicing of mitochondrial transcript (Lambert 2015). Even with confirmation of knockdown of PNPass expression, our findings contradict previous studies. However, since our results were not statistically significant, we were unable to conclude if polycistronic transcript accumulation decreases with knockdown of PNPass. Results produced could be attributed to a reduced but functional level of PNPass expression even though knockdown of PNPass expression occurred. The residual level of PNPass expression could allow transport of RNase P RNA to the mitochondria, thus allowing processing of polycistronic transcripts and a decrease in its accumulation.

For future directions, optimization of RNAi protocol could be carried out to improve penetrance of the knockdown phenotype. Such changes could include increasing IPTG inducer to 1 mM and adding it to the worm plate rather than in the bacterial cultures, and grow liquid cultures without induction and allow seeded cultures to grow at room temperature (Kamath et. al., 2001). Additionally, comparisons could be made between staging worms at the L4 stage versus staging embryos to determine which protocol produces the greatest knockdown effect.

**Mutant Studies**

Previous studies of mutations in PNPass in cultured skin fibroblasts saw a 50-60% decrease in PNPass protein but no change in PNPT1 transcripts (Vedrene et al., 2012). Researchers identified a disruption in the trimerization of the protein when the missense
mutation was located in the second RNase PH catalytic domain (Vedrenne et. al., 2012). Our findings were consistent with previous reports but were limited to pnpt-1 transcripts. We found that pnpt-1 mRNA expression was unchanged in the mutants. We were able to conclude that three specific missense mutations in the first RNase PH domain do not affect PNPase expression since the mutations were located in the RNase PH domain and not in a regulatory region associated with pnpt-1.

Mutant strains resulted in no significant change in ROS production compared to their wildtype counterpart. Previous studies have noted that phenotypic effects from PNPase mutations vary and severity of functional deficits of PNPase is dependent on the severity of the mutation (Golzarroshan et. al., 2018; von Ameln et. al., 2012). One study noted that a p.E475G missense mutation in human PNPase did not affect PNPase expression but resulted in a disease phenotype consisting of hearing loss (von Ameln et. al., 2012). Our results proved that these specific mutations in pnpt-1 produced no significant phenotypic change in ROS production, suggesting that these mutations were not severe.

All three strains with mutations in the first RNase PH domain resulted in non-significant changes in polycistronic transcript accumulation and mutant strains G58E and G74E resulted in an increase trend for total mitochondrial transcripts. These findings suggested that certain mutations in PNPase could vary in their contribution to mitochondrial homeostasis. Previous findings of bacterial PNPase determined that the second RNase PH domain carried out most of the catalytic activity of the protein, and mutations in the second domain resulted in a severe non-functional phenotype that impaired phosphorolysis, polymerization, and exchange (Jarrige et. al., 2002). However, mutations in the first RNase PH domain did reduce specific activity of PNPase, but not at the same severity or degree as the mutations in the second RNase PH domain (Jarrige
et. al., 2002). Also, studies in human PNPase determined that both RNase PH domains possessed similar phosphorylitic properties with equal enzymatic activity. Presence of either one of them was sufficient for total enzymatic activity (Sarkar et. al., 2005). Our findings did agree with previous studies. The results produced showed that mutations in the first RNase PH domain do not contribute to significant increase in polycistronic transcript accumulation. This could partly be due to the fact that the second RNase PH domain contained no mutations and compensated phosphorylitic activity for the first mutated RNase PH domain. One study identified a patient with two biallelic pathogenic missense variants in PNPT1 (R136C and P467H), one in each RNase PH domain of PNPase (Rius et. al., 2019). When assessing PNPase mitochondrial RNA processing activity, investigators reported that the patient’s fibroblasts presented a significant increase in accumulation of unprocessed mitochondrial transcripts (Rius et. al., 2019). Individual mutations could vary in degree of disrupting normal PNPase function without altering PNPase expression even if they were located in the first catalytic domain. However, conserved amino acids present in orthologs of PNPase could suggest a higher degree of importance as determining residues. Mutations affecting these amino acids would be more likely to impart a disease phenotype. Among the mutant strains selected, the G58E missense mutation occurs at a conserved residue while G74R and G74E are missense mutations in an unconserved residue. Despite this, all three mutant strains did not produce a significant change in polycistronic transcript accumulation nor ROS production. As a result, we can conclude that the studied mutations in pnpt-1 do not produce a deleterious phenotype within the areas of polycistronic transcript accumulation, total mitochondrial transcript accumulation, and ROS production.
For future studies, using other mutant strains that contain missense mutations in the second catalytic RNase PH domain would determine if both catalytic domains work equally or if the second domain carries out most of the enzyme activity. Additionally, there is another mutant strain that has a missense mutation in the S1 RNA binding domain of pnpt-1. Using this strain would determine if the mutation affects RNA binding activity of PNPase and if that change leads to observable phenotypic effects. Also, CRISPR could be used to knock in human mutations at conserved residues in wPNPT1 such as A510P and Q387R in the human studies. Doing so could determine if similar phenotypes seen in human studies would be present in C. elegans. Using only Polyphen-2 and SIFT for predicted effects of mutations limited our knowledge of the effect the missense mutations had on protein catalytic function. Having protein structure analysis would give insight on the structural changes occurred and if those changes inhibit normal protein function. Non denaturing or native gel electrophoresis would also determine if those mutations affected trimerization of the protein.

**Overexpression Studies**

Creation of an overexpression model for PNPase first required design and synthesis of the transgenic construct. Initial attempts at cloning PNPase into the pPD49.83 vector were not successful and it is likely that certain cloned fragments of pnpt-1 were toxic to transformed bacteria. Varied attempts consisted of adjusting different steps of the cloning protocol to bypass this issue. Different competent cells, BL21 DE3, ABLE C, and ABLE K, were used for their specific properties at retrieving clones that were toxic to E. Coli since initial cloning attempts with DH5alpha and DH10B were unsuccessful leading us to hypothesize that the fragment being cloned was toxic to the bacteria. ABLE C and ABLE K strains are designed to increase the
probability of retrieving toxic clones by reducing the plasmid copy number. This results in decreased levels of the cloned gene product and enhances the probability for toxicity to the host. BL21 DE3 cells have T7 RNA polymerase controlled by Lac regulatory construct that will only be active in the presence of IPTG, thus limiting toxic protein expression. Temperature conditions were lowered and transformed bacteria were grown at room temperature (20°C - 30°C) for several days to reduce toxicity of the hypothetically toxic cloned gene. Any attempt at cloning a 3’ fragment of pnpt-1 resulted in the fragment ligating in the opposite orientation or ligating in the correct orientation but with mutations. A third party manufacturer (ThermoFisher GeneArt Gene Synthesis) was used to design a fragment of pnpt-1 hypothesized to be toxic to E. coli. While they were able to clone the fragment and synthesize the plasmid, using that pnpt-1 fragment for subsequent ligating and cloning protocols was unsuccessful. Gibson assembly protocol was also used to minimize the number of cloning and transformation steps but also failed. As a result, we conclude that the 3’ fragment is toxic to E. coli, thus preventing successful cloning of this gene. PCR Fusion allowed for annealing of overlapping ends between multiple individual segments and using a two-step method to create the full-length clone (Hobert et. al., 2002; Luo et. al., 2012). It was vital to use a polymerase with 3’ - 5’ proofreading activity and high fidelity to fix and prevent, respectively, sequence errors during each amplification cycle (Hobert et. al., 2002). This technique allowed for bypassing of intermediate steps associated with cloning such as overnight cultures, mini-preps, and transformations. As a result, generating the full-length clone in vitro did not require the construct to be propagated in E. coli. This ultimately streamlined the assembly process.

Overexpression of PNPase was achieved through heat shocked protocols at two hour intervals at 30°C for exogenous PNPase expression. A combination of qRT-PCR}s and Western
blots allowed identification and differentiation of exogenous PNPase from endogenous PNPase due to the added 3X FLAG Tag in the Fusion PCR construct. We determined that transcription activation only occurred at a certain temperature and degree of expression correlated with increasing temperature. Also, heat shock activation led to protein formation of exogenous PNPase. Activation the hsp-16.41 promoter minimally required a temperature of 29°C to 31°C while temperatures as low as 25°C saw a little as 1% of cells staining (Stringham et. al., 1991). Additionally, transgenic nematodes maintained at 20°C results in no transgenic expression (Bacaj et. al., 2007). Our findings matched these predictions and demonstrated that heat shocked at 30°C activated the hsp-16.41 promoter for transcription of ectopic pnpt-1 that eventually led to exogenous protein formation. If there was no heat shock or if a certain temperature threshold was not reached, the promoter would not activate and transcription would not occur. Additionally, no leakage of ectopic expression could be detected when samples were not heatshocked. Successful activation of the hsp-16.41 produced increased expression levels of exogenous PNPase, thus contributing to elevated levels of total PNPase expression. Also, these findings concluded that heat shocked protocols saw no significant influence in changing endogenous PNPase expression.

When pnpt-1 was induced to be overexpressed, quantification of ROS production saw no significant difference in any of the transgenic strains. Previous findings determined overexpression of hPNPase resulted in an increase in ROS production (Sarkar et. al., 2004). Overexpression of PNPase also led to a senescent like growth arrest phenotype with elevated levels of ROS being vital for induction and maintenance of the cell senescence process (Sarkar et. al., 2004; Davalli et. al., 2016). Our results did not agree with previous findings and suggested that the degree of overexpression of PNPase was not sufficient enough to induce a
senescent-like phenotype that would lead to an increase in ROS production. One potential complication with this experiment is that the *pnpt-1* overexpression transgene is not present in every cell preventing uniform expression of PNPase throughout the nematode body and instead resulting in a more mosaic pattern of expression. Although the transgenic lines proved to be stable, extrachromosomal arrays, such as the microinjected Fusion PCR construct, can be unstable to cell division with varying degrees of mitotic instability and incomplete inheritance (Evans et. al., 2006). Specifically, some of the disadvantages of creating extrachromosomal arrays through microinjection are difficulty in predicting and controlling the level of expression, variable expression among siblings of a single strain, and variable expression due to mitotic instability of the arrays (Evans et. al., 2006). As a result, lack of uniform exogenous PNPase overexpression in the transgenic models would prevent induced senescence in every cell. Finally, we could not be seeing the same results as previous studies due to not running enough trials. A different approach to allow uniform expression of exogenous *wpnpt-1* in all cells and consistent inheritance with each generation of progeny would be to use CRISPR to insert the Fusion PCR construct in the *C. elegans* genome in a region that does not disrupt any endogenous gene function.

When *pnpt-1* was overexpressed, wildtype transgenic lines (VC2010) resulted in an increase in polycistronic transcript accumulation while *rrf-3* mutant transgenic lines (CF3152) saw a decrease when compared to their non-heat shocked counterparts. Total mitochondrial transcripts presented elevated trends in heat shocked wildtype and in heat shocked *rrf-3* mutants. Despite producing similar trends in total PNPase and exogenous PNPase expression, opposite results between transgenic wildtype and *rrf-3* mutants could be due to CF3152 strains containing a *rrf-3* deletion. The *rrf-3* gene codes for an RNA dependent RNA polymerase that is involved in
chromatin silencing by small RNA and spermatogenesis. While the *rrf-3* deletion is often associated with temperature sensitivity in regards to sterility and fertility, and enhanced RNAi (Han et. al., 2009), deletion of this gene could also be imparting some phenotypic effect that leads to decreased polycistronic transcript accumulation in conjunction with overexpression of PNPase. Heat shock treatment to already temperature sensitive *rrf-3* mutant strains to activate exogenous PNPase expression could influence the onset of additional phenotypes beyond what is already known.

**Temporal Regulation of PNPase Expression in the Germline**

Exogenous PNPase expression was only detected in the nematode when the transgenic worm was heat shocked. Immunofluorescence labeling for FLAG Tag in gonads for exogenous PNPase resulted in staining only in CF3152 FSN 3B3 transgenic worms after heat shock activation in the pachytene region of the nematode gonad. Our findings matched our expectations that *hsp-16.41* and *unc-54* 3` UTR both served as transcriptional and post-transcriptional regulators that limit expression to a specific region of the gonad. The combination of both the promoter and UTR spatially controlled expression of exogenous PNPase in cells, limiting it to cells in the late pachytene region of the gonad. Previous studies had indicated that the *hsp-16.41* promoter was expressed in most somatic cells with a higher incidence of expression in intestinal and pharyngeal tissue so was not germline specific (Merrit et. al., 2010). In addition to staining in the nematode gonad, intestinal cells stained brightly for FLAG Tagged exogenous PNPase expression, matching with previous reports (data not shown). Unpublished data had observed weak expression in late pachytene of gametogenesis from the *hsp-16.41* promoter (Merritt et. al., 2010; Bacaj et. al., 2007). The *unc-54* 3` UTR restricts
expression to all germ cells (Merritt et. al., 2008). These reports on spatial influence of transcriptional regulators were consistent with our findings of predictions of PNPase expression in the middle region of the gonad, approximately where pachytene occurs.

Although FLAG Tag expression for exogenous PNPase was consistent with each attempt, the polyclonal antibody used created so much background in the gonad that it interfered with image quality. It led to concerns that staining the germline was an artifact and not a positive result. However, it was confirmed that the staining for exogenous PNPase in the germline was not an artifact after a monoclonal FLAG Tag antibody produced similar results (data not shown).

**PNPase Senescence or Differentiation in the Germline**

The goal of this study was to determine if overexpression of ectopic PNPase in the germline would result in induction of senescence or differentiation of germline tumor cells. Immunofluorescence labeling for germline specific P granules and exogenous PNPase identified cells in the gld-1 cancer model that expressed exogenous PNPase but did not present P granule staining. All six groups presented successful cancer phenotype formation with knockdown of gld-1. Gonads resulted in mass mitotic proliferation from the distal to proximal end of the germline with no oocyte production. Previous studies have indicated that knockdown or mutation of gld-1 results in brief entry of germline cells into meiosis but quickly reverting to mitotic proliferation. As a result, no oogenesis nor oocytes were produced with mitotically proliferating cells taking its place (Kirienko et. al., 2014). These reports were consistent with our findings and allowed us to conclude that gld-1 results in mitotic proliferation from distal to proximal ends of the germline with no oocyte development.
Only heat shocked CF3152 FSN 3B3 nematodes presented staining for exogenous PNPase in the middle of the gonad. Specifically, cells that contained exogenous PNPase did not present P granule staining suggesting terminal differentiation of a germline tumor cell into a non-germline cell. PGL-1, or P granules, is a germline specific marker that is essential for fertility in *C. elegans* (Kawasaki et. al., 1998, Nayak et. al., 2004). Previous studies had noted that knockdown of *gld-1* resulted in development of non-germline cells in the nematode gonad. Specifically, 40% of gonads presented both differentiated neuronal and muscle cells and 55% of gonads presented differentiated neuronal cells in germline of 1.5 day adults. When differentiated, the cells lost P granule expression with the nuclei not resembling germline cells (Ciosk et. al., 2006). While the nuclei formation could not be observed to differentiate somatic and germ cell nuclei, these reports were consistent with our finding that exogenous PNPase induces differentiation into a non-germline cell. However, the type of differentiated cell, whether it was a neuronal or muscle-like cell, could not be determined. Antibodies and markers used limited our scope to only determining if the cell belonged in the germline or not. Also, high concentration of cells as a result of the *gld-1* knockdown cancer phenotype prevented individual cellular analysis of nuclei morphology. Based on this, we proposed that ectopic overexpression of PNPase has a role in differentiation inducing mechanisms in a tumor setting.

While the initial data look promising, additional attempts need to be carried out to determine a consistent and significant result. Additionally, our experimental design allows us only to determine if germline cells differentiated into non-germline cells. It does not allow confirmation if the differentiated cells are senescing or not. While long term exit from the cell cycle is the main marker for cellular senescence, terminal differentiation results in replicative arrest triggered by physiological cues while cellular senescence withdraws from the cell cycle as
a result of activation of tumor suppressor networks and other associated factors (Kui\lman et. al., 2010). Another encountered shortcoming occurred when using gld-1 knockdown cancer models. The gonads of these models were extremely brittle and prone to bursting and ripping upon dissection. Also, the cancer model gonads had a greater tendency to wash off the Poly-L-Lysine coated slides than wildtype gonads. Analyzing and locating individual cells in the proximal region of the gonad for differentiated non-germline cells proved to be unfeasible. Large concentrations of mitotically dividing cells prevented individual cellular analysis, making it difficult to determine if P granule staining was present or not.

For future cancer model experiments, using the double knockdown cancer model, gld-1 and mex-3 in N2 worms has been reported to produce a higher incidence of non-germline cells compared to only gld-1 knockdown worms (Ciosk et. al., 2006). Using the V2010 FSN 9C3 transgenic line, strain synthesized from the N2 wildtype derivative, with gld-1 and mex-3 knockdown should produce a modest over proliferation phenotype rather an extreme one that inhibited individual cellular analysis. Rather than solely relying on K76, P granule antibody, to differentiate between non-germline and germline cells, using antibodies for UNC-119 or HLH-1 would allow detection of neuronal or muscle like cells, respectively. This would provide quantifiable results and determine if overexpression of PNPase induces a higher incidence of non-germline cell differentiation.

Since hsp-16.41 expression was greatest in intestinal and pharyngeal tissue, using a cancer model that causes hyperplasia in those tissues would be advantageous. Such cancer models include lin-35;fzr-1 double mutants, and gain of function mutations in cdc-25.1 for hyperplasia in intestinal tissue (Kirienko et. al., 2014). Rather than relying on higher heat shock temperatures and the associated risk of killing the worms, it would be easier to achieve higher
levels of exogenous PNPase in these tissue regions with the current heat shock protocol.

Examples of protocols include heat shock treatment at 33°C for two 2 hour intervals with a 30 minute break at 20°C inbetween (Stringham et. al., 1992) or 34°C for 30 minutes (Bacaj et. al., 2007). Reports for the second protocol indicated presence of the transgene product up to 24 hours post heat treatment (Bacaj et. al., 2007).

**Additional Future Directions**

Future directions for phenotypic analysis of varied PNPase expression would include optimizing current protocols and adding new ones. For current protocols, qRT-PCR for polycistronic transcript accumulation and the ROS assay require optimization. Designing and testing new primer designs for the *chtb-I* and COIII junction to measure polycistronic transcripts would determine if the machine errors are a result of primer design. For the ROS assay, increasing the number of worms per sample to 100 would allow direct comparison with the previous studies. Ultimately after optimization, multiple trials, or a minimum of three trials, of each experiment need to be performed to allow statistical analysis.

Other experiments to analyze phenotypic effects from varied PNPase expression would include analysis of mitochondrial morphology, identification of markers of senescence in nematode, and quantification of 8-hydroxyguanin (8-oxoG) RNA molecules to correlate PNPase and its role in protecting against oxidative stress. Analysis of mitochondrial morphology would allow more correlations to be drawn between PNPase expression and mitochondrial homeostasis. Also, it would allow direct comparison with previous knockdown studies. Although overexpression of exogenous PNPase is confirmed, using markers for senescence would assess the senescent inducing phenotype associated with PNPase such as age-dependent increased
expression of lysosomal enzymes, acid phosphatase, beta-N-acetyl-D-glucosaminidase, beta-D-glucosidase, and alpha-D-mannosidase (Bolanowski et. al., 1983).

Rather than solely quantifying ROS, recent studies in *Escherichia coli* have identified PNPase to function as a protector against oxidative stress. PNPase specifically binds to synthetic RNA containing oxidative lesions called 8-oxoG, suggesting a role in removing oxidative damage (Wu et. al., 2009). 8-hydroxyguanine is an oxidized form of guanine that has potential to pair with both adenine and cytosine at equal efficiencies, causing translational errors if this molecule was present in messenger RNA. Additionally, 8-oxoguanine can cause mispairing during DNA synthesis (Hayakawa et. al., 2001). RNA oxidation can be quantified to determine the degree of accumulated oxidative damage in each expression profile of PNPase. These experiments will provide better insight on PNPase function and association with mitochondrial homeostasis.

In addition to identifying phenotypes associated with varied expression and mutation of PNPase, further experiments can be carried out to investigate molecular mechanisms of PNPase on these phenotypes. RNA-seq can be used with different expression profiles of PNPase to analyze the *C. elegans* transcriptome of gene expression patterns encoded within RNA to identify which genes are differentially expressed and are influenced by PNPase. Co-immunoprecipitation for protein-protein interactions can be used to determine if mutations in PNPase affect protein binding activity to helicase SUV3, which forms a complex to carry out mitochondrial RNA surveillance. Immunofluorescence can also be used to determine if localization of PNPase is affected within a cell when the protein is overexpressed, knocked down, or mutated.
Chapter 5: Conclusion

The mutant studies confirmed that different functional deficits of PNPase is dependent on the severity of the mutation. For our specific mutant strains, results suggest that these mutational changes do not affect PNPase function. Although PNPase expression was not altered in strains containing the mutation, the mutation appeared to not have contributed to protein function within the realms of ROS production, and polycistronic and total mitochondrial transcript accumulation with all mutant studies data presenting non significant results. For knockdown studies, trends observed with ROS production was similar with previous studies. However, inconsistencies and contradictions with previous data in regard to polycistronic transcript accumulation require further optimization and testing to determine a significant trend. Our studies have produced a stable transgenic overexpression model for PNPase that presented exogenous PNPase expression in transgenic lines upon heat shock activation. In a cancer setting with this model, germline cells expressing ectopic PNPase did not present P granule staining, suggesting differentiation into a non-germline cell type. This finding expands on the differentiating inducing mechanisms associated with PNPase. Although much of the data was not statistically significant, creation and confirmation of a working overexpression animal model for PNPase provides a strong foundation in better understanding protein functions, and its role in cancer.


VITA

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Appendix: Supplementary Protocols and Information

NGM Plates (Nematode Growth Media)

To a flask, 1.5 g of NaCl, 1.25 g of BactoPeptone (Difco), 8 g of BactoAgar (Difco), and 500 mL of ddH20 were added and autoclaved to sterilize the reagents. Additional reagents of 12.5 mL of 1M K3PO4 pH 6.0, 0.5 mL of CaCl2, 0.5mL of MgSO4, and 0.5 mL of 5 mg/mL Cholesterol were added to the autoclaved NGM solution. NGM media was poured by hand into 60 mm x 15 mm medium plates and allowed to solidify at room temp prior to long term storage in 4°C.

For NGM Carbenicillin Plates, 1 mL of 50 mg/mL filter sterilized carbenicillin was added to the autoclaved 0.5 L of NGM media prior to pouring into 60 mm x 15 mm plates to bring the final concentration of carbenicillin at 0.1 mg/mL.

Gels for western

<table>
<thead>
<tr>
<th>Reagents</th>
<th>7.5% Acrylamide Resolving Gel</th>
<th>3.9% Acrylamide Stacking Gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>30% acrylamide/0.8% bisacrylamide</td>
<td>2 mL</td>
<td>0.52 mL</td>
</tr>
<tr>
<td>4X Tris-HCl/SDS pH 8.8</td>
<td>2 mL</td>
<td>N/A</td>
</tr>
<tr>
<td>4X Tris-HCl/SDS pH 6.8</td>
<td>N/A</td>
<td>1 mL</td>
</tr>
<tr>
<td>ddH20</td>
<td>4 mL</td>
<td>2.48 mL</td>
</tr>
<tr>
<td>10% APS</td>
<td>30 uL</td>
<td>14 uL</td>
</tr>
<tr>
<td>TEMED</td>
<td>6 uL</td>
<td>4 uL</td>
</tr>
</tbody>
</table>

M9 Buffer

M9 consisted of 3 g KH₂PO₄, 6 g Na₂HPO₄, 5 g NaCl, 1 mL 1M MgSO₄, and H2O to 1 litre.

1X Transfer Buffer

1X Transfer Buffer consisted of 50 mL of 10X Transfer Buffer (15.2 g of Tris, 72.1 g of Glycine, 5.0 g of SDS, and ddH2O to 500 mL), 150 mL of methanol, and 300 mL of ddH20.
Knockdown Studies of PNPase in *C. elegans*

The goal for the knockdown studies of decreased expression of PNPase was to confirm previous results, expand the findings, and observe how they compared to overexpression and mutant studies of PNPase.

PNPase Expression

Knockdown of PNPase was generated through an RNAi clone (Exon 3), that spanned the region from 5' of exon 1 through exon 3. Previously knockdown of PNPase through RNAi resulted in a 63% reduction of mRNA and a 58% reduction in protein levels (Lambert 2015). PNPase expression was measured using a qRT-PCR protocol for PNPase expression at the exon 9 and exon 10 junction. Six replicate qRT-PCRs were performed to measure PNPase expression and the fold change results are listed as follows: Exon 3 (a) produced 0.36x (64% reduction), Exon 3 (b) produced 1.44x (44% increase), Exon 3 (c) produced 1.03x (3% increase), Exon 3 (d) produced 0.25x (75% reduction), Exon 3 (e) produced 1.03x (3% increase), and Exon 3(f) produced 0.74x (26% reduction) (Figure 31). Worms from samples a, d, and f were used for measuring polycistronic transcript accumulation for Figure 33. The results from this experiment identified issues with the knockdown model that required troubleshooting.
Figure 31: *pnpt-1* expression of Knockdown Samples. Exon 3 knockdown produced inconsistent knockdown results: (a) 0.36x (64% reduction), (b) 1.44x (44% increase), (c) 1.03x (3% increase), (d) 0.25x (75% reduction), (e) 1.03x (3% increase), and (f) 0.74x (26% reduction). L4440 served as the empty vector control (n=6).

Polycistronic Transcript Accumulation

Previous work with PNPase knockdown samples resulted in an increased trend in polycistronic transcripts with knockdown of PNPase expression (Lambert 2015). The goal was to verify previous results after confirming samples had decreased expression of PNPase through RNAi. qRT-PCR for PNPase expression determined knockdown of PNPase expression prior to measuring polycistronic transcript accumulation with a statistically significant 45X fold change in Exon 3 (55% knockdown) (p = 0.02) (Figure 32). Knockdown samples used for measuring both PNPase expression and polycistronic transcript accumulation included knockdown samples a, d, f. Polycistronic transcript accumulation was quantified using qRT-PCR protocols that measured expression using primers that amplified the *ctb-1* and COIII junction. Exon 3, knockdown of PNPase, resulted in a decreased trend for polycistronic transcripts with an 86.1%
decrease compared to L4440 control (Figure 33). Statistical analysis could not be carried out since technical difficulties limited this experiment to two working attempts with samples a and d.

Figure 32: **PNPase Expression in Knockdown Samples a, d, f.** Exon 3 knockdown results in a 0.45x (55% decrease) fold change in PNPase expression. (*p = 0.02*) (n=3)

Figure 33: **Knockdown of PNPase.** Exon 3 knockdown (Samples a and d) results in a decrease trend for polycistronic transcript accumulation. (n=2)
Total Mitochondrial Transcripts

Total mitochondrial transcripts were quantified using qRT-PCR by measuring the expression of COIII. The COIII gene was used since it includes the ctb-1 and COIII junction that was used to quantify the polycistronic transcripts. Knockdown of PNPase resulted in a decrease trend in total mitochondrial transcripts with a 37% decrease compared to the L4440 control. Due to technical issues with this experiment, the experiment was only conducted once and no statistical analysis could be carried out (Figure 34).

**Figure 34: Total Mitochondrial Transcripts in Knockdown Animals.** Knockdown of PNPase (Exon 3) produced a decreased trend in total mitochondrial transcripts. (n=1)
ROS Assay

Previous work studying knockdown of PNPase in *C. elegans* had identified a significant increase in ROS production (Lambert 2015). To reconfirm the findings, ROS production was measured in both knockdown (Exon 3) and control (L4440) strains. Knockdown of PNPase was initially confirmed through qRT-PCR prior to running the assay with two out of the three Exon 3 samples showing decreased expression of PNPase (samples d and f). Knockdown samples used for this assay consisted of samples d, e, and f. As a result, Exon 3 presented a 0.67x fold change (32.5% decrease) in PNPase expression compared to L4440 control but was not statistically significant ($p = 0.22$) ($n=3$)(Figure 35). Knockdown of PNPase (Exon 3) displayed an increased trend for ROS production compared to the control, matching previous results. However, statistical analysis determined the result to be not significant ($p = 0.20$) ($n=3$)(Figure 36).

![PNPase Expression (Pre-ROS Assay)](image)

**Figure 35: PNPase expression in RNAi strains associated with ROS Assay.** Exon 3, knockdown of PNPase, shows a decreased trend for PNPase expression but was determined to be non-significant ($p = 0.22$) ($n=3$)
Figure 36: ROS Production in Knockdown Animals. Knockdown of PNPase (Exon 3) showed no difference in ROS production compared to the control (L4440) (p = 0.20)(n=3)
Figure 37: Total PNPase Expression of all 3 expression profiles of PNPase
Figure 38: Polycistronic Transcript Accumulation from all 3 expression profiles of PNPase. (Knockdown samples, Exon 3 and L4440, were obtained from RNAi samples of ROS Assay and RNAi trouble shooting experiments.) (n=3)
Figure 39: Total Mitochondrial Transcripts from all 3 expression profiles. (n=1)
Figure 40: ROS Assay for all 3 expression profiles of PNPase (n=3)