NRDE-1 IS REQUIRED FOR GERMLINE IMMORTALITY

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A dissertation submitted to the faculty at the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Genetics and Molecular Biology in the School of Medicine.

Chapel Hill
2015

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ABSTRACT

Jacinth Mitchell: NRDE-1 is required for germline immortality.
(Under the direction of Shawn Ahmed)

NRDE-1 is required germline immortality and is a critical component of the transcriptional silencing pathway known as nuclear RNAi. Loss of the *nrde-1* gene activity results in progressive accumulation of transgenerational stress which compromises the germline tissue and results in germ cell loss, ending ultimately in sterility.

Using *nrde-1*, we paint a sketch of a germline stress and identify DAF-2, a component of the insulin signaling pathway, as well as piRNAs as significant contributors to this stress. Additionally, we explore the accumulation of stress by defining the developmental stage at which the sterility occurs. Due to its role in silencing, we conducted RNAseq on early vs sterile generation *nrde-1* mutant animals to investigate the cause of its Germline Mortality and outline results in the expression of selfish genetic elements such as retrotransposons. Although these elements are expressed in sterile generation *nrde*-1 they were similarly upregulated in another *nrde* mutant that does not become sterile. Additionally, we have uncovered a list of 19 genes whose transcription is >2 fold upregulated in late generation *nrde-1* mutants that have accrued damage. Of these genes 42% correspond to the process of spermatogenesis, in agreement with other Mrt studies involving epigenetic transgenerational sterility. We explain this by showing that sperm are not necessary for sterility and that developmental delay occurs and may explain spermatogenesis gene misregulation.

ACKNOWLEDGEMENTS

Special thanks to Cori Bargmann for provision of the original *yp4* allele. Eric Miska, Ph.D. and Perter Sarkies Ph.D. for collaborative efforts and assistance with RNAseq data analysis. Thanks to the Caenorhabditis Genetics Center (CGC) for providing worm strains. Thanks to Ashley Hedges, Malik, Aisa Sakaguchi and Alicia for mapping and verifying *yp4* and *yp5* alleles of *nrde-1*. Thanks to my committee for continued support through this process.

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LIST OF ABBREVEIATIONS

ARD	Adult Reproductive Diapause
L4	Fourth larval stage of *Caenorhabditis elegans*
MRT	Mortal Germline phenotype
UNC	Uncoordinated phenotype
RNAi	RNA interference
siRNA	short interfering RNA
piRNA	PIWI interacting RNA
miRNA	microRNA
RdRP	RNA-dependent RNA polymerases
Ago	Argonaute
dsRNA	double-stranded RNA
dsDNA	double-stranded DNA
endo	endogenous
exo	exogenous
GSC	Germline stem cell

Chapter 1: Introduction

Small RNA mediated silencing pathways have proven invaluable to the biology of many organisms by offering a broad array of regulatory mechanisms to the cell. In mammals, small RNA mediated silencing plays a crucial role in X chromosome inactivation where non coding RNAs from the Xist locus coat the chromosome and initiate silencing (Gendrel et al., 2014). In the fruitfly, Drosophila, and the nematode, *C. elegans*, small RNAs aid in maintaining genome stability by silencing the expression of foreign genetic elements such as transposons (Aravin et al, 2007).

Crucial to our understanding of how small RNAs lead to silencing is RNA interference (RNAi). During RNAi, a double-stranded RNA (dsRNA) trigger leads to silencing of a complementary sequence (Fire, et al., 1998; Kennerdell JR, 1998). Key components of the RNAi machinery are well conserved. Double-stranded RNAs are first processed into 21-26 nucleotide species by the ribonuclease III type enzyme Dicer (Carmell et al., 2004). Dicer contains four key characteristic motifs conserved across most of the model organisms: a C-terminal double-stranded RNA binding domain, two tandem RNase III motifs, a PAZ domain, and the DExH-box helicase domain (Zhang et al., 2004; Kavi et al., 2008). Dicer cleaves the initial dsRNA in a polarity dependent manner leaving a 5’ phosphate group and a 3’ two nucleotide overhang (Bernstein et al.,2001; Lee, et al., 2001).

Another class of well conserved RNA binding proteins are the Argonautes. Most Argonautes contain the characteristic PAZ and PIWI domains, where the PAZ domain allows for binding of the 3’ end of an RNA (Song et al., 2003) . Argonautes are crucial for instigating the effects of RNAi and can be categorized into one of two clades: the Ago Clade and the Piwi clade (Carmell et al.., 2002). Dicer generated small RNAs work as RNA guides in combination with Argonautes to form the core components of effector complexes such as the RNA-induced silencing complex (RISC). These effector

complexes go on to carry out the function of silencing the target sequence in a pathway dependent manner. Argonaute proteins can vary greatly in function, with some containing nuclease activity allowing them to cleave the target sequence for destruction, whereas others lack this function such as the PIWI proteins in *C. elegans*.

Although RNAi is conserved among many species, much has been done to elucidate the mechanisms in the roundworm *C. elegans*. In *C. elegans*, after forming a complex with an Argonaute, many of the small RNAs are transported downstream to undergo additional rounds of amplification. This amplification, further explored down below, is mediated by RNA-dependent RNA polymerases (RdRPs) which act on single-stranded RNA templates to create more double-stranded RNA (Sijen, et al., 2001) (Martienssen, 2004). During this amplification the guide RNA can act as a primer for the synthesis of oligonucleotides 5' of the initial mRNA template targeted (Sijen, et al., 2001; Paddison, 2008). This allows for a more robust signal and can create effective small RNAs that can recruit the Argonaute to spread silencing along the entire region of the targeted gene. RdRP amplification step is not well conserved as it is seen specifically in *C. elegans* and plants.

Since their discovery, many new species of small noncoding RNA have been found to mediate silencing in the *C. elegans*. The best characterized of these RNAs are the microRNAs, the piRNAs, and the siRNAs. MicroRNAs (miRNA) were discovered first in *C. elegans* with *lin-4* and the subsequent identification *let-7,* which was found to be conserved in many eukaryotes including humans (Lee RC, 1993; B.J., et al., 2000). In *C. elegans*, microRNAs are ~22 nucleotides in length and are formed from primary miRNA transcripts, called pri-mRNA, that are cleaved into precursor miRNA (pre-mRNA) in a sequential manner by the RNase III enzymes Drosha and Dicer respectively (Lee, et al., 2003). The pri-miRNAs transcripts contain a stem loop structure composed of self-complementary sequence found within itself.

The siRNA (short interfering RNA) class of small non coding RNA has been alluring over the years. In *C. elegans* much progress has been made in identifying both the mechanism of biogenesis and the role of siRNAs. In other eukaryotes, such as flies and mouse, only portions of siRNA biogenesis are understood and focus on function depends on the organism being studied. In *C. elegans*, siRNA can be separated into two categories: exogenous siRNA (exo-siRNA) and endogenous siRNA (endo-siRNA). Exo-siRNAs are generated in response to external challenges such as viruses or feeding bacteria expressing dsRNA in the laboratory setting. The major endo-siRNAs in *C. elegans* can be broken into two categorizes based on size; and these are 26G- and 22G- siRNA. Each endo-siRNA species starts with a 5'-Guanine, accounting for their designation. 26G siRNA are 26 nucleotides in length and can be further subdivided into two groups based on function. The first group of 26G siRNA are enriched during spermatogenesis and depend on the activity of the argonautes ALG-3/4. In the *alg-3/4* double mutant this species is lost but fertility can be rescued with the introduction of wild-type male sperm into the hermaphrodite worm (Han, et al., 2009; Conine, et al., 2010).The second set of 26G siRNA associate with the argonaute ERGO-1 and is enriched in the oocyte and embryo (Han, et al., 2009; Vasale, et al., 2010; Fisher, et al., 2011). The 22G siRNA are considered secondary RNA and are the product of the RdRP dependent amplification via RRF-1 and EGO-1. These siRNAs can also be separated into groups based on function. The first group of 22G RNA go on to bind to a worm specific group of argonautes called WAGO, named WAGO-1 through WAGO-12 and aid in the identification of self vs foreign genetic elements via gene silencing (Gu, et al., 2009). The second set of these small 22G RNAs bind to the argonaute CSR-1 and aid in chromosome segregation and the licensing of genes for expression.

The third class of well-studied small RNAs are the PIWI-interacting RNAs (piRNAs). As the name implies, piRNAs complex with the PIWI clade argonautes when acting in the cell. Piwi proteins are highly conserved, being first identified as a factor crucial to germline stem cell maintenance in Drosophila (Carmell M. A., 2002; Lin & Spradling, 1997). Since then Piwi proteins are shown to be required for

germline function in many animals. In mice, three Piwi proteins; MIWI (PIWIL1), MILI (PIWIL2), and MIWI2 (PIWIL4); are necessary for spermatogenesis with none being required for female murine fertility. In contrast, the three Drosophila PIWI proteins; Piwi, Aubergine (Aub), and Argonaute3 (Ago3); are required for male and female fertility (Kennerdell, 2002; Brennecke, 2007; Gunawardane, 2007). In zebrafish, mutation of the Piwi proteins, ZILI and ZIWI, results in the loss of all germ cells with *ziwi* mutants showing pre-meiotic apoptosis and *zili* mutants unable to undergo oogenesis and female development (Houwing S. K., 2007; Houwing S. B., 2008). In higher mammals such as humans, macaque and bovine, immunofluorescence and qRT-PCR data reveal the presence of PIWIL1 and PIWIL2 proteins in adult oocyte and testis with more work being done to define function (Roovers, et al., 2015). The *C. elegans* genome encodes two PIWI orthologues, PRG-1 and PRG-2. PRG-1 is the only PIWI protein whose absence proves it has activity in the worm (Batista, 2008; Das, et al., 2008; Wang, et al., 2008). PRG-1 is present in the germline during all developmental stages and can be seen in the cytoplasm localized to perinuclear structures called P-granules (Batista, 2008). From here piRNA mediated silencing gets quick access to transcripts at the nucleus.

piRNAs contain certain highly conserved features. Unlike miRNAs and siRNAs, piRNA biogenesis is not Dicer dependent and mature piRNAs contain a 5’ Uracil and a 3’ 2-O-methylation (Houwing S. K., 2007; Das, et al., 2008; Vagin, 2006). In Drosophila, mature piRNAs species are between 24-29 nucleotides long. This includes the special subset of piRNAs known as repeat-associated (rasi) RNAs that target satellite and repetitive elements in the genome. In mice piRNAs are 26-30 nucleotide products. In *C. elegans*, piRNAs are 21 nucleotides long with the 5’ Uracil and are thus referred to as 21 U-RNAs. When compared, piRNA biogenesis shows much diversity between animals [reviewed in (Weich & Miska, 2014)]. In Drosophila, piRNAs are transcribed from the heterochromatic pericentromeric and telomeric regions as well as from repeat element sequences and can be bi-directional coming off both strands (Brennecke, 2007). In mice, pre-pachytene sites in the germline serve as the source of

transcription for piRNAs targeting transposon and genic sequences and are developmentally regulated with pre-pachytene expression shown in the newborn. Additionally, pachytene sites give rise to piRNA species that correspond to intergenic sequences (Aravin A. A., 2008; Li, et al., 2013). piRNAs from the *C. elegans* are predominantly transcribed from two chief clusters on chromosome *IV*. The piRNA sequences start as 26-29 nucleotide precursors transcribed by POL II via an upstream bipartite motif, termed the Ruby motif, which is recognized as an autonomous promoter of each piRNA precursor (Ruby, 2006; Billi, 2013; Cecere, 2012; Goh, et al., 2014). Mature piRNA are thought to work via "imperfect base pairing" with their target sequence which gives them the ability to evoke targets of many genes and makes them highly adaptive against the invasion of new foreign selfish genetic elements (Shirayama, et al., 2012).

PIWI proteins and piRNAs are known for their joint role in silencing of transposable genetic elements. In Drosophila, one such example is the interaction between the endogenous retrotransposon *gypsy* and the heterochromatic gene loci *flamenco*. In the absence of piRNA activity from the *flamenco* loci, *gypsy* is mobile and inserts itself throughout the genome with expression present in imaginal discs and adult ovaries. Furthermore, *flamenco* based suppression of *gypsy* transposition is PIWI dependent as seen by expression of a *gypsy-lacZ* transgene in the *piwi* mutant background (Sarot et al.,2004; Brennecke, 2007; Desset, 2003). In *C. elegans*, the Tc3 transposon is active in *prg-1* mutants and causes a 100- to 1000-fold increase of Tc3 excision rates (Das, et al., 2008). In addition to silencing transposable elements many new functions have been elucidated from the PIWI pathway via the *C. elegans*. Newly described roles of PIWI and piRNA function include establishing a role for self non self in the germline and maintenance of transgenerational inheritance.

Chapter 2: piRNAs Can Trigger a Multigenerational Epigenetic Memory in the Germline of *C. elegans*[1]

INTRODUCTION

Since August Weismann (1834–1914) formulated the distinction between innate and acquired characteristics at the end of the 19th century, the debate relating to the inheritance of acquired traits has raised many controversies in the scientific community (Weismann, 1891; Bateson, 1919; Haig, 2006). August Weismann himself theoretically rejected this type of hereditability, arguing that, even though environmental stimuli could provoke adaptive responses in the somatic lineage, no evidence suggested that these changes could be communicated to the germline (Weismann, 1891). However, a number of epigenetic phenomena involving RNA, histone modification, or DNA methylation in many organisms have renewed interest in this area (Varmuza, 2003; Haig, 2006; Daxinger and Whitelaw, 2012). Paramutation is a prime example. In this phenomenon, a silenced allele can act in trans on a homologous sequence to cause stable and heritable silencing. This newly silenced allele can now itself act in a paramutagenic fashion to silence other alleles. Paramutation has been described in multiple species, and it seems likely that small RNAs play a key role in the process, although the full mechanisms involved still remain unclear(Stam and Mittelsten Scheid, 2005; Chandler, 2010; Suter and Martin, 2010).

C. elegans has emerged as a key model for the analysis of several related pathways that regulate genes via small RNAs. *C. elegans* is well suited to the analysis of multigenerational effects, due to its

[1] This chapter previously appeared as an article in the journal Cell. The original citation is as follows:
Ashe A, Sapetschnig A, Weick E-M, Mitchell J, Bagijn MP, et al. (2012) piRNAs can trigger a multigenerational epigenetic memory in the germline of C. elegans. Cell 150: 88–99.

Imaging and analysis of *nrde*; piRNA sensor data were contributed by myself as well as participating in defining *nrde-1 yp4 and yp5*. *prg-1*; piRNA sensor data contributed by Ana-Lisa Dobley. Remaining portions contributed by efforts of Ashe A, Sapetschnig, A.; Weick, E-M. and Bagin, MP.

short generation time (~3 days) and the ease with which they can be maintained under tightly controlled experimental conditions. In eukaryotes, 20–30 nucleotide (nt) RNAs bound to Argonaute (AGO) protein cofactors are the effectors of a number of gene regulation pathways (Carmell et al., 2002). The discovery of the process of RNA interference (RNAi) has been a major milestone (Fire et al., 1998). While 21–22 nt small interfering RNAs (siRNAs) are the small RNA effectors of RNAi, RNAi can be induced by injection of long double-stranded RNA (dsRNA) or by providing dsRNA environmentally in the food of *C. elegans* (Timmons et al., 2001). In both instances, dsRNA is processed by the RNase Dicer to give rise to primary siRNAs. RNAi effects are generally systemic (soma and germline) and are observed in the F1 generation, but the latter requires the generation of secondary siRNAs (Grishok et al., 2000; Pak and Fire, 2007; Sijen et al., 2007; Gu et al., 2009). Secondary siRNAs represent the most abundant class of endogenous small RNAs in *C. elegans*, are RNA-dependent RNA polymerase products, have a 5' triphosphate, and are predominantly 22 nt in length with a 5' guanosine (22G-RNAs). Secondary siRNA pathways and RNA-dependent RNA polymerases (RdRPs) have not been found in vertebrates or Drosophila, but have been found in many other organisms, including nematodes, plants, fungi, and viruses. Secondary siRNA pathways in *C. elegans* are complex, can involve many different AGO proteins, and are only partly understood (Yigit et al., 2006).

Several studies have reported inheritance of environmental RNAi beyond the F1 generation (Fire et al., 1998; Grishok et al., 2000; Vastenhouw et al., 2006; Alcazar et al., 2008; Gu et al., 2012). In one transgenerational paradigm, small RNA inheritance and histone H3K9me3 marks were observed for at least two generations (Gu et al., 2012). In addition, transgenerational inheritance of viral immunity (Rechavi et al., 2011) and longevity (Greer et al., 2011) were recently reported for *C. elegans*. These data suggest that the biological roles of transgenerational inheritance could be diverse but remain largely speculative. In addition, whether this transmission involves transgenerationally transmitted RNAs or modifications of chromatin is still controversial.

Piwi-interacting RNAs (piRNAs) are distinct from siRNAs and have an evolutionarily conserved role in transposon silencing in the germline in many animals, including nematodes (Malone and Hannon, 2009; Bagijn et al., 2012). *C. elegans* encodes two Piwi clade, AGO superfamily proteins, PRG-1 and PRG-2, although PRG-2 has likely little or no function (Batista et al., 2008; Das et al., 2008; Bagijn et al., 2012). *C. elegans* piRNAs are absent in *prg-1* mutant animals, which exhibit fertility defects. PRG-1 and piRNA expression is restricted to the male and female germline (Batista et al., 2008; Das et al., 2008; Bagijn et al., 2012). The piRNAs of *C. elegans* are 21 nucleotides in length with a 5' uracil (21U-RNAs) (Ruby et al., 2006; Batista et al., 2008; Das et al., 2008; Wang and Reinke, 2008). *C. elegans* piRNAs derive from two broad clusters on chromosome IV(Ruby et al., 2006)and act intrans to regulate endogenous targets in the germline(Bagijn et al., 2012).

Here, we report how transgenerational inheritance of environmental RNAi and the piRNA pathway converge on the same germline nuclear RNAi/chromatin pathway. Both nuclear RNAi factors and chromatin regulators are essential for silencing. This pathway can elicit a long-term epigenetic memory for more than 24 generations. Once established, the initial silencing trigger is no longer required.

RESULTS

A Reporter-Based System to Investigate Transgenerational Gene Silencing in *C. elegans*

To genetically dissect multigenerational gene silencing in *C. elegans*, we developed a heritable environmental RNAi paradigm. Taking advantage of the recent advance in technologies to generate single-copy intrachromosomal transgenes in *C. elegans* (Frøkjaer-Jensen et al., 2008, 2012), we generated a reporter transgene expressing a histone-GFP fusion protein in the germline of *C. elegans* (Figure 2.1A and Figure S2.1 available online). The use of such a defined artificial locus combines the ability to map small RNA populations, which was previously not possible using multicopy transgenes (Vastenhouw et al., 2006), with a high-throughput, quantifiable approach that was not possible using an

endogenous locus (Alcazar et al., 2008). Eliciting environmental RNAi by feeding transgenic animals with bacteria expressing dsRNA corresponding to the GFP mRNA results in gene-specific silencing of this GFP transgene (P0, Figures 1.1A and 1.1B), as expected(Timmons et al., 2001). Transfer of these animals to a neutral environment results in a high level of silenced animals in the F1 generation, again as expected (Figure 2.1B) (Fire et al., 1998; Grishok et al., 2000). Furthermore, silencing of the transgene is maintained for at least four additional generations in a subpopulation of animals. We quantified this phenomenon in thousands of animals for each generation using flow cytometry and found that inheritance of transgene silencing was maintained in more than 60% of animals for at least four generations (Figures 1.1C and S1.2). We conclude that we have established a reporter-based paradigm for the investigation of transgenerational inheritance, the ‘‘*h*eritable *R*NAi *de*fective,’’ or ‘‘Hrde,’’ sensor.

Multigenerational Gene Silencing Is Associated with Continued Small RNA Expression

As the mechanisms of transgenerational inheritance are currently not understood, we first asked whether the Hrde sensor silencing that we observed is due to posttranscriptional regulation of mRNA or (co-)transcriptional gene regulation. Using quantitative RT-PCR, we tested whether Hrde sensor silencing in the F2 generation affected either. We were able to robustly detect both primary transcript (pre-mRNA) and Hrde transgene mRNA. However, mRNA levels were significantly repressed ($p < 0.05$) in silenced animals as compared to nonsilenced animals (Figure 2.1D). pre-mRNA levels showed a similar trend. These data suggest that posttranscriptional mechanisms of silencing are required in the Hrde paradigm. We postulated that Hrde sensor transcript availability might result in continued small RNA pathway activity in silenced animals. Therefore, we profiled small RNAs using high-throughput sequencing from animals undergoing environmental RNAi (P0), control RNAi (P0), or at the F4 generation after RNAi. Small RNA libraries were prepared using protocols that did not necessitate the presence of a 5’ monophosphate to capture primary and secondary siRNAs. We detected abundant sense and antisense small RNAs during environmental RNAi (P0 generation) (Figure 2.1E). These small

RNAs had a peak length of 21–22 nt and little bias for the 5'- most nucleotide and likely represent Dicer cleavage products (primary siRNAs) (Figure 2.1F). In contrast, four generations later, only antisense small RNAs remain with the characteristic signature of secondary siRNAs (22 nucleotide length with a 5' guanosine bias, 22G RNAs). Given that each generation represents at least a hundred-fold dilution in volume (with more than 200 offspring generated by each hermaphrodite), these secondary siRNAs must be generated de novo in each generation. Animals undergoing control RNAi displayed a peak of small RNAs homologous to cloning sequences flanking the GFP minigene. These are homologous to cloning sequences that are present in the RNAi vectors and have no apparent effect on Hrde sensor activity (Figure 2.1C).

Multigenerational Gene Silencing and piRNA Silencing Depend on Common Nuclear Factors

We recently reported that piRNA-mediated silencing in the *C. elegans* germline results in secondary siRNA-dependent silencing of a ''piRNA sensor'' and endogenous piRNA targets (Bagijn et al., 2012). Thus, piRNA-mediated silencing might converge on a common downstream multigenerational gene silencing pathway. To this end, we carried out forward genetic screens to identify genes required for either phenomenon using the Hrde and piRNA sensors. Using these two distinct sensors (Figure S2.1), one silenced by a single endogenous piRNA (piRNA sensor) and the other silenced by heritable environmental RNAi (Hrde sensor), we identified, mapped, and cloned new alleles of three known genes in small RNA pathways: *nrde-2*, *nrde-4*, and *hrde-1/wago-9* (Table 1). Next, combining forward genetic screens with a candidate gene approach, we were surprised to identify a total of eight small RNA or chromatin pathway genes to be required (Table 1). For example, the Hrde sensor was desilenced in *nrde-2*, *hrde-1/wago-9*, and *set-25* mutants (Figure 2.2A and Table 1). The products of all of these genes are either known to be, or are predicted to be, nuclear. NRDE-2 is a conserved protein involved in nuclear RNAi that is expressed in the nucleus (Guang et al., 2010); SET-25 is a putative histone H3 lysine-9 methyltransferase with a C-terminal SET domain. To our knowledge, this is the first time that a

histone-modifying enzyme has been identified as required for multigenerational inheritance. HRDE-1/WAGO-9 is an Argonaute protein. Using immunostaining, we show that it is expressed in the germline (Figure 2.2B), where it localizes to the nucleus (Figures 1.2C and 1.2D). NRDE-2 was recently shown to be important in a similar inheritance paradigm (Gu et al., 2012). However, some genes that were previously reported to be involved in transgenerational effects appeared not to be required for our transgenerational inheritance paradigm, including *hda-4*, *mrg-1* (Vastenhouw et al., 2006) or *spr-5*, *lsd-1*, and *amx-1* (Katz et al., 2009) (Table 1). For the piRNA sensor, aside from proteins that were defined in Hrde screens such as NRDE-2, HRDE-1/WAGO-9, and SET-25, we also identified additional nuclear small RNA components and chromatin factors, including NRDE-1, NRDE-4, SET-32, and one of the *C. elegans* heterochromatin protein 1 (HP1) orthologs, HPL-2 (Table 1 and Figure S2.3). We conclude that there exists a common and specific nuclear RNAi/chromatin pathway in the germline that is required for environmentally induced heritable RNAi- and piRNA-induced silencing.

The Nuclear RNAi/Chromatin Silencing Pathway Acts Downstream of Small RNA Expression in Gene Silencing

To establish a hierarchy in the silencing pathways described here, we asked whether nuclear RNAi/chromatin components are upstream or downstream of secondary siRNA expression and/or stability. First, we analyzed small RNA expression in the Hrde sensor paradigm in wild-type and a *nrde-2* mutant background. In both cases, we find abundant 22G secondary siRNAs that map to the Hrde sensor (Figure 2.3A). Thus, NRDE-2 is not required for secondary siRNA generation. Next, we tested a chromatin factor using the piRNA sensor. The HP-1 ortholog HPL-2, but not HPL-1, is required for silencing of the piRNA sensor (Table1 and Figure 2.3B). Therefore, we asked whether secondary siRNAs are expressed and stable in *hpl-2* mutant animals. Using northern blotting, we show that the piRNA 21UR-1 and a piRNA-sensor-specific 22G RNA (siR22G-1) are dependent on the Piwi protein PRG-1 (Figure 2.3C).

However, both RNAs are present in *hpl-2* and *hpl-2; hpl-1* mutant backgrounds, although possibly at reduced levels for siR22G-1.

These observations are in agreement with similar observations made for siRNAs in *S. pombe* lacking Swi6/HP1 (Bühler et al., 2006). In addition, we analyzed endogenous targets of the piRNA pathway that we recently identified (Bagijn et al., 2012). We generated small RNA libraries from wild-type and *prg-1* or *hpl-2* mutant animals. 22G secondary siRNAs at endogenous piRNA targets *bath-45* and *zfp-1* are dependent on PRG-1, but not HPL-2(Figures 1.3Dand 1.3E). Again, we observed some reduction in RNA levels, consistent with a positive interaction between nuclear RNAi and chromatin regulation. We conclude that the nuclear RNAi/chromatin pathway described here is not essential for secondary siRNA expression or stability.

Multigenerational Gene Silencing and piRNA Silencing Does Not Spread into the Soma

As the nuclear RNAi/chromatin pathway that we describe here utilizes small RNAs, it might act in trans on transcripts that share significant sequence similarity. Indeed, using the piRNA sensor, we were able to test this directly. The piRNA sensor is under the transcriptional control of a germline-specific promoter (*mex-5*). Silencing of the piRNA sensor is established through an endogenous piRNA (21-UR-1) with perfect complementarity to a corresponding sequence in the piRNA sensor (Bagijn et al., 2012).A cross of the piRNA sensor strain to a different transgenic strain with a ubiquitously expressed GFP transgene that is not regulated by piRNAs (Figure 2.4A) results in dominant silencing of both transgenes in the germline of heterozygous F1 animals (Figure 2.4B), likely via a process termed transitive RNAi (Alder et al., 2003). Thus, the nuclear RNAi/chromatin pathway can silence in trans. We postulate that this effect is mediated via secondary siRNAs. As exogenous and endogenous RNAi are systemic in *C. elegans* (Fire et al., 1998; Winston et al., 2002), we therefore wondered whether the germline nuclear RNAi/chromatin silencing pathway that we describe here could transcend the germline/soma boundary. We do not find this to be the case, as GFP expression in the trans-

heterozygous animals (*dpy-30::his-58::gfp::tbb-2*/piRNA sensor) described above remains unaffected in the soma (Figure 2.4B). We made the same observation using the Hrde-1 sensor and another somatic transgene in an analogous experiment (data not shown). We conclude that, though the nuclear RNAi/chromatin pathway that we describe here can be vertically transmitted, it does not trigger systemic RNAi. This is consistent with recent work demonstrating that secondary siRNAs are not systemically transmitted in the soma of *C. elegans* (Jose et al., 2011). We note that results based on multicopy transgenes that possibly involve dsRNA intermediates could be different from those reported here (Jose et al., 2011).

piRNAs Can Trigger Long-Term Multigenerational Gene Silencing

Our data demonstrate that environmentally induced multigenerational gene silencing and piRNA silencing converge on a common germline silencing pathway. Can a piRNA therefore trigger multigenerational gene silencing? To address this question, we carried out genetic crosses in which we removed PRG-1 and thereby piRNA function from the piRNA sensor strain (Das et al., 2008; Bagijn et al., 2012). In these circumstances, the piRNA trigger is removed but silencing might be maintained. In a cross of animals homozygous for the piRNA sensor (GFP silenced) with an animal homozygous for the piRNA sensor but in a *prg-1* mutant background (GFP expressed), we generated F1 animals homozygous for the piRNA sensor but heterozygous for the recessive mutation in *prg-1* (Figure S2.4). Such animals are GFP silenced for several generations, as expected. These heterozygous animals segregate progeny that are homozygous, heterozygous, or wild-type with respect to *prg-1*. We observed piRNA sensor reactivation in *prg-1* homozygous mutants or their immediate offspring. Because all piRNAs are eliminated in *prg-1* mutants (Batista et al., 2008; Das et al., 2008), these data suggested that a piRNA trigger may be required to maintain multigenerational silencing memory.

Next, we recreated a piRNA sensor strain that was mutant for *prg-1* by outcrossing the piRNA sensor and then performing several crosses using mutations that cause visible phenotypes to mark the

positions of *prg-1* or the piRNA sensor transgene (see Experimental Procedures). Unexpectedly, 11 *prg-1*; piRNA sensor strains failed to reactivate the piRNA sensor (n = 8 or 3 independent strains created per trial for 2 trials) (Figures 1.5A and 1.5B). GFP expression of these *prg-1; piRNA sensor* strains failed to materialize even though many successive generations were scored, which were last analyzed at F16, F17 (three strains), F20, and F24 (six strains) generations. We also observed that silencing can become PRG-1 independent using a second piRNA sensor construct integrated on a different chromosome (the piRNA mCherry sensor; Figure S2.5). We conclude that germline silencing can persist for many generations even in the absence of a piRNA trigger. It is of interest to note that all crosses that led to trigger independent maintenance of silencing involved the piRNA sensor transgene being heterozygous for 3–5 generations due to outcrossing.

In contrast to multigenerational silencing of piRNA sensor transgenes in the absence of *prg-1*, mutation of *nrde-1* (*yp4* or *yp5*) or mutation of *nrde-2* (*gg95*) triggered reactivation of outcrossed piRNA sensors (n = 3, 2, and 3, respectively, independently isolated F3 or F4 strains scored) (Figure 2.5C). All *nrde-2* mutant lines expressed bright GFP from F3 onward. Of five piRNA sensor; *nrde-1* lines, three lines expressed weak GFP signal in all germ cells in the F3 generation, whereas germ cells of all animals scored in piRNA sensor; *nrde-1* lines were uniformly positive for a weak GFP signal by the F4 generation. We conclude that nuclear small RNA factors are required to maintain the silenced state over many generations, whereas the piRNA trigger that initiates silencing becomes dispensable if the silent locus is outcrossed multiple times.

A Tudor domain protein RSD-6 and a novel protein RSD-2 have previously been shown to be required for RNAi responses to environmental dsRNA triggers that target genes expressed in the germline and are proficient for RNAi to some somatic targets, possibly due to dose-dependent RNAi defects (Tijsterman et al., 2004; Merritt et al., 2008; Zhang et al., 2012). The strong germline RNAi defects of *rsd-6* or *rsd-2* suggested that they could function to promote systemic spreading of RNAi from

soma to germline (Tijsterman et al., 2004). To determine where *rsd-6* functions to promote germline RNAi, single-copy *rsd-6* transgenes driven by the germline-specific *pgl-3* promoter or by the ubiquitous promoter *dpy-30* were created (Frøkjaer-Jensen et al., 2008; Han et al., 2008). Both transgenes rescued an *rsd-6* mutant for the response to dsRNAs targeting the germline-expressed genes *pop-1* or *par-6* (Figure S2.6), indicating that RSD-6 functions in a cell autonomous manner within the germline. We created *rsd-6*; piRNA sensor and piRNA sensor; *rsd-2* strains using outcrossed sensor transgenes and observed that these strains were GFP negative when initially created and for many generations thereafter (Figure 2.5C). These results suggest that piRNA sensor silencing may not depend on systemic RNAi effects (possibly mediated by expression of dsRNA in somatic cells). They also suggest that the response to dsRNA generated in germ cells is unlikely to promote sensor silencing (Tabara et al.,1999; 2002).

DISCUSSION

Here, we show that piRNA and environmental RNAi pathways converge on a common germline nuclear RNAi/chromatin pathway. This pathway can induce stable, multigenerational inheritance. Previous work found evidence for inheritance of small RNAs, chromatin, or both in related transgenerational inheritance paradigms (Burton et al., 2011; Rechavi et al., 2011; Gu et al., 2012). However, here we demonstrate that both small RNA and chromatin factors are essential for multigenerational inheritance and do not act redundantly (Table 1 and Figures 1.2, 1.3, S1.3). We also show that small RNA biogenesis occurs upstream of nuclear RNAi and chromatin factors (Figures 1.3). Recent work has proposed that somatic nuclear RNAi acts at the level of transcriptional elongation (Guang et al., 2010). These observations opened the possibility that chromatin changes observed in transgenerational inheritance paradigms (Gu et al., 2012) might simply be correlative without being functional in silencing. However, our data show that chromatin factors, such as HPL-2 and SET-25/32, are required (Table 1 and Figures 1.3 and S 1.3). SET-25/32 are putative histone H3K9me3

methyltransferases. This histone modification, a hallmark of silenced chromatin, has been correlated with small RNA-mediated transgene silencing (Shirayama et al., 2012 [this issue of Cell]; Gu et al., 2012) and is enriched on the Hrde sensor reported here (data not shown). In addition, multigenerational silencing of transgenes is promoted by HPL-2 and SET domain proteins (Shirayama et al., 2012). Though this related study did not examine the requirement for SET-25 or SET-32, it did find that MES-4, a histone H3K36 methyltransferase that participates in silencing of the X chromosome (Bender et al., 2006; Rechtsteiner et al., 2010), is also required for multigenerational inheritance. These observations suggest that the chromatin states involved in multigenerational silencing might be complex and could include a hierarchy, which merits further investigation. We have summarized a model of our current understanding of this pathway in Figure 2.6.

Multicopy versus Single-Copy Transgenes

Multicopy transgenes, intra- or extrachromosomal, are generally efficiently silenced in the germline of *C. elegans* (Kelly et al., 1997). This has been interpreted as an example of the RNAi machinery distinguishing self from nonself (Vastenhouw and Plasterk, 2004). In this model, repetitive DNA such as endogenous transposable elements or multicopy transgenes would give rise to dsRNA that is processed by Dicer to generate siRNA triggers to induce silencing.

As the pathways silencing multicopy transgenes and transposable elements share common factors, this phenomenon is of biological interest. However, it has also been a major technical roadblock for researchers studying germ cell biology who rely on reproducible transgene expression in the germline. The advent of MosSCI technology to produce single-copy transgenes has the promise to overcome this problem (Frøkjaer- Jensen et al., 2008, 2012). Interestingly, we and others observed that, in some cases, individual transgenes remain silenced even when present as single, intrachromosomal entities (N.J.L. and E.A.M., unpublished data) (Frøkjaer-Jensen et al., 2008). Indeed, an accompanying paper reports a collection of MosSCI transgenes that remain silenced (Shirayama et al., 2012).

Generating MosSCI transgenes in animals in which germline nuclear RNAi pathways are impaired, such as *mut-7*, or ‘‘curing’’ silenced transgenes by outcrossing first to germline RNAi mutants strains and then back to wild-type often results in loss of transgene silencing (N.J.L., A.S., and E.A.M., unpublished data). These results suggest that the original RNAi model of multicopy transgene silencing needs to be revised. Indeed, there appears to be no requirement for dsRNA intermediates in the silencing phenomena reported here either, as factors required for dsRNA-induced RNAi in the germline such as RSD-2 and RSD-6 (Figure 2.5 and Table 1) or RDE-1 and RDE-4 (Shirayama et al.,2012) are dispensable for single-copy transgene silencing.

Self versus Nonself

How does *C. elegans* detect single-copy transgenes and target them for silencing in the germline, or how does the animal distinguish self from nonself? The answer might lie in a combination of three factors: scanning germline gene expression by the piRNA pathway (nonself RNA recognition), licensing of germline transcripts (self RNA recognition), and unpaired genomic DNA in meiosis. Based on this and related work, we propose that the piRNA pathway can detect transgenes as sources of foreign RNA (nonself) and initiates targeted silencing of these transgenes (Bagijn et al., 2012; Lee et al., 2012 [this issue of Cell]). The piRNA pathway is perfectly suited for this task, as it provides a diverse and large set (~15,000) of small RNA triggers that are mismatch tolerant but do not depend on dsRNA generation (Ruby et al., 2006; Batista et al., 2008; Das et al., 2008; Wang and Reinke, 2008; Bagijn et al., 2012). Furthermore, endogenous germline transcripts are generally depleted in piRNA target sites (Bagijn et al., 2012). In addition, germline licensing pathways might act in the opposite manner to protect bona fide germline transcripts. A recent study reported such a phenomenon in mutants of the *fem-1* locus in *C. elegans* (Johnson and Spence, 2011). In this case, maternal transcripts were required to overcome silencing of an endogenous locus in a mutant background. Furthermore, the germline Argonaute CSR-1 associates with secondary siRNAs that map to many germline-expressed genes without inducing

silencing (Claycomb et al., 2009). Interestingly, CSR-1-bound 22G RNAs appear to match abundantly to single-copy transgenes that evade silencing (Shirayama et al., 2012). Taken together, a balance of nonself recognition by the PRG-1/piRNA pathway and self-recognition by licensing factors such as the CSR-1 pathway might determine the outcome of gene expression in the germline. This model helps to explain the apparent discrepancy between the facultative multigenerational inheritance that we observe here in our piRNA sensor and the obligatory multigenerational inheritance observed for a related piRNA sensor in a parallel study (Lee et al., 2012; Shirayama et al., 2012). Differences in the composition of the sensors, e.g., the inclusion of the coding region of the *his-58* gene in our sensor, might tip the PRG-1/CSR-1 pathway balance. However, this model fails to explain the ability of our piRNA sensor to be silenced or active depending on its multigenerational ancestry. In our crosses, the piRNA sensor became stably silenced when present in a heterozygous state for several generations (Figures 1.5 and S1.5). We propose that unpaired chromatin that has been subjected to silencing by a piRNA trigger can be subjected to an additional layer of silencing during meiosis that then makes the original piRNA trigger dispensable (Figure 2.6). Unpaired DNA silencing responses have been observed in *C. elegans* in the case of the unpaired X chromosome (Kelly et al., 2002; Bean et al., 2004) and for a mutant *fem-1* locus (Johnson and Spence,2011) and has also been found in other organisms (Hynes and Todd, 2003; Lee, 2005; Matzke and Birchler, 2005). Establishment of heritable silent chromosome domains that can be robustly maintained in the absence of the original piRNA trigger could be relevant to populations in which sources of piRNAs are polymorphic and may evolve rapidly in response to novel transposons or retroviruses.

Related Phenomena in Other Phyla

The core molecular pathway described here is reminiscent of related (co-) transcriptional pathways both in yeasts and plants (Moazed, 2009). Many yeasts and all plants and nematodes share key factors, such as the RNA-dependent RNA polymerases involved in secondary siRNA generation.

Though transgenerational phenomena have been reported in many animals, including humans (Hitchins et al., 2007), this class of polymerases and secondary siRNAs appears to be absent in Drosophila and vertebrates. However, it is interesting to note that Drosophila and vertebrates have a more complex piRNA system that includes an amplification loop termed ‘‘ping-pong,’’ which could function in a manner analogous to secondary siRNA pathways (Brennecke et al., 2007; Gunawardane et al., 2007). Despite differences in details of piRNA and secondary siRNA systems, common downstream silencing mechanisms may exist.

One, Few, or Many Generations?

Transgenerational phenomena have been observed over one or multiple generations (Grishok et al., 2000; Vastenhouw et al., 2006; Alcazar et al., 2008; Burton et al., 2011; Rechavi et al., 2011; Gu et al., 2012). In some cases, inheritance is stochastic; in others, Mendelian. Here, we report that piRNAs can trigger silencing that lasts for more than 20 generations (Figures 1.5A, 1.5B, and S1.5). Although maintenance of this memory is observed in 100% of offspring, establishment of strong piRNA-independent memory is not obligatory (Figure S2.5) and only occurs if a silent locus is heterozygous for several generations. This is reminiscent of ubiquitous yet stochastic inactivation of repetitive germline transgenes in many organisms, including *C. elegans*. Our study of transgenes targeted by an endogenous piRNA may recapitulate the fate of transposons that are transmitted in rare horizontal transfer events, in which a single transposon insertion could be subjected to dual layers of silencing, as the locus would likely remain heterozygous for a number of generations before potentially becoming fixed. It will be of great interest to identify the factor(s) that determines these distinct states of silencing.

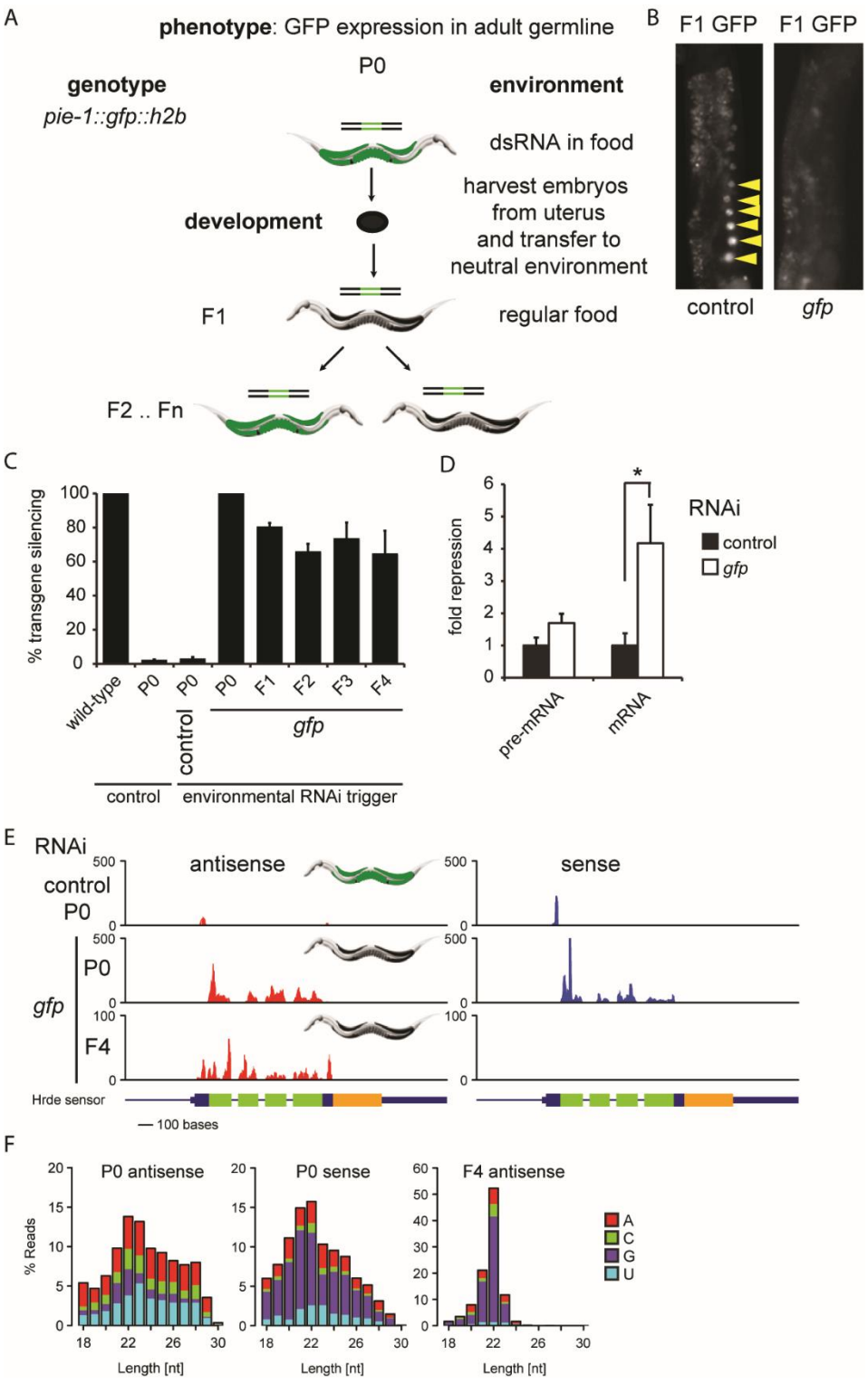


Figure 2.1. A Novel Inheritance Paradigm Demonstrates that Transgenerational Inheritance Is Associated with Continued Small RNA Production. (A) A diagram of the Hrde sensor inheritance paradigm. Green animals illustrate the germline expressed GFP sensor, whereas black worms represent

silenced animals. (B) Representative images showing the germline expressed transgene. The left panel shows the germline of an animal fed control vector, whereas the right panel shows the germline of an animal whose parent was treated with dsRNA targeting the GFP transgene. Arrows show the developing oocytes. (C) Graph showing the percentage of GFP silenced animals following exposure to GFP RNAi. Wild-type worms do not contain the *hrde* sensor;P0-F4 animals carry the sensor and differ only in their exposure to dsRNA. GFP fluorescence of the transgene and the percentage of silenced animals per plate were determined using a large particle biosorter and FlowJo. Ten silenced worms were selected from each plate to produce the next generation. At least 1,000 worms were analyzed per plate with the following number of replicates: P0 (GFP vector) n = 3, F1 n = 18, F2 n = 11, F3 n = 8, F4 n = 5. Error bars represent the SEM. Silencing was normalized to wild-type to account for autofluorescence of the intestine. (D) qRT-PCR showing levels of nascent, unspliced pre-mRNA and mRNA for the GFP transgene in silenced, GFP RNAi treated F2 and control worms. Fold change is shown relative to control and normalized to ama-1 expression. n = 4, 3, 4, 4 for pre-mRNA control, gfp, mRNA control and gfp, respectively. (E) Small RNA reads with unique perfect match in the transgene construct and no perfect match in the reference genome are shown for P0 and F4 L4 stage animals. F4 animals are the offspring of silenced animals in previous generations. Antisense and sense reads are shown in red and blue, respectively. Profiles indicate number of reads per million. Schematic indicates the transgene structure. Blue bars are pie-1 genomic DNA, 50 and 30 UTR, and exon (thin, medium, thick), respectively. Thick green/yellow bars represent GFP/his-58, respectively. (F) Size distribution of small RNA reads in (E). For each size, the relative contribution of small RNAs with a particular 50 nucleotide is represented in colors as indicated.Error bars represent SEM. See also Figures S1.1 and S1.2.

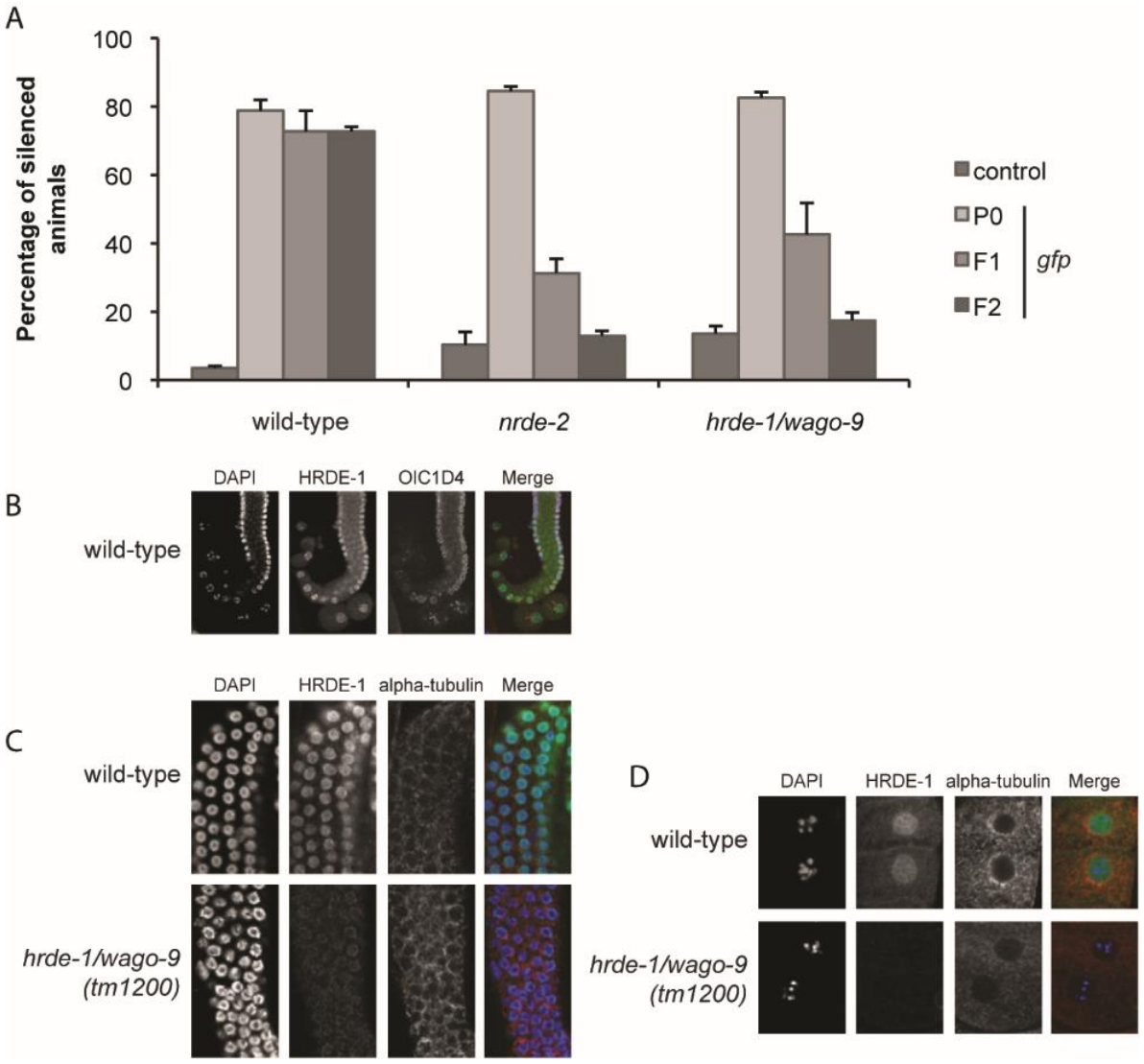


Figure 2.2. Transgenerational Inheritance Requires NRDE-2 and the Germline- Specific Nuclear Argonaute HRDE-1/WAGO-9. (A) Biosorter analysis of WT, *nrde-2*, and *hrde-1/wago-9* animals showing the failure of heritable silencing in these mutant strains. GFP fluorescence of the transgene and the percentage of silenced animals per plate were determined using a large particle biosorter and FlowJo. Ten silenced worms were selected where possible from each plate to produce the next generation. At least 500 worms were analyzed per plate with the following number of replicates (empty vector, GFP RNAi, F1, F2, respectively). WT, n = 7, 6, 4, 4; nrde-2, n =3, 3, 3, 3; *hrde-1/wago-9*, n = 12, 6, 6, 6. Error bars represent the SEM. (B) HRDE-/WAGO-9 is expressed in the germline. Wild-type dissected germlines (adults) were stained with anti-HRDE-1/WAGO-9 (green) and a P-granule-specific antibody (OIC1D4, red). DNA was costained with DAPI (blue). Images on the right are merged from all three channels. (C and D) HRDE-/WAGO-9 is a nuclear protein. Immunostainings were performed on dissected gonads from adult wild-type (N2) or *hrde-1/wago-9 (tm1200)* animals using anti-HRDE-1/WAGO-9 (green) and anti-a-tubulin antibodies (red). DNA was costained with DAPI (blue). Images on the right are merged from all three channels. Images shown are germ cells in the transition zone/pachytene region (C) and oocytes (D).

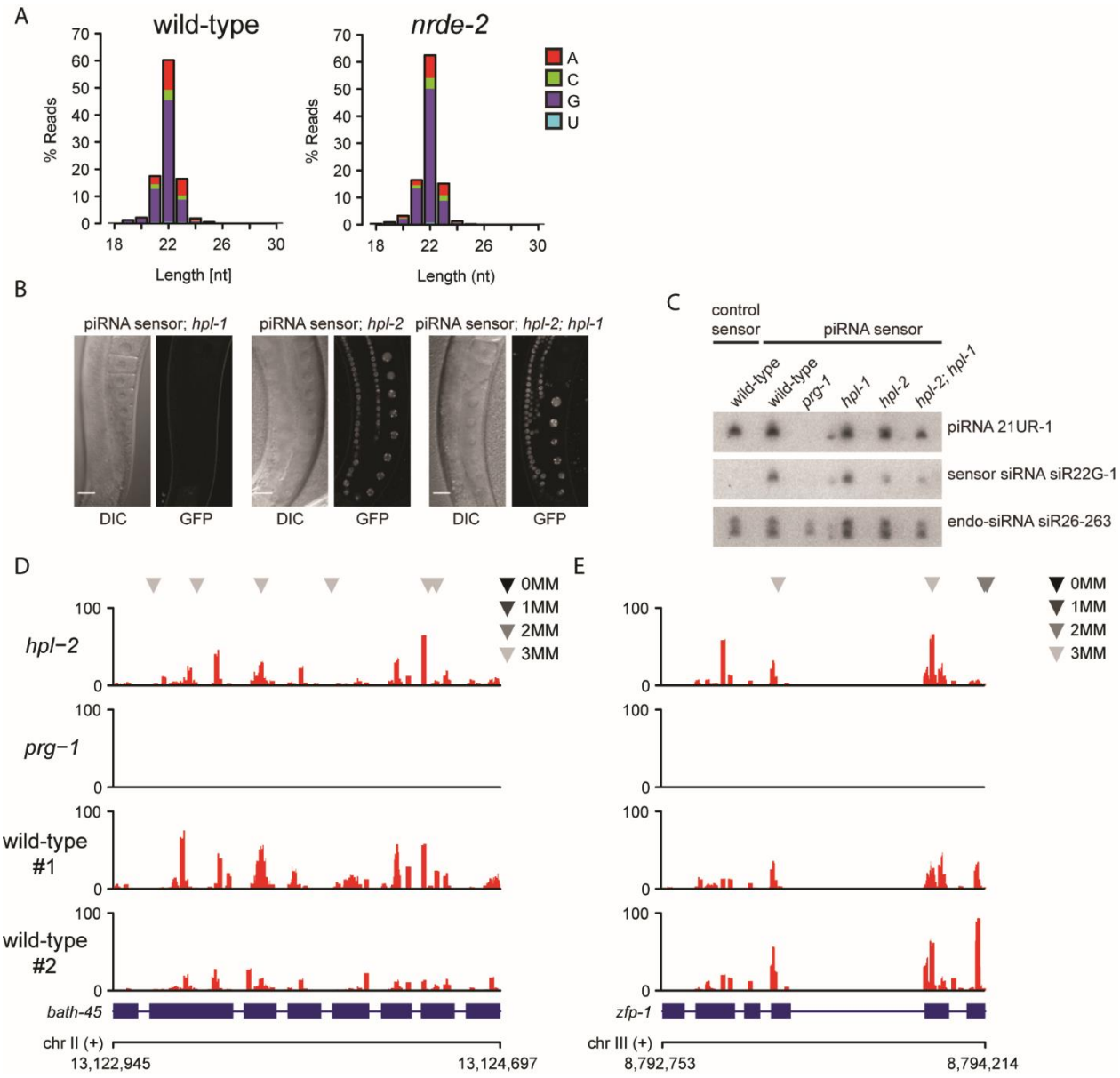


Figure 2.3. The Germline Nuclear RNAi/Chromatin Pathway Acts Downstream of Small RNA Production and Stability. (A) Size distribution of small RNA reads with unique perfect match in the transgene construct and no perfect match in the reference genome are shown for wild-type and *nrde-2* F2 animals. For each size, the relative contribution of small RNAs with a particular 50 nucleotide is represented in colors as indicated.(B) The heterochromatin protein HPL-2 is required for piRNA sensor silencing. DIC and fluorescence microscopy of piRNA sensor germlines in indicated mutant genotypes. Scale bars, 20 mm. (C) HPL-2 acts downstream of 22G-RNA biogenesis. Northern blot of total RNA from control sensor and indicated piRNA sensor strains. Probes were against piRNA 21UR-1, a piRNA sensor-specific 22G-RNA, and the Piwi-independent endo-siRNA siR26-263. For oligonucleotide sequences, see Bagijn et al., 2012. (D and E) Antisense 22G-RNA profiles are shown for selected elements. Profiles indicate number of reads per million. piRNA target sites are indicated above each profile as explained in the color key. See also Figure S2.3.

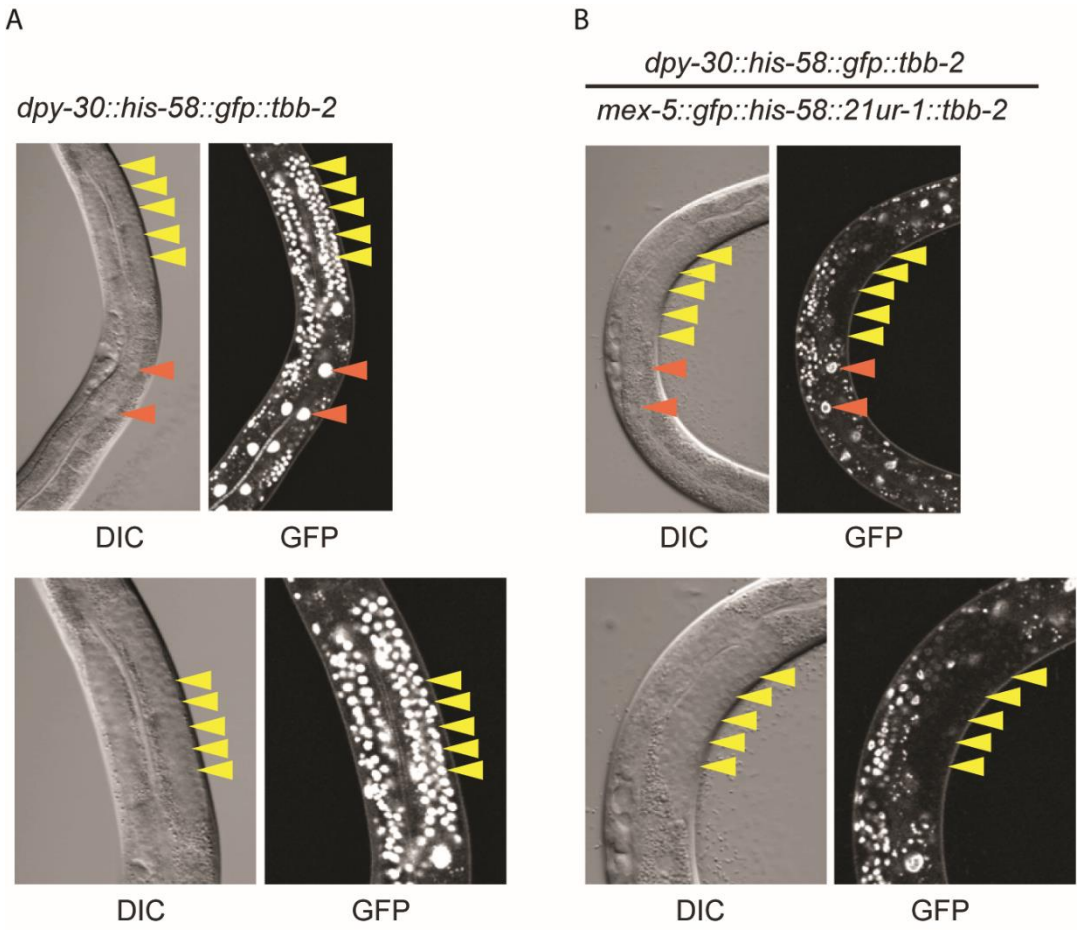


Figure 2.4. The Germline Nuclear RNAi/Chromatin Pathway Acts in trans but Cannot Exit the Germline. (A and B) trans-heterozygous animals were generated by crossing SX1866 hermaphrodites with piRNA sensor males. Strain SX1866 expressing H2B-GFP under control of the ubiquitous dpy-30 promoter was generated by MosSCI into ttTi5606 on chromosome II (mjSi1[*dpy-30::his-58::gfp::tbb-2*]). DIC and fluorescence microscopy of animals from the parental line (A) or of transheterozygous animals from the cross (B). Note that the parental line expresses H2B-GFP from two copies in the genome and is therefore brighter. Yellow arrowheads indicate germ cell nuclei; red arrowheads indicate somatic (intestinal) cell nuclei.

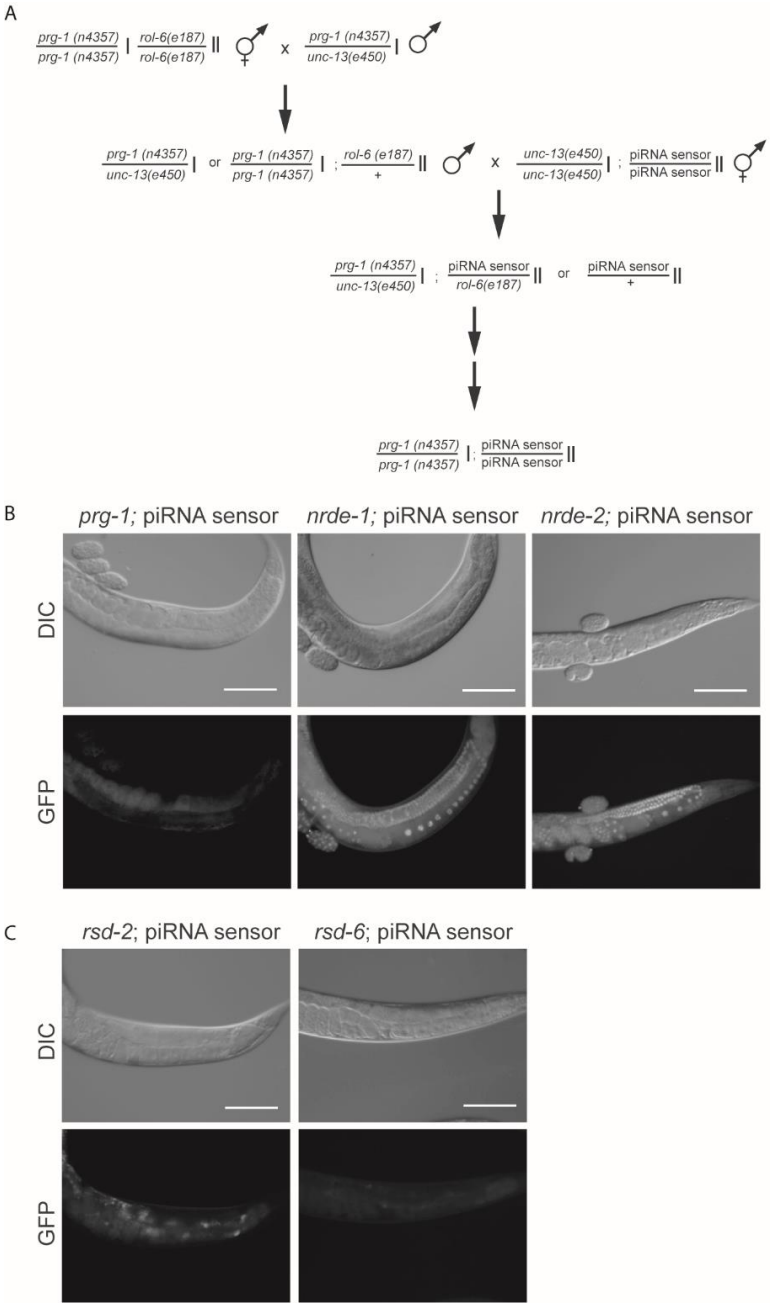


Figure 2.5. piRNAs Can Induce Stable Multigenerational Inheritance that Does Not Require PRG-1 for Maintenance. (A) Schematic showing generation of *prg-1*; piRNA sensor strain, which has lost the requirement for PRG-1 to maintain transgene silencing (for details of previous outcrosses, see Experimental Procedures). Analogous crosses were performed for nuclear RNAi factors, with *nrde-2* requiring further intermediate steps. (B) Following a number of crosses in a *prg-1* sufficient background, the piRNA reporter is desilenced in *nrde-1* and *nrde-2*, but not *prg-1*, mutant backgrounds. Differential interference contrast or GFP epifluorescence photos are shown. White bars correspond to 100 mm. (C) Outcrossed piRNA sensors fail to express GFP in *rsd-2* or *rsd-6* mutant backgrounds. Higher autofluorescence is observed for these strains, which were raised at 25°C, than for those in (B), which were raised at 20°C. See also Figures S1.4, S1.5, and S1.6.

Table 1. Multigenerational Environmental RNAi and piRNA Silencing Require a Common Nuclear Pathway

Genotype	Gene Product(s)	Reference Allele Tested	Alleles Described in This Study	Heritable Environmental RNAi Defective	piRNA Silencing Defective
wild-type	NA	NA	NA	–	-
nrde-2	novel	gg91, gg95	mj168 (Q135Stp)	+	+[a]
nrde-1	novel	gg88, yp4, yp5		ND	+
nrde-4	novel		mj249 (Q707Stp) mj259 (Q663Stp)	ND	+
hrde-1/wago-9	nuclear Argonaute	tm1200	mj278 (P720L)	+	+[b]
nrde-3	nuclear Argonaute	tm1116		ND	-[b]
sago-1	Argonaute	tm1195		–	–[b]
sago-2	Argonaute	tm894		–	–[b]
hpl-2	chromo domain	tm1489		ND	+
hpl-1	chromo domain	tm1624		ND	–
hpl-1; hpl-2	chromo domains	tm1489; tm1624		ND	+
set-25	SET domain	n5021		+	+
set-32	SET domain	ok1457		ND	+
met-2	SET domain	n4256		-	–
met-1	SET domain	n4337		ND	–
lin-59	SET domain	n3192		ND	–
set-2	SET domain	n4589		ND	–
set-6	SET domain	ok2195		ND	–
set-9	SET domain	n4949		ND	–
set-11	SET domain	n4488		ND	–[a]
set-12	SET domain	n4442		ND	–
hda-4	histone deacetylase	ok518		–	ND
mrg-1	chromo domain	qa6200		–	ND
spr-5	histone demethylase	by134		–	ND
lsd-1	histone demethylase	vr12		–	ND
amx-1	amine oxidase	ok659		–	ND
prg-1; prg-2	Piwi	n4357, n4358		–	+[b]
prg-1	Piwi	n4357		ND	+[b,d]
rsd-2	novel	pk3307		ND	–
rsd-6	Tudor domain	pk3300		ND	–
mut-7	RNase D	pk204		+[c]	+[b]

For heritable environmental RNAi and piRNA silencing assays, intrachromosomal single-copy transgenes were used as reporters. For heritable environmental RNAi, animals were scored at the F2 generation (see Figure 1A). For piRNA silencing, a sensor for the endogenous piRNA 21UR-1, the "piRNA sensor," on chromosome II was used (Bagijn et al., 2012). ND, not done.

[a]Tested using the piRNA cherry sensor on chromosome I, as described in Bagijn et al., 2012 .

[b]Previously reported in Bagijn et al., 2012 .

[c]These mutants were already defective in silencing in the F1 generation.

[d]Results are dependent on multigenerational ancestry of the animals (this study).

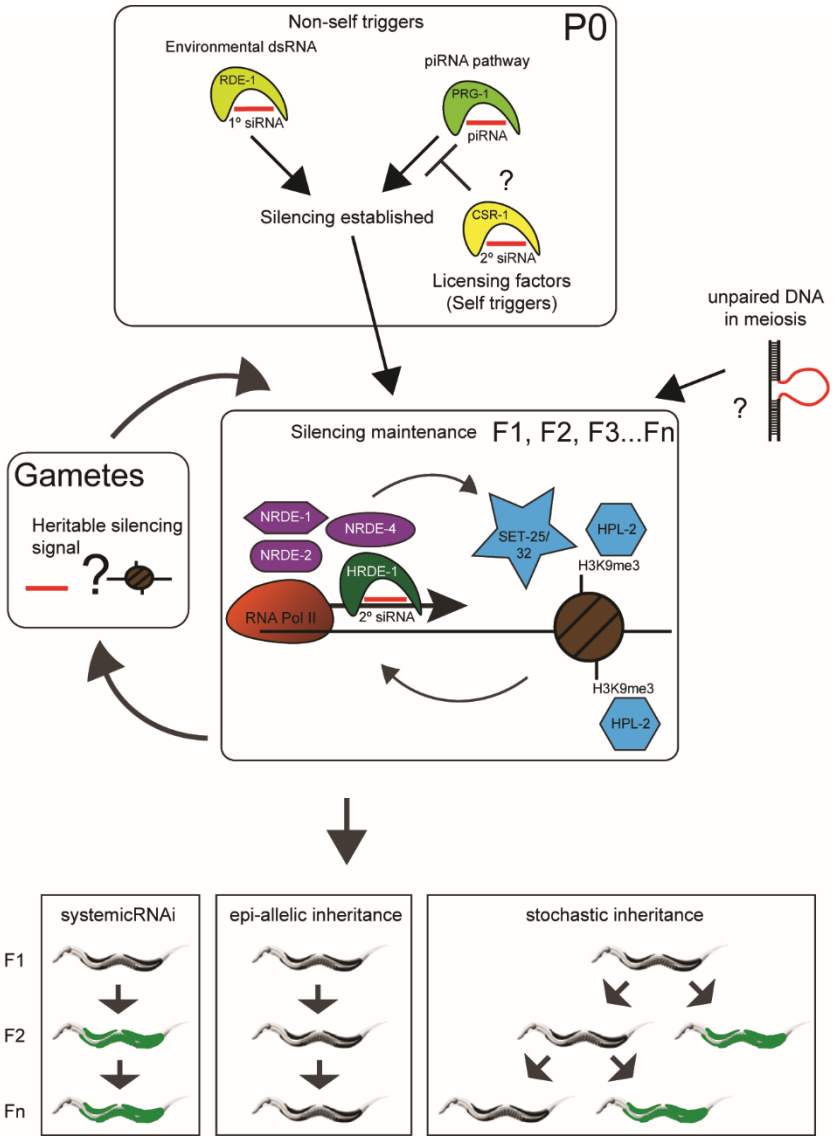


Figure 2.6. A Model of Transgenerational Silencing in the Germline of *C. elegans*. Triggers such as environmental RNAi and endogenous piRNAs lead to the establishment of a nuclear RNAi/chromatin pathway. Maintenance of silencing requires nuclear RNAi factors, including the germline-specific nuclear Argonaute HRDE-1/WAGO-9 and chromatin proteins such as the HP1 ortholog HPL-2 and the putative histone methyltransferases SET-25 and SET-32. Silencing can be maintained into the F1 for multiple generations (F1–F5) or can become epi-allelic with multigenerational, nonstochastic inheritance. Silencing might be suppressed by a germline licensing pathway that recognizes bona fide germline transcripts (CSR-1 22G-RNA pathway) or might be enforced through the recognition of unpaired DNA during meiosis.

CHAPTER 3: Analysis of the Mortal Germline mutant *nrde-1*.

Introduction

Understanding how the germline maintains both its proliferative capacity and the ability to self-renew over multiple generations could lead to a bevy of discoveries that can aid in ameliorating human aging and disease. Such mechanisms may illuminate measures of cellular rejuvenation or halting detriment from normal cellular processes. Sustained maintenance of the genome is imperative to the germline. Even between generations it must ward off threats both endogenous and exogenous while keeping the integrity of the DNA sequence. In order to do this, the germline has developed numerous mechanisms of cell survival; one of which among these is transgenerational epigenetic inheritance. Transgenerational epigenetics allows for the germline of the parent to transmit traits and information to their offspring without introducing DNA sequence mutations and compromising the genetic code. This allows a germline to relay acquired resistances to detrimental genetic elements to a whole new set of tissues in the progeny without the broad and prolonged screening of natural selection. In *C. elegans*, small RNA mediated silencing is crucial to transgenerational epigenetics and, as discussed above, imperative to that is the Piwi-piRNA pathway and the downstream nuclear RNAi silencing pathway.

The nuclear RNAi pathway was discovered in a screen to identify genes involved in the phenomena of silencing nuclear localized RNAs (Guang, et al., 2008). Initially, the pathway was described via the argonaute NRDE-3 a protein that contains a nuclear localization signal (NLS) and a characteristic PAZ and PIWI domain found in most argonautes. In contrast to most argonautes, NRDE-3 lacks the DDH catalytic domain predicted to be responsible for the slicer activity necessary for the cleavage of target mRNA. NRDE-3 has since been further characterized as being a somatic argonaute

responsible for shuttling 22G RNAs from the cytoplasm into the nucleus for target transcriptional silencing (Guang, et al., 2008; 2010).

Through genetic evidence two other components, NRDE-2 and NRDE-4, are demonstrated as downstream of NRDE-3 and required for the dsRNA triggered chromatin modifications associated with NRDE mediated transcriptional gene silencing. NRDE-2 works close to the site of the argonaute and aids in localizing small RNAs into the nucleus (Guang, et al., 2010). Although NRDE-4's specific role in the nucleus is not known, it has been shown to be required for NRDE-1 mediated recruitment of factors responsible for histone H3 lysine 9 trimethylation (H3K9 me3) at the chromatin (Burkhart, 2011).

NRDE-1 has dual associations in the nuclear RNAi pathway. One of NRDE-1's roles is to act in association with the other Nrde components on nascent pre-mRNA transcripts at the site of RNA Pol II activity. When assayed, immunoprecipitation of RNA using a gfp tagged version of NRDE-1 showed a ~30-70 fold increase in pre-mRNA associated with NRDE-1::GFP protein following RNAi induced silencing (Burkhart, 2011). This activity is dependent on the components NRDE-2 and the initial presentation of the targeting RNA via an upstream argonaute.

NRDE-1 additionally has a downstream role at the chromatin acting closer to the site of H3K9me3 silencing (Burkhart, 2011). ChIP experiments show that after exposure to a dsRNA trigger, NRDE-1 shows a ~6 fold enrichment of chromatin associated with the targeted loci while neither NRDE-2 nor NRDE-4 show significant enrichment (Burkhart, 2011).

A nuclear silencing process promotes germ cell immortality

Strains containing the *tir-1 ky388* mutation show progressive sterility but can be rejuvenated after outcrosses with N2 wild type (C. Bargmann, personal communication). Analysis of two other *tir-1* mutations, *tm1111* and *tm3036*, failed to reveal progressive sterility (n=2 strains per allele, 30 generations of growth), suggesting that *tir-1 ky388* contained a linked mutation responsible for its Mortal Germline phenotype. Three-factor crosses with *+ tir-1 ky388 + / unc-93 + dpy-17* heterozygotes

revealed a novel *mortal germline* mutation located just to the left of *tir-1*, that we named *mrt-4 yp4* (Figure 3.1A).

Analysis of ethylmethane sulphonate-induced *mrt* mutants that cause progressive sterility (Y. L. and S. A., unpublished data) revealed one mutation, *yp5*, with a map position similar to that of *mrt-4 yp4*. *unc-93 yp4 + / + yp5 dpy-17* but not *unc-93 + + / + yp5 dpy-17* or *unc-93 yp4 + / + + dpy-17* trans-heterozygotes resulted in progressive sterility (n=4, 5 and 6 strains propagated, respectively), indicating that *yp5* fails to complement *mrt-4 yp4* and that their Mrt phenotypes are likely to be caused by mutations in the same gene. Sequencing of ~120 kb in the genetic map interval of *mrt-4* revealed 4 mutations in genomic DNA from *mrt-4 yp4*, one of which caused a stop codon at amino acid Q692 in the predicted protein product of C14B1.6 (Figure. 2.1a). Genomic DNA of *mrt-4 yp5* possessed a G to T point mutation that in C14B1.6 is predicted to cause a G650D substitution in an amino acid that is conserved in C14B1.6 homologs in closely related nematodes (Figure. 2.1b). BLAST analysis failed to reveal homologs of this protein in more distantly related organisms.

C14B1.6 was shown to encode NRDE-1, a nuclear RNAi protein that promotes histone methylation at loci targeted by exogenous or endogenous dsRNA triggers (Burkhart, 2011). Nuclear RNAi proteins are ubiquitously expressed in *C. elegans* tissues and can function in both germ and somatic cells. To further understand the role of nuclear RNAi in germline immortality we tested whether the remaining components of the Nrde pathway show Mrt phenotypes. Like MRT-4/NRDE-1, a second nuclear RNAi protein NRDE-4 has no homologs outside of closely related nematodes and *nrde-4* mutants become progressively sterile at 20^{o}C or 25^{o}C (median germline lifespan of 10+/-2.7). However, *nrde-2* mutants remain fertile indefinitely at 20^{o}C and only become progressively sterile if propagated at 25^{o}C, whereas mutations in the Argonaute protein required for nuclear RNAi, *nrde-3*, does not result in progressive sterility at any temperature. Thus, the nuclear silencing proteins MRT-4/NRDE-1, NRDE-2 and NRDE-4 promote germ cell immortality (Figure 3.1c).

NRDE-1 represses trans-generational stress that triggers embryonic lethality, sterility and apoptosis

Sterility in *nrde-1* nuclear silencing mutants could occur as a consequence of gradual derepression of toxic genomic loci over the generations or as a consequence of an epigenetic switch that is unleashed after growth for many healthy generations to trigger sterility. To test these models, we examined brood sizes of freshly established maternally depleted *nrde-1* or *nrde-4* F3 siblings derived from outcrosses. Most F3 animals had modestly smaller brood sizes than wild type and failed to display embryonic lethal or sterile F4 progeny (mean percent lethality of 0.1 for N2; 1.07 *nrde-1 yp5* and 3.0 for *nrde-1 yp4*)(Figure 3.2). Analysis of successive generations of *nrde-1* animals revealed increased levels of embryonic lethality. The frequency of embryonic lethality was almost always more severe as individual animals aged, such that embryos laid during the first two days of adulthood were more likely to hatch. Analysis of individual worms from the penultimate generation, giving rise to only sterile progeny show lower brood size and a persistence of embryonic lethality. These data suggest that stochastic levels of heritable stress present in mid-generation *nrde-1* strains may accumulate naturally as hermaphrodite's age and manifests in inviable embryos.

Levels of embryonic lethality dropped in the final generation of *nrde-1* animals and cohorts of dark adults that possessed a reduced number of germ cells could be seen and could not be propagated further because they were completely sterile. Stochastic levels of sterile animals can be seen in earlier generations for both *yp4* and the *yp5* alleles, and the mean number of these germline defective animals increased in later generations (Figure 3.2). Although the heritable stress transmitted by *nrde-1* animals can cause low to moderate levels of embryonic lethality or sterility, these phenotypes can occur independently of one another in progeny of a single animal. These results are coincident with data in the literature whereby declining brood size over multiple generation was scored as a measure of germline mortality.

The Germline Specific Argonaute HRDE-1 Shows a Progressive Sterility Phenotype as Measured by the Mrt assay.

The heritable RNAi defective gene *hrde-1* was identified in a screen to uncover genes involved in multigenerational RNAi. As indicated by the localization of a GFP tagged version of the gene, HRDE-1 is a nuclear argonaute that is specific to the germline (Buckley, et al., 2012). HRDE-1 binds a subset of 22G siRNAs that can be triggered either exogenously, by exo-siRNA, or endogenously via endo-siRNA. endo-siRNA targets of HRDE-1 include germ cell specific coding genes as well as pseudogenes. To determine if *hrde-1* functions in maintaining genome integrity transgenerationally, animals were propagated for multiple generations and brood size was scored (Buckley, et al., 2012). HRDE-1 shows a temperature sensitive Mrt phenotype with animals scored showing no progeny by F5 when reared at 25°C. When assayed using the Mortal Germline assay as described by S. Ahmed et al, the median time to sterility is 13 (+/- 3.7) with complete sterility by F22 (Figure 3.4). The difference in these two techniques highlight the robust nature of the message being transmitted over the generations.

Germ Cell Remodeling Events in Sterile *nrde-1* Animals

To better assess the events taking place in the germline at the time of sterility, cohorts of sterile *nrde-1* adults were obtained and stained using the DNA binding dye 4',6-diamidino-2-phenylindole (DAPI), to reveal germline nuclear morphology. The *C. elegans* adult germline can be divided into four major sections. The distal region (or proliferative zone) defines the beginning of the germline and this is where the somatic distal tip cell forms a niche that forces a small set of germline stem cells (GSCs) to remain in a mitotic state. These GSCs undergo cell divisions that give rise to undifferentiated germ cells that continue to undergo mitosis. As the undifferentiated germ cells traverse the germline they move away from the signals from the distal tip and enter into meiotic S-phase at the border of what is called the transition zone. At the transition zone the nucleus of the cell takes on a distinctive crescent shape indicating early meiotic prophase. After the transition zone, germ cells encounter the meiotic zone. The

meiotic zone is defined by the cells progressing into pachytene and beginning to grow in size. As the germ cells travel the meiotic zone they encounter a bend in the germline that concludes the meiotic zone and leads to the last part of the germline, the proximal zone. The proximal zone is defined by oocytes going through diakinesis and subsequently arrest until fertilization by sperm. DAPI staining of late generation fertile *nrde-1* adults shows the average number of chromosomes is normal when scoring for bivalents in oocytes in the proximal portion of the germline, but abnormal oocytes carrying an increased number of chromosomes are occasionally encountered (FigureS2.2). DAPI staining of sterile *nrde-1* revealed four distinct phenotypes: germline occupying similar space as normal germline, atrophied germline made up of a small number of germ cells in the distal region, germline with germ cells that encounter signals from the transition zone to the meiotic zone, termed "short", and germline that appear completely devoid of any germ cells termed "empty" (Figure 3.3B). Further analysis of the distribution of each phenotype shows: 9% normal, 23% short, 44% atrophy, 24% empty for the *yp4* allele (n=206) and 22% normal, 32% short, 34% atrophy and 12% empty for the *yp5* allele (n=239).

Discerning the Source of the Stress via RNA Regulation.

Given *nrde-1*'s role in transgenerational epigenetic maintenance via transcriptional gene silencing; loss of *nrde-1* could result in long-term multigenerational loss of transcriptional regulation. To determine the effects of *nrde-1* loss on gene expression, we conducted RNAseq analysis. Whole animal RNA extracts were collected from three different lines of early and late generation *nrde-1* L4 animals. In addition, RNA from a wild type strain and a *nrde-2* mutant grown at the permissive temperature were collected as controls. Our results show, late generation *nrde-1 yp4* and *yp5*, in comparison to early generation populations, demonstrate a general dysregulation of gene expression. The dysregulation in gene expression includes genes showing increases in expression as well as other genes showing decreases in expression at some loci. The general misexpression is seen to be significantly greater in both the *nrde-1 yp4* and *yp5* late generations alleles than *nrde-2* (Figure 3.5a). Further analysis of the

late generation samples show the *nrde-1* alleles have a bias toward gene expression being upregulated with a large proportion showing greater than two-fold increase in expression. This is in contrast to *nrde-2* late generation populations, which display an overall greater downregulation of gene expression (Figure 3.5b). Due to the preponderance of genes having a general upregulation in late generation *nrde-1* and considering *nrde-1* has a role in silencing, we analyzed what genes showed the greatest amount of upregulation in the late generation *nrde-1*. In comparison to N2, both *nrde-2* and *nrde-1* show an increase in the expression of genes involved in spermatogenesis when comparing early generations versus late. When comparing the increase seen in spermatogenesis in the *nrde-2* grown at permissive temperature against that observed in the Mrt associated *nrde-1* mutants, a significant increase can be seen in the expression of spermatogenesis genes in late generation *nrde-1* alleles (Figure 3.5c and 2.5d). These results together illustrate that as *nrde-1* mutants progress over multiple generations, the absence of transcriptional gene silencing results in the misregulation of gene expression globally with spermatogenesis regulation being most affected at the fourth larval stage (L4) prior to sterility. Furthermore, when viewing the top nineteen genes that are upregulated in the late generation *nrde-1*, 42% correspond to genes involved in spermatogenesis (Table2.1).

As previously discussed, other members of the nuclear RNAi process show the Mrt phenotype and have been used to study the mechanistic underpinnings of the nuclear RNAi phenomena. ChIP-seq targeting H3K9me3, a marker of transcriptional gene silencing, in *nrde-2* and *nrde-4* mutants revealed 320-358 predicted sites regulated by nuclear RNAi when compared to wild type (Buckley, et al., 2012; Ni & Chen, 2014). When assaying *hrde-1* using this method, the authors found approximately 60% of those sites showed overlap (Ni & Chen, 2014). These regions have been designated germline nuclear RNAi heterochromatic loci or GRH. Additional studies using Pol II ChIP-seq performed on the *hrde-1* mutant further uncovered native targets by assaying Pol II occupancy in wild type versus *hrde-1*. Via Pol II ChIP-seq 191 1-kb sites were unveiled and these have been designated germline nuclear RNAi dependent

transcriptional silencing or GRTS (Ni & Chen, 2014). When correlating the *hrde-1* dependent germline nuclear RNAi dependent transcriptional silencing loci with germline nuclear RNAi heterochromatic loci the authors observed 50% of the germline nuclear RNAi heterochromatic loci overlapped with or were adjacent to the germline nuclear RNAi dependent transcriptional silencing loci.

Mechanistic insights of *hrde-1*, *nrde-4* and *nrde-2* outline the possibility that specific portions of the genome are regulated by the nuclear RNAi pathway. Furthermore, the lack of Nrde components from the germline causing germline mortality reveals that regulation of germline nuclear RNAi dependent transcriptional silencing loci and germline nuclear RNAi heterochromatic loci is important to transgenerational genome stability. To test whether any given gene or pathways were at the root of the germline mortality phenotype we tested whether reduced or loss of expression from genes upregulated in late generation animals could ameliorate the sterility phenotype seen in the late generation *nrde-1*. Initial analysis of the top nineteen genes upregulated in *nrde-1* revealed two genes that overlap with genes identified in germline nuclear RNAi heterochromatic loci clusters in *nrde-2/4* and *hrde-1* analysis (Table 2.1). No genes have been identified from our studies that overlap with the data provided for the germline nuclear RNAi dependent transcriptional silencing loci. The genes C01F6.2 and Y43C5B.3 are both uncharacterized in *C. elegans*. C01F6.2 has no known function in the *C. elegans* but domain analysis shows that C01F6.2 contains a potential SNF1 protein kinase subunit beta. To test whether reduction of C01F6.2 in late generation *nrde-1* would ameliorate the germline mortality phenotype, we conducted RNAi knockdown of C01F6.2. Early stage fourth larval late generation *nrde-1* yp4 animals from populations that contained sterile adults were fed either vector or C01F6.2 and scored for the presence of sterility through the third day of adulthood (Table 2.2). Initial analysis of C01F6.2 RNAi on adult's show no rescue of Germline Mortality as all parents fed RNAi showed complete sterility with one adult showing a brood size of 1 offspring. Further propagation of the one offspring showed no rescue of the sterility in the next generation. In comparison, siblings fed vector alone displayed 50% sterility (2/4

experiments) in the parental generation. For those animals that bore progeny, during the peak laying periods, number of offspring was an average of 25% of that seen in wild type. Analysis of the subsequent generation showed a similar 50% of the population as sterile.

aak-2 is one of two homologs of the catalytic alpha subunit of AMPK. We tested whether knockdown of AMPK, via *aak-2*, would alter the state of the Mortal Germline phenotype. Feeding vector control to *nrde-1 yp5* did not show any sterility in the parent generation. In the F1 40% showed sterility across the entire population. When sibling cohorts of *yp5* were fed *aak-2* brood size was two-fold higher and the average number of sterile animals measured to 25%.

Revisiting the idea that the Mortal Germline phenotype could be due to a specific gene being misregulated or a specific pathway being misregulated, we also further analyzed the phenomena that genes from the top nineteen upregulated genes in late generation *nrde-1* were involved in spermatogenesis. To this end, additional RNAi were conducted using the genes, *B002.4*, *fog-2*, *spe-26*, and *csr-1.* B0024.4 is an uncharacterized protein with unknown roles in spermatogenesis. B0024.4 appears the most upregulated in our analysis of late generation *nrde-1*. RNAi constructs targeting B0024.4 fed to late generation *yp4*, show 50% sterility in the adult parent. Progeny counts show 31% of that seen when the RNAi construct is fed to wild type and the average amount of sterility in this generation is 46%. Additionally, the gene *spe-26*, a cytoskeletal protein with a putative role in guiding components around the cell during spermatogenesis, appears upregulated. Parents fed *spe-26* RNAi did not result in complete sterility but did show very low brood (39 and 9 offspring at the peak time of fertility). Of those animals that survived an average of 46% showed sterility in the next generation.

In addition to addressing specific genes of spermatogenesis, to remove the entire process of spermatogenesis, we knocked down either *fog-2* or *csr-1*. *fog-2* is an F-box protein that is active during the transition between third to fourth larval stages of development. FOG-2 is crucial to the sex determination step whereby L4 hermaphrodites generate sperm based on autosome to sex

chromosome ratio. FOG-2 knockdown in *yp4* animals showed 60% sterility in the offspring with no immediate germline mortality in the parental generation. Brood counts were again low showing less than 40 offspring per plate during peak fertility. CSR-1 is an argonaute that has a primary role characterized in chromosome segregation but has an additional role in spermatogenesis in the licensing of genes for transcription (Conine, et al.,2010). For *csr-1* RNAi in late generation *yp4*, greater than 50% of the parents showed sterility and the brood size was 3 offspring. Of the 3 offspring laid all became sterile at adulthood. In the *yp5* background with experimental knockdown of CSR-1, animals show 17% the average brood size seen in wild type that were fed the same. One experimental plate became complete sterile in the parent generation and for the remaining population, the subsequent generation showed an average of 90% sterility. ALG-3/4 is also an argonaute responsible for binding a subset of 26G RNAs and its absence results in spermatogenesis defects in the adult hermaphrodite. When knocking down activity of ALG-3/4 in *yp5*, animals show an average of 42% sterility, similar to the 40% sterility seen in vector alone controls but the average brood size is only 15% of that seen in wild type control fed *alg-3/4* RNAi.

In addition to the genes mentioned above other genes from the list of top nineteen genes upregulated in late versus early generation *nrde-1* were also tested using the *yp4* allele. In all of these cases the experimental parent animals all showed complete sterility showing that knockdown of these genes does not provide any immediate rescue.

To determine whether an increase in activity of the genes found to have upregulated expression in late generation *nrde-1* could be involved in triggering Mortal Germline sterility we took a genetic approach. We generated double mutants with *nrde-1* and genes from the list of top upregulated genes and assayed for rescue or extension of germline mortality. When tested the median time to sterility for the *nrde-1 yp4* and *yp5* single mutant alleles are ~10 (+/- 2.89) and ~8 (+/- 2.33) generations respectively. Introduction of the *aak-2 rr48*, point mutation does not rescue germline mortality. *aak-2;*

nrde-1 yp4 has a median germline lifespan of 14 generations while *aak-2; nrde-1 yp5* allele shows a median generation time of 9 (+/- 1.4). To assess whether lack of *aak-2* and *nrde-1* alters the phenotype of the germline, DAPI of late generation sterile adults was conducted*. aak-2; nrde-1* show four categories of the germline arrangement similar to *nrde-1*. Of the 187 germline arms scored 27% appear normal, 28% can be characterized as "short", 33% are atrophy and 11% are empty. In addition to the testing effects of the mutation in the alpha catalytic subunit, we also procured a copy of a mutation of the uncharacterized gene *c01f6.2*. *c01f6.2*, *nrde-1* has a median germline lifespan of 9 generations (+/- 1.6) and sterile animals germ arms are distributed as 31% normal, 32% "short", 28% atrophy, and 9% empty (n=161).

The gene *parg-2* is a poly (ADP-ribose) glycohydrolase and is required for the normal resistance to ionizing radiation (www.wormbase.org). *parg-2* was introduced into the *nrde-1* background and two independent lines were assayed for Germline Mortality. *parg-2; nrde-1* show a median mortal germline phenotype of ~9 (+/-3.4) generations indicating that increased activity of parg-2 is not directly responsible for the resulting mortality phenotype.

Based on conservation of structure the gene *f53h4.6* is a predicted orthologue of the human gene SMARCAD1 and ERCC6. An *f53h4.6; nrde-1* double mutant strain could not rescue germline mortality. At the sterile generation, 28% of germ arms scored were normal, 43% were "short", 21% were atrophied and 7% could be classified as empty. Evidence from *hrde-1*, and *nrde-2/4* show that targets of the nuclear RNAi machinery lie in close proximity to one another, forming clusters that are targeted for silencing by endo siRNA (Sapetschnig et al. 2015). *f53h4.6* is 685 base pair upstream of the gene csb-*1*, a gene that is essential to overcome the deleterious effects of UV irradiation in somatic cells. *csb-1* is non-essential to the germline as it functions redundantly with *rad-23* and *xpc-1*, but increased expression and activity of the gene could have deleterious effects on the germline tissue. Removal of CSB-1 activity

via *csb-1; nrde-1* show a median time to mortality of 14 (+/- 3.8) generations indicating its activity is not the sole source of the mortality phenotype.

C. elegans has two sexes: the hermaphrodite and the male. Male *C. elegans* are the by-product of a sex chromosome non-disjunction and can occur 1 in every 500 animals. The hermaphrodite contains two copies of the X chromosome and use dosage compensation to down-regulate one copy. For the hermaphrodite to development a proper germline, a core sexual determination pathway must make way for processes mediating gametogenesis. When a hermaphroditic sexual fate is chosen, the germline will go through a period of spermatogenesis between the third and fourth larval stages and will transitions to strictly oocyte development in the adult. As previously noted 42% of the genes in Table 1 are genes that correspond to spermatogenesis. Misregulation of spermatogenesis genes in an epigenetic mutation showing Germline Mortality is not unfounded. The argonaute mutant's *csr-1* and *alg-3/4* have been cited as having spermiogenesis defects due to lack of activation of transcription in absence of CSR-1 and ALG-3/4. The histone H3K4me2 methyltransferase, MET-2, and the LSD1 H3K4me2 demethylase, SPR-5, work synergistically to promote cell fate in sperm. In these absence of these two genes H3K4me2 transgenerational inheritance is perturbed and typically somatic genes will express spermatogenesis genes ultimately leading to sterility.

Phenotypes that emerge as a consequence of germline mortality in *nrde-1* also appear around the L4 stage. In populations that show sterility, evidence of animals that show defects in spermatogenesis can be observed. The identification of chromosomal abnormalities such as multinucleated sperm cells can be seen but thus far the detection of gross loss of the germ cells during the beginning of the fourth larval stage has not been clearly detected (screened 100 DAPI stained young- to mid- staged fourth larval animals from populations of *nrde-1 yp4* and *nrde-1 yp5* that contained sterile adult siblings from medium populations). Additionally, screening of late generation third larval

stage *nrde-1 yp4* by Normarski DIC did not reveal the presence of any vacuole-like structures (99/100 animals show no germline morphology defects with 1/100 showing a defect near the vulva) Figure S3.1.

To test whether loss of the process of spermatogenesis could ameliorate the onset of sterility in late generation *nrde-1* animals we genetically removed *fog-3*. FOG-3 is required for germ cells to take on sperm fate and in its absence a genetically hermaphroditic *C. elegans* will only produce oocytes. We generated a *fog-3, unc-29/+; nrde-1* and assayed for Germline Mortality. Homozygous *fog-3,unc-29; nrde-1* animals obtained from late generation populations showing sterility were outcrossed to wild type males and assayed for amelioration of germline malfunction via the ability to rescue sterility. Outcross to a control late generation *fog-3, unc-29/+; nrde-1* that undergo normal hermaphrodite germline development show 88% sterility (n=27). *fog-3; unc-29* outcrossed to wild type showed a 32% (n=19) inability to mate with males. When late generation *fog-3, unc-29; nrde-1* homozygotes were outcrossed to wild type males 82% (n=36) of the animals remained sterile (Figure 3.8). Comparing *fog-3, unc-29; nrde-1 and fog-3, unc-29/+; nrde-1* to one another show they are statistically the same (Z=0.7, p=0.541). Comparing either population to the *fog-3; unc-29* control shows a difference between the populations (Z=3.7, p=0.000251 for *fog-3, unc-29; nrde-1* and Z=3.9, p= 0.001 for *fog-3, unc-29/+; nrde-1*). Outcrossing single *nrde-1* sterile adults to age matched wild-type males resulted in 98% and 97% sterility (41/42 and 26/27) in *yp4* and *yp5* alleles respectively.

A Histone MCHERRY Transgene Reveals a Developmental Delay in Late Generation *nrde-1*

The strain *EG4883* contains the *spe-11:: histone:: mCherry* transgene insertion which has been characterized as expressing mCherry in the sperm of hermaphrodite germline. As a means to mark sperm generated in late generation *nrde-1* animals, a copy of *EG4883* was placed in the *nrde-1* background. *EG4883; nrde-1* was propagated until signs of Germline Mortality and germline were screened for the presence of HISTONE::MCHERRY. Late generation adults of both *EG4883* alone and the *EG4883; nrde-1* double mutant show similar expression of HISTONE::MCHERRY (Figure 3.9). When L4

larvae of late generation animals of both these strains are compared MCHERRY fluorescence can be seen in the *EG4883* strain but not in the *EG4883; nrde-1* double mutant (Figure 3.9).

Discussion

Here we have been able to document the multigenerational decline in function of the germline in the epigenetic mutant *nrde*-1. Where somatic tissues only function during a single generation and die; the germline serves to bridge the divide between parent and offspring, establishing tissues and maintaining and recording changes in the genome for each generation. As a consequence, *nrde-1*, as well as the other components of the germline nuclear RNAi machinery, and their role in maintaining silencing of small RNA targeted loci serve as key components of maintaining the germlines function.

One possible scenario evident explaining the Germline Mortality in absence of Nrde function could be that lack of silencing at the germline nuclear RNAi heterochromatic loci and germline nuclear RNAi dependent transcriptional silencing loci opens up chromatin and slowly causes upregulation of expression of a gene(s) that cause sterility in the late generation animals. This scenario presents an appealing situation as a specific gene or process would serve as a source of stress for a highly robust and replicative tissue such as the germline. This scenario presents an obvious target for therapeutics to possibly reduce toxic load. Recent findings have uncovered that *hrde-1* and *nrde-1/2/4* are required for the generation and/or stability of tertiary 22G siRNA that are generated downstream of 21U piRNA and 22G secondary RNA (Sapetschnig, et al. 2015). These tertiary 22G RNA are required for the distal spreading of the silencing signal in a 3’ to 5’ manner. Bearing in mind that Nrde targeted germline nuclear RNAi dependent transcriptional silencing loci and germline nuclear RNAi heterochromatic loci lie in clusters, it is conceivable that massive loss of the heterochromatic state over collectively large areas of the chromatin could make way for transcription factors to bind and upregulate a hazardous target from either within the germline nuclear RNAi dependent transcriptional silencing loci and

germline nuclear RNAi heterochromatic loci or in a neighboring area. Since there is some consistency to the germline nuclear RNAi dependent transcriptional silencing loci and germline nuclear RNAi heterochromatic loci, it is conceivable for this to happen reproducibly, subsequently leading to sterility of a whole population. Much of the literature suggests that the major culprit of damage that small RNA silencing pathways target are the selfish genetic elements such as transposable elements. Of note is the fact that Ni *et al* were able to identify an enrichment for LTR retrotransposons but not DNA transposons in the germline nuclear RNAi dependent transcriptional silencing clusters. Analysis of our RNAseq data revealed that although we were able to see an increase in the expression of *C. elegans* transposable elements, expression of these elements did not correlate with Germline Mortality as *nrde-2* animals grown at the permissive temperature showed the highest levels of expression (Figure 3.7).

Individual knockdown of some of the more common and best characterized genes from those we found most closely correlated with Germline Mortality did not consistently ameliorate phenotypes affiliated with declining function of the germline. Neither did functional disruption of these pathways via genetic ablation. This does not exclude the possibility that there is a targetable gene or set of genes as the rescue may rely on a gene whose expression was not within the top nineteen genes assayed but with whom even the slightest misregulation has dramatic repercussions. Alternatively, the large upregulations of these genes may require downregulation via RNAi of a combination of genes at once.

Some endogenous small RNAs serve critical roles in the timing of development of the animal. The loss of the ability to respond to a subset of endo-siRNAs in the absence of *nrde-1* may result in loss of proper regulation of development. In this scenario there may be a slow loss of germline maintenance due to the misexpression of genes in or around the germline nuclear RNAi dependent transcriptional silencing loci or germline nuclear RNAi heterochromatic loci at inappropriate times. Inspection of many of the genes listed in the germline nuclear RNAi heterochromatic loci and germline nuclear RNAi dependent transcriptional silencing regions with 3-fold enrichment in ChIP-seq experiments do not show

any obvious genes responsible for the regulation of germline development. Incidentally, many of the genes regulated by Nrde mediated silencing seem to be highly uncharacterized. Another interesting observation is that if this scenario is true, we cannot see the effects of this developmental abnormality until after the fourth larval stage. During this time the germline of those animals that are going sterile undergo gross morphological changes including the appearance of vacuole-like structures. Lack of earlier sign of this phenotype may outline a lack of sensitivity from the Mrt assay or it may be evidence that the Nrde pathway requirement is initiated near the end of the fourth larval stage. Certainly, a GFP tagged *hrde-1* transgene does show expression in the oocyte and sperm, but there is also expression of *hrde-1::GFP* in the undifferentiated portion of the germline and in the germline of earlier larval stages as well. Possibly *hrde-1* plays multiple functions in different areas of the germline and at different larval stages. This issue may also help shed light on an additional inconsistency where *hrde-1* is a temperature sensitive Mrt and *nrde-1/4* show progressive sterility at all temperatures tested. In *nrde*-1 late generation sterile animals, sperm can be seen in the proximal portion of the germline despite the absence of germ cells in the distal portion of the germline arm. Ideally, this means that the gross morphological disruptions present at adulthood are somehow triggered after the completion spermatogenesis in the L4 hermaphrodite. This information somewhat coincides with observations seen in the *nrde-1* late generation mutants. It alternatively argues that transgenerational stress may be sensed and responded to in a different manners during different times of development. For instance, the process of spermatogenesis may somehow be safeguarded against the accumulation of damage or sperm may be robust enough to withstand the chromosomal abnormalities that result from accumulated damage and less fit sperm will be outcompeted. For unknown reasons accumulated damage during oogenesis results in a great deal of absence of germ cells in the germline. Hypothetically this could be due to downregulation of a process that normally guards the germline from the effects of accumulated transgenerational stress or it could be that quality of the oocyte is so imperative that when

the germline senses it cannot maintain a standard it ceases germline function. As evidenced in Figure 3.8, experiments that try to rescue fertility by creating feminized *nrde-1* that only generate oocytes show that damage in the female germline may also play a role in the sterility that is seen in Germline Mortality. One caveat that could possibly explain the lack of fertility is a lack of willingness or ability to mate. Incidentally, penultimate generations of *nrde-1* show increased amounts of embryo lethality prior to complete sterility. This may be evidence of an imperfect metric the germline uses to see how fit it is to generate offspring. Coincident with the idea that the germline may be able to sense and respond to accumulated stress is the idea that it may be able to sense the alleviation of that stress and resume function. Ideally this would present itself as any of the sterile germline phenotypes being able to repopulate the germline and resume function.

A large number of genes found to be misexpressed in the late generation *nrde-1* animals showed to be affiliated with spermatogenesis yet we did not find that removal of spermatogenesis nor competition from wild type sperm were able to rescue the sterility of *nrde-1* mutants. This leads us to believe that problems that occur at the sterile generation of *nrde-1* present themselves not only in the male germline as seen by misexpression during spermatogenesis but also in the female germline which in feminized *nrde-1* did not show complete rescue. One possible explanation of this is that the misexpression of spermatogenesis associated genes we see at the L4 stage still show continued misexpression into the adult where oogenesis takes place. A means to better assess this problem would be to conduct qRT-PCR on early versus sterile generation L4 larvae and adults and wild type controls and check whether genes from the list of top 19 misregulated genes are inappropriately expressed in the germline of adults. It would additionally be interesting to see whether feminized sterile generation *nrde-1; fog-3* L4 larva and adults show expression of the misregulated spermatogenesis genes as well. It would logic then that despite the lack of FOG-3 activity to instruct the germline to undergo spermatogenesis lack of NRDE-1 function still causes these spermatogenesis genes to be expressed.

These two pieces of evidence would lend to how important it is to have tight regulation of transcription during development.

In addition to the qRT-PCR experiment presented above, to better define the source of the stress and its transmittance, reciprocal crosses can be made using *nrde-1* males and feminized *nrde-1;fog-3* animals. Mating late generation *nrde-1* males to early generation feminized *nrde-1; fog-3* mutants and tracking their time to sterility it would be possible to see whether the stress of Germline Mortality is passed through the male germline. Reciprocally, mating late generation *nrde-1; fog-3* females to early generation *nrde-1* males would give an indication if stress is passed via the female germline. Additionally, RNA deep sequencing on the first and second generations of either of these crosses and assaying whether progeny from a particular cross yields an upregulation of the genes we have already identified as misexpressed would lead us to better understand how the *nrde-1* accumulated stress is transmitted.

Analysis of the feminized *nrde-1* show that there is no rescue of the sterility defect in the animals when comparing the *nrde-1; fog-3, unc-29* animals to their siblings that are *nrde-1; fog-3, unc-29/+*, or the control *fog-3;unc-29*. This data parallels what is seen when the sterile *nrde-1* single mutants are outcrossed to wild type males and argues that neither sperm defects nor non cell autonomous influences from spermatogenesis are the sole reason for the Germline Mortality phenotype that is present.

Lack of *nrde-2* Mrt affiliated increase in transposable elements, distinctions between *hrde-1* Mortality and *nrde-1/4* Mortality and the obvious effect that loss of Nrde components have on gametogenesis argue that there may be multiple sets of functions that the Nrde pathway controls. Recently, much work has been conducted outlining possible rationales for the observed germline mortality of the PIWI argonaute *prg-1*. *prg-1* acts upstream of *nrde-1* in the gene silencing pathway. Independently two groups have identified a synthetic sterility phenotype reminiscent of the types of

phenotypes seen in both *prg-1* and *nrde-1* animals at sterile generation (Phillips et al, 2015; de Albuquerque et al. 2015). In both instances the authors removed piRNAs, via *prg-1* mutations, and were able to deactivate the pathway necessary for the production of downstream secondary siRNA to produce these immediate phenotypes. Using this as a tool the authors were able to conclude that the absence of piRNA-dependent siRNA pools may free up an Argonaute which is responsible for binding these small RNAs in the germline and allow it to take on inappropriate binding of a less favorable small RNA, siphoning off pools meant for specific binding. They propose one such Argonaute is *hrde-1*. The authors show that *hrde-1* is capable of binding known *csr-1* small RNAs and conclude that it may cause inappropriate silencing of their target genes once bound. Furthermore, the authors show, independently, that reducing the activity of *hrde-1*, can ameliorate phenotypes associated with sterility linked to loss of piRNAs including enacting a ~50% increase in fertility at the sterile generation.

HRDE-1 is the upstream argonaute of NRDE-1. To identify if there are any ameliorative effects that *nrde-1* may have on *prg*-1, a *nrde-1* mutation was introduced into two different alleles of a *prg-1* mutant. Loss of NRDE-1 activity does not rescue the Germline Mortality phenotype associated with both *nrde-1* and *prg-1* in the *prg-1; nrde-1* background (Figure 3.10A).

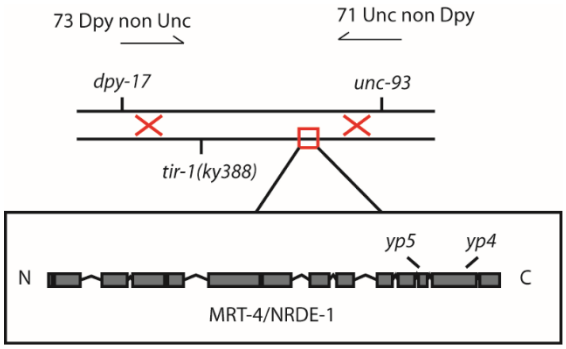
DAF-2 is a receptor tyrosine kinase and is the *C. elegans* orthologue of the insulin/IGF receptor. DAF-2 negatively regulates the forkhead transcription factor DAF-16 and mutations in *daf-2* endow somatic longevity. In the PIWI deficient background of *prg-1* mutants, the loss of insulin signaling via *daf-2* mutation results in a rescue of Germline Mortality that is DAF-16 dependent (Simon, et al., 2014). *nrde-1* was identified as required downstream of *prg-1* in the silencing of a piRNA sensor. The absence of DAF-2 activity in a *nrde-1* background results in an extension of the time to Mortality (Figure 3.10B) in contrast to the rescue seen in *prg-1; daf-2* animals as reported in the literature. To test whether the absence of piRNAs could restore germline immortality a *prg-1; nrde-1, daf-2* triple mutant was develop. Initial analysis of *prg-1; nrde-1, daf-2* populations show there is an extension on the onset of Germline

Mortality greater than that of *nrde-1, daf-2* but not a complete rescue as some strains do show Mortality (Figure 3.10B).

Given some of the evidence presented here we can postulate that late generation *nrde-1* animals undergo delayed development in the germline and that developmental delay coincides with an increased misexpression of a number genes in the germ that potentially are toxic to the tissue and either hinder its further development or triggers the degeneration of the germline.

FIGURES

A



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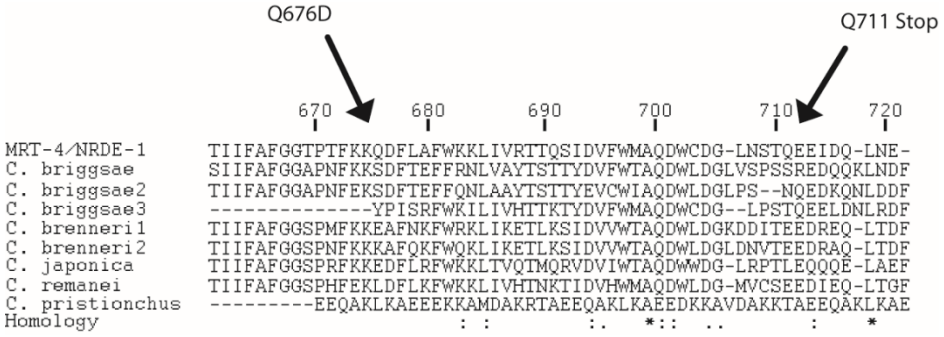

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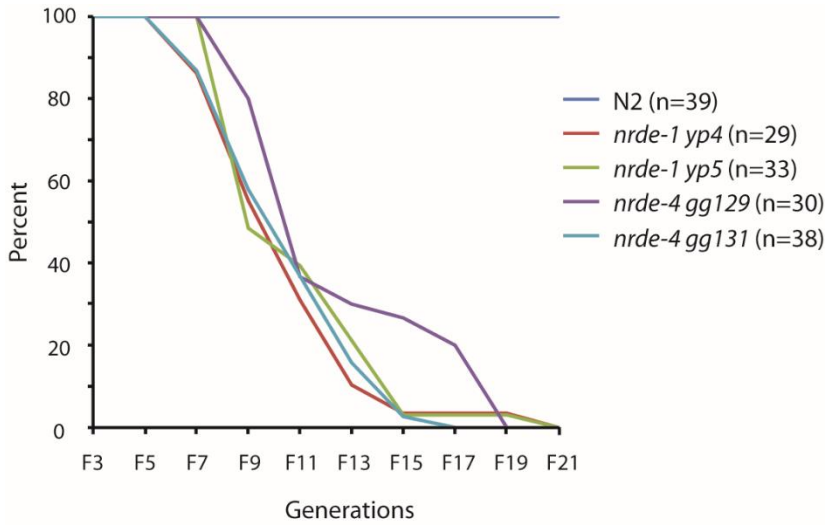
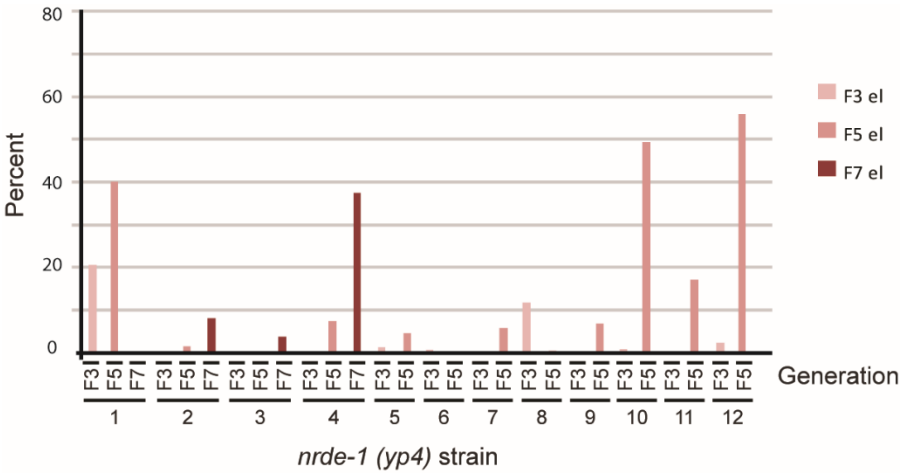


Figure 3.1. The MRT-4/NRDE-1 nuclear silencing protein promotes germ cell immortality. (A) Three-factor mapping of *nrde-1* revealed a genetic map position at -4.6 on Chromosome *III*. Sequence analysis of this region of the genome revealed mutations in the gene C14B1.6 for two *nrde-1* alleles. (B) Alignment of NRDE-1 homologs in five Nematode species. c) Progressive sterility is observed for *nrde-1 yp4, nrde-1 yp5, nrde-4(gg129) and nrde-4(gg131)* (*yp4*=8 ±4, *yp5*=8±2.9, *gg129*=10±3.7, *gg131*=10±2.7 generations).

A



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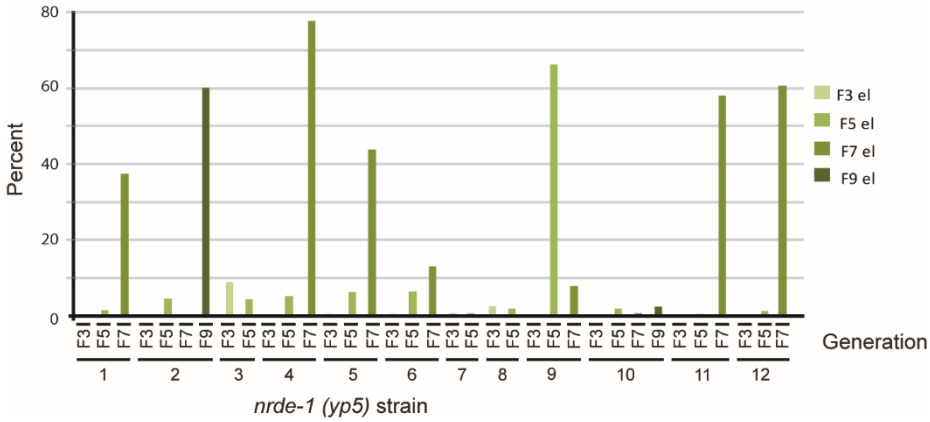


Figure 3.2. Embryonic Lethality in *nrde-1*. Phenotypes for progeny of individual *nrde-1* lines for *yp4* (A) and *yp5* (B). Lines underneath each grouping of bar denotes an individual line initiated by a single parent. Generation time is measured up until the penultimate generation as lines became completely sterile at final generation.

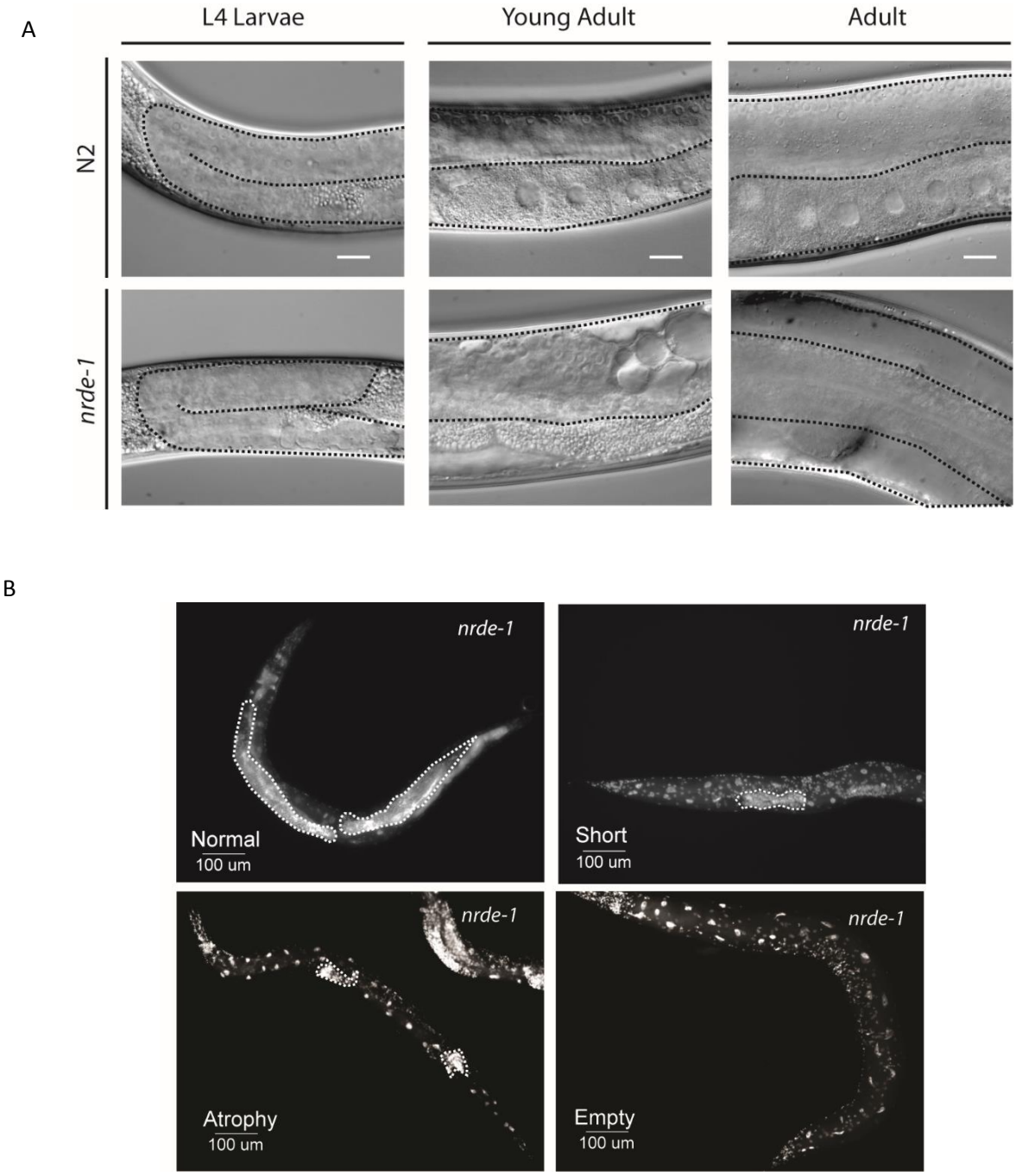


Figure 3.3. Germ cell remodeling occurs in sterile *nrde-1* adults. (A) Differential Interference Contrast images of late generation *nrde-1 yp4* L4 larvae and young adults. (B) Representative images of "Normal" (upper left), "Short" "Atrophy" and "Empty" DAPI-stained late-generation sterile *nrde-1* adults.

A

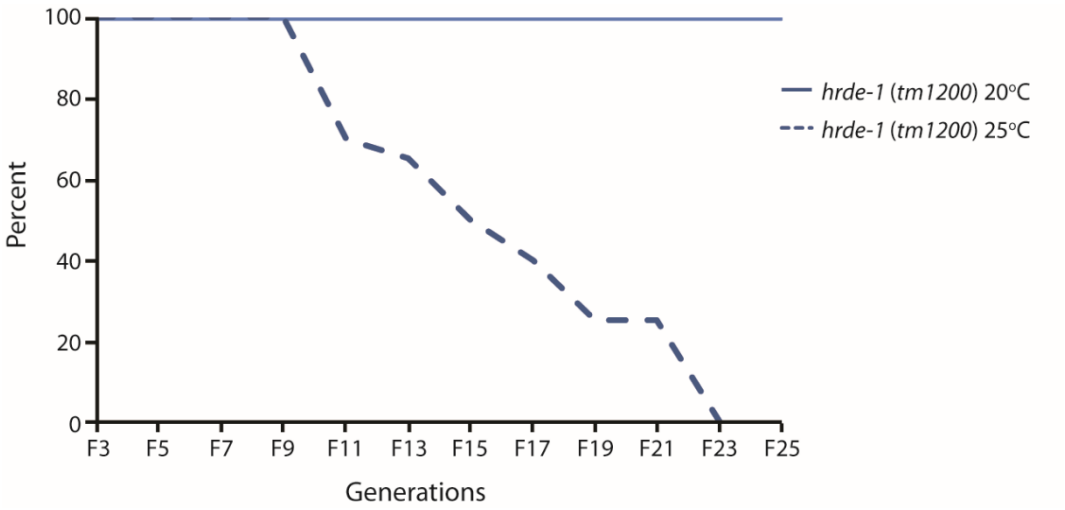


Figure 3.4. Hrde-1 displays temperature sensitive mortal phenotype. Strains of *hrde-1*(tm1200) were propagated at 20°C and 25°C and scored for germline mortality using the Germline Mortality assay developed by Shawn *et al.*

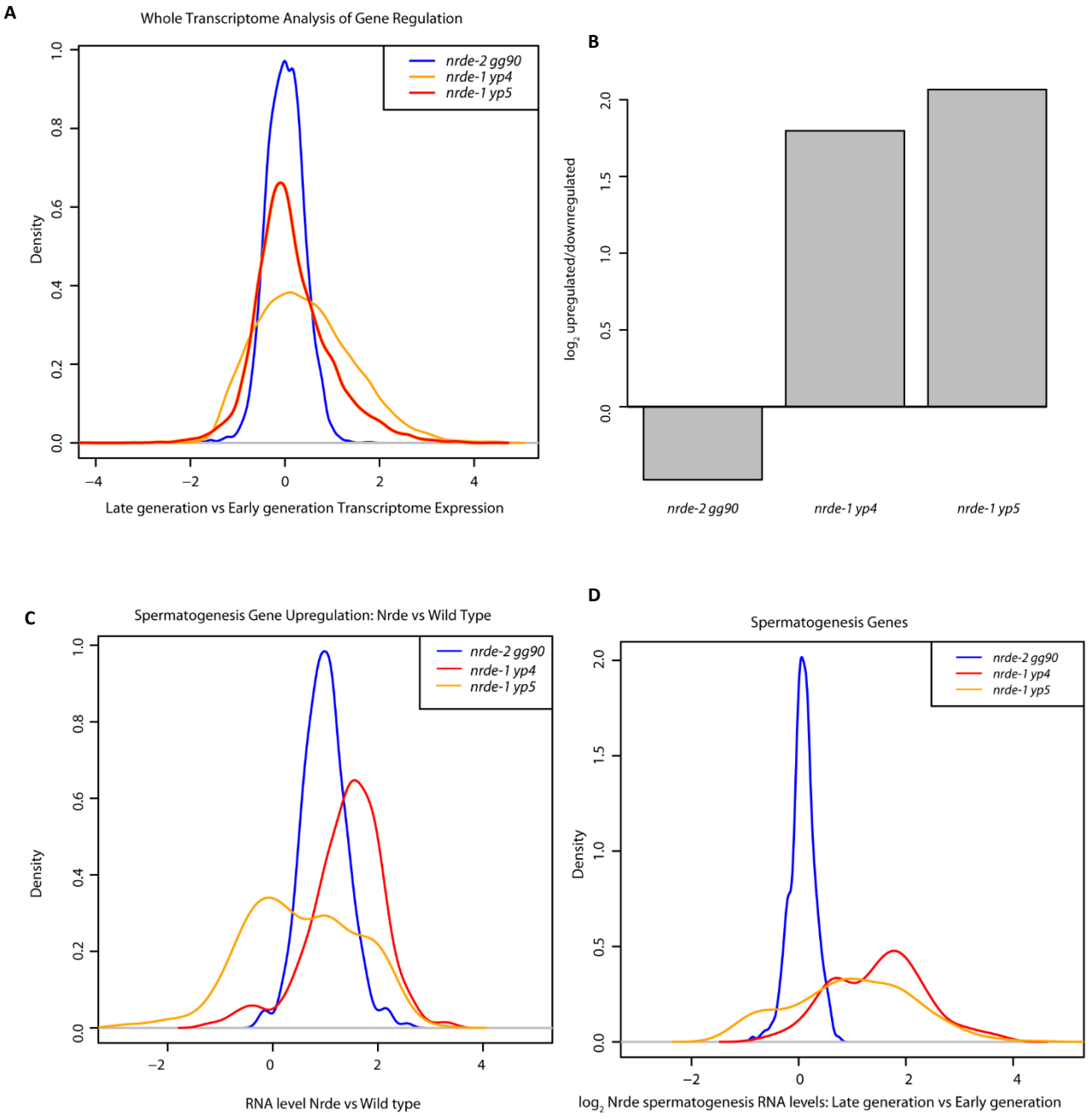
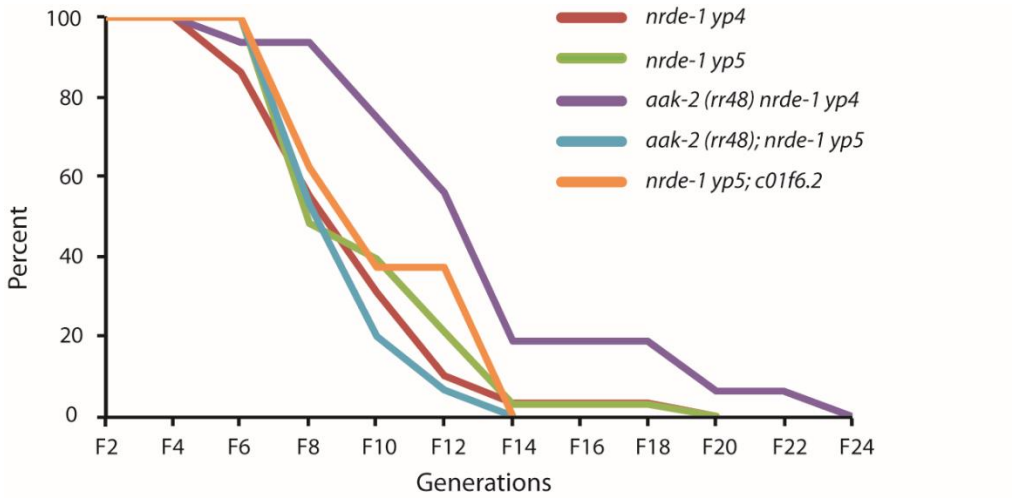


Figure 3.5. RNAseq analysis of gene expression in late generation Nrde animals. (A) Density plot from whole transcriptome expression RNAseq contrasting late generation strains of nrde-2 *gg90* at permissive temperature and *nrde-1* alleles *yp4* and *yp5* against the early generation corresponding genotypes. (B) Bar plot analysis showing fold increase/decrease of genes >2 fold change in late generation vs early generation *nrde-2* and *nrde-1* genotypes. (C) Density plot analysis comparing the change in levels of spermatogenesis of late generation *nrde-1 yp4* and *yp5*, and *nrde-2 gg90* to a wild-type counterparts. (D) Density plot examining spermatogenesis gene expression levels in late generation *nrde-2 gg90* and *nrde-1 yp4* and *yp5* alleles against the early generation corresponding genotypes.

A



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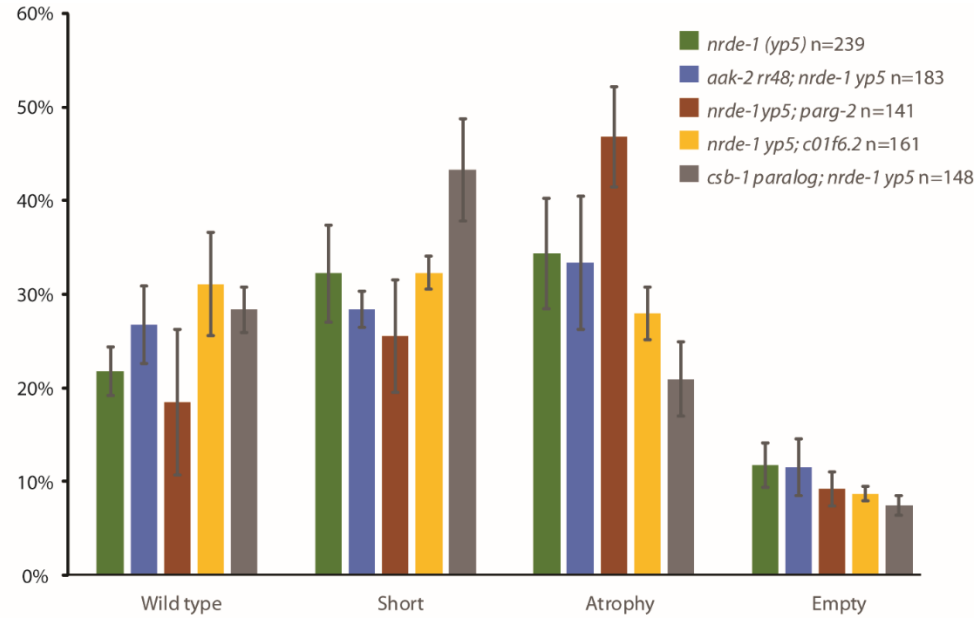


Figure 3.6. Genetic inactivation of genes upregulated in late generation *nrde-1*. (A) Germline lifespan analysis of double mutants from *nrde-1 yp5* background carrying a mutation in a gene from the list of the 19 highest upregulated genes in the late in generation *nrde-1* L4 larvae. The gene *c01f6.2* showed no significant amelioration (log rank test, p=0.205) of the Mortal Germline phenotype of *nrde-1 yp5*. In the *nrde-1 yp4* but not the *nrde-1 yp5* allele, *aak-1 rr48* shows a significant amelioration of the time to sterility (log rank test p=0.0106 and p=0.419 respectively). (B) Quantification of the germline phenotypes seen at the time of sterility for *nrde-1 yp5* mutants that lacked activity of one of the top 19 upregulated genes at the time of sterility. Error bars represent standard deviation.

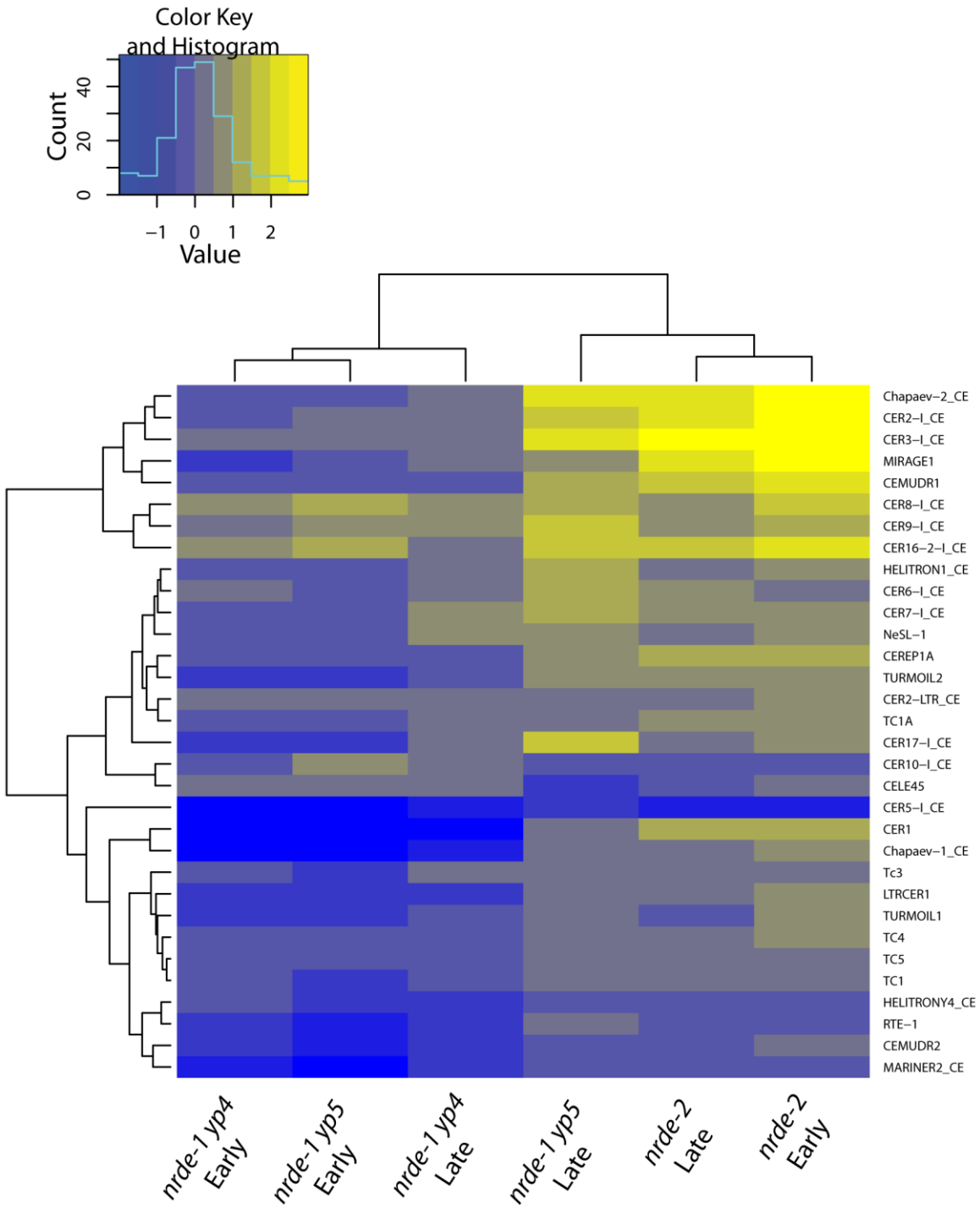
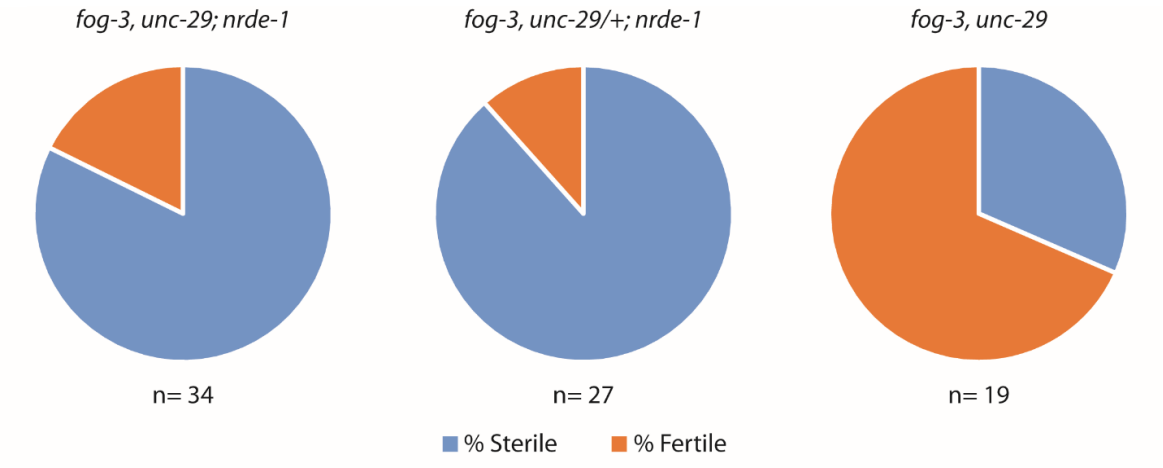


Figure 3.7. Measurement of the behavior of transposon expression for late and early generation *nrde-1*, *nrde-2* and wild-type L4. Heatmap of transposon expression in *nrde-1* (*yp4* and *yp5)* and *nrde-2* relative to wild-type at indicated generation. Blue represent decreased expression and yellow represents high expression. *nrde-1* data acquired from 3 independently isolated strains and expanded to 20 plates each. The *nrde-2* data is from one independent isolate expanded over 5 plates.

A.



B.

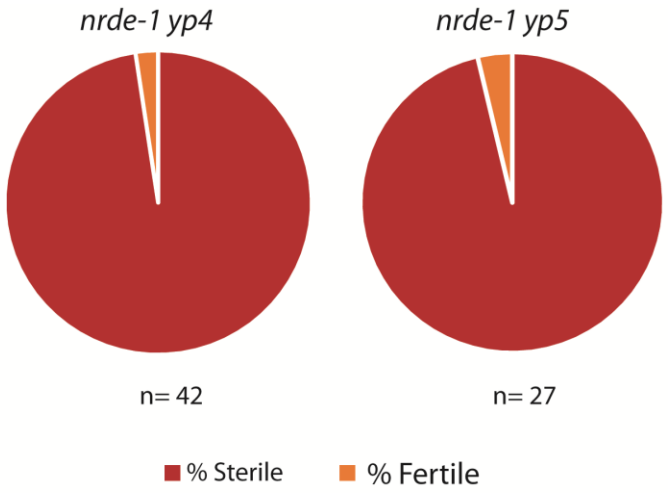


Figure 3.8. Measure of the effect of loss of spermatogenesis on the fertility of late generation *nrde-1*. Pie graph showing the percent of adult sterile animals that produce progeny upon outcross to wild-type adult males. Each experiment conducted by: 1- isolating single L4 hermaphrodites of the respective genotype, 2-waiting to 2 days adulthood, 3- Selecting against plates with embryo or larvae, 4-Outcrossing to 2 wild type males. (A) Sterile generation *nrde-1; fog-3* animals and their siblings that are hemizygous for the *fog-3* mutation do not show significant rescue when outcrossed to wild type males in comparison to *fog-3* mutants alone. Z=3.7, p= 0.000251 for *fog-3; nrde-1* and Z=3.9, p=0.0001 for hemizygous *fog-3/+; nrde-1* siblings show significantly difference between them and the control. (B) Male outcross of two alleles of *nrde-1* yield similar yet minor levels of rescue in the single mutant control. Z score=0 and p=1 show no difference between the two alleles behavior.

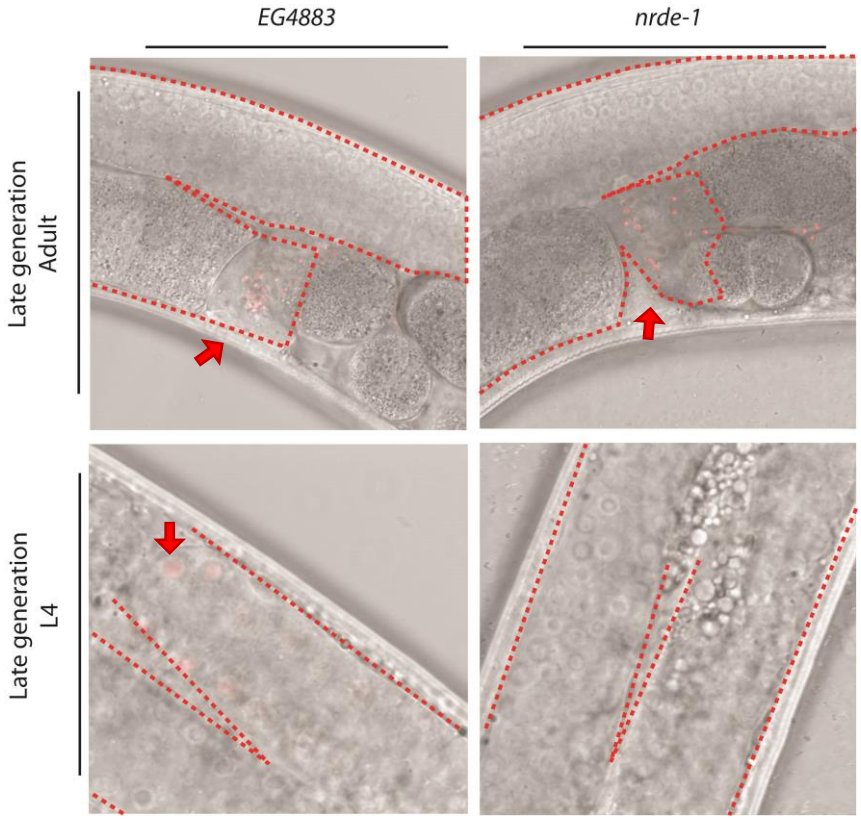
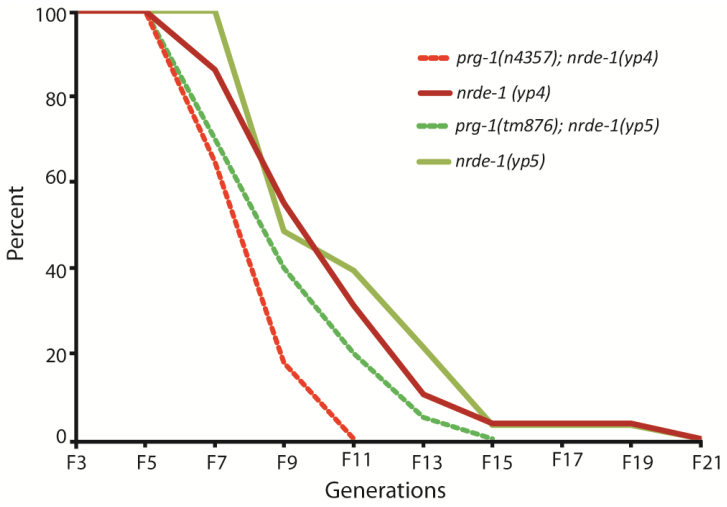


Figure 3.9. *spe-11* promoted histone mCHERRY in late generation *nrde-1* L4 and adults. EG4883 consists of a spe-11p::mCherry::histone + unc-119(+) and fluoresces as a dim mCherry in the hermaphrodite sperm. Top panel is confocal image of sperm cells in the EG4883 late generation adults (left) and EG4883; *nrde-1* (right). Bottom left panel shows confocal image of L4 larval EG4883 germ cells with dim mCherry fluorescence and bottom right show the *EG4883;nrde-1* late generation L4. Red arrows point to mCherry fluorescent cells.

A



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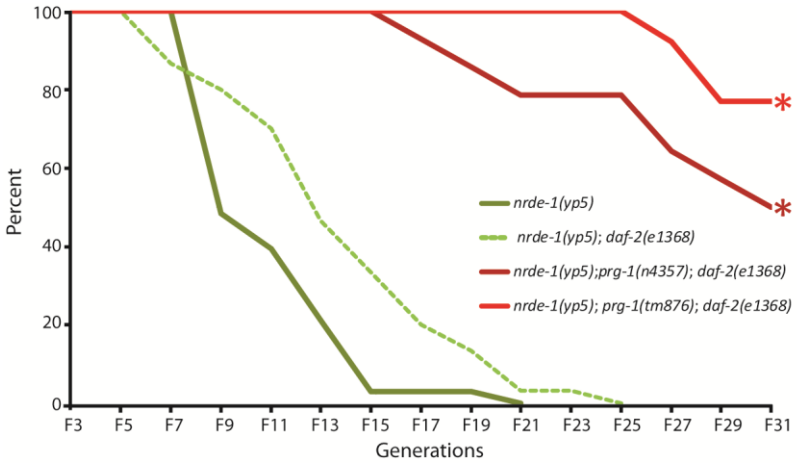


Figure 3.10. A *nrde* mutation in the *prg-1* background does not rescue *prg-1*. Both Phillips *et al.* and de Albuquerque *et al.* show that mutating the germline nuclear RNAi pathway results in amelioration of phenotypes associated a with synthetic *prg-1* sterility including increased fertility. The mortal germline mutant *prg-1* has been shown to have a germline lifespan median of ~34 generation (Simon et al. 2014). (A) A *nrde-1* mutation in the *prg-1* background does not rescue its Germline Mortality phenotype and shows a significant decrease in time to sterility for the *prg-1; nrde-1 yp5* double mutant (log rank test p=0.021) but not for *yp4* allele (p=0.086). (B) A mutation in the insulin signaling pathway via *daf-2* can extend the time to sterility of a *nrde-1* mutant (log rank test p=0.044). Loss of piRNAs in the *nrde-1, daf-2* background endows longer time to sterility (log rank test p=0.00001 in each case).

Table 3.1

Gene Name	Common Name	Spermatogenesis related	Location
B0024.4		Y	V:10297399..10298982
C01F6.2		N	IV:9099263..9101585
C05E11.4	*amt-1*	N	X:4571448..4573914
C15H7.3		N	III:9650421..9651959
C32D5.4		N	II:6321340..6323079
F36D3.5		Y	V:16510369..16515060
F47C12.4	*clec-79*	N	IV:3973349..3975935
F53B2.5		N	IV:12528563..12531142
F53H4.6		Y	X:15852935..15860608
H23L24.5	*parg-2*	N	IV:8712413..8715027
K04G2.4		Y	I:8035886..8037141
M70.3b		Y	IV:2237663..2242417
R09E10.2		Y	IV:10317557..10318299
R10H10	*spe-26*	Y	IV:10389577..10391945
T08B6.9		Y	IV:4890844..4894087
W10G11.3		N	II:3561747..3562565
Y43C5B.3		N	IV:10353168..10355091
ZK622.1		N	II:5293817..5295628
ZK795.2		N	IV:12557074..12558958

Table 3.2

	N2			*nrde-1 yp4*		
RNAi Clone	No. Offspring at peak fertility* (avg)	Sterility Parent (%)	Sterility F1 (%)	No. Offspring at peak fertility* (avg)	Sterility Parent (%)	Sterility F1 (%)
vector	133.5	0	0	34	50	50
csr-1	80.5	0	0	3	50	100
boo2.4	190	0	0	59	50	46
c011F6.2 (5' amp protein kinase)	125.5	0	0	1	50	100
fog-3	102.5	0	0	17.5	0	60
spe-26	113	0	0	24	0	46
w10g11.3	156	0	0	5	50	100
k04g2.4	187.5	0	0	N/A	100	N/A
c32d5.4	151.5	0	0	N/A	100	N/A
f47c12.4	175	0	0	N/A	100	N/A
par-6	0	0	0	0	0	0

	N2			*nrde-1 yp5*		
RNAi Clone	No. Offspring at peak fertility* (avg)	Sterility Parent (%)	Sterility F1 (%)	No. Offspring at peak fertility* (avg)	Sterility Parent (%)	Sterility F1 (%)
vector (L440)	190	0	0	74.75	0	40
csr-1	180.5	0	0	31.25	0	90
alg-3/4	264.75	0	0	39	0	42
fem-3	187.5	0	0	99.75	0	19
aak-2	287.5	0	0	142.25	0	17
par-6	0	0	0	0	0	0

* The average number of offspring is a calculation of the average of the offspring counted for 3-4 experiments.

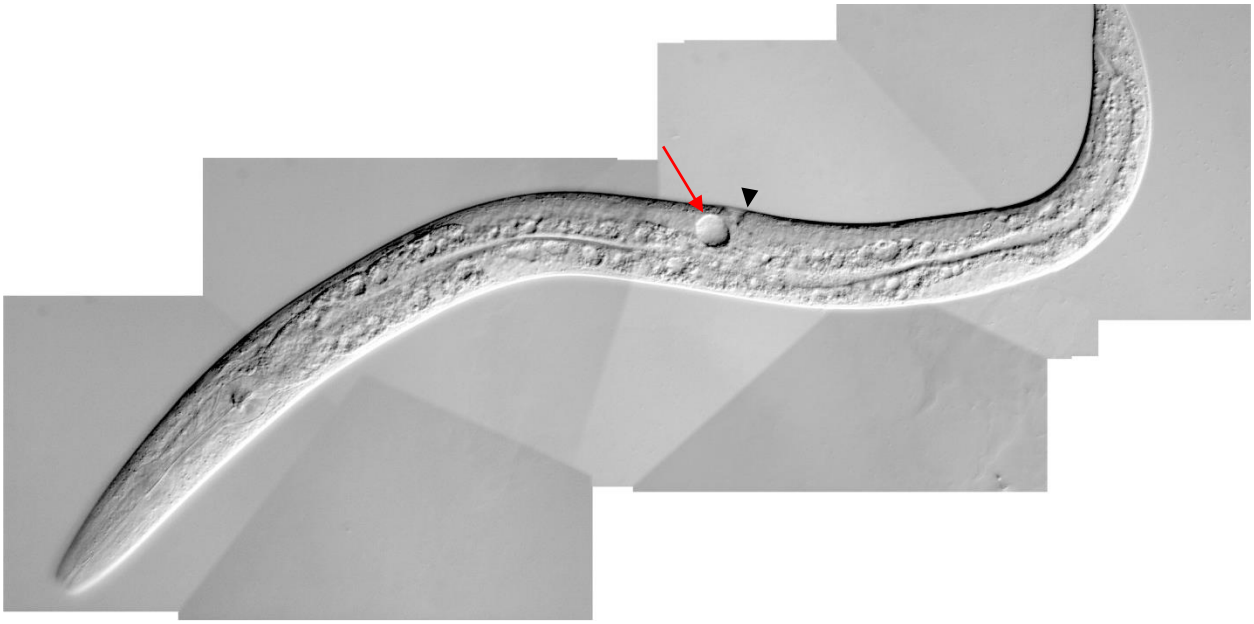
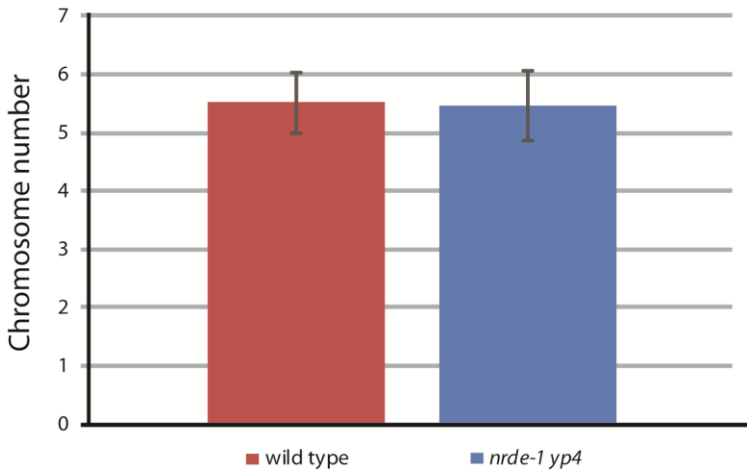


Figure S3.1 DIC image of the individual *nrde-1 yp4* L3 that was seen to have a morphological defect in the germline. The red arrow indicates the defect in the germline and the arrowhead indicates the vulva. Although no other L3 animals scored showed any germline defects it can be noted the phenotype seen here may illustrate an early read out of accumulated damage or a body morphology mutation that is the result of being born of a parent with accumulated damage.

A



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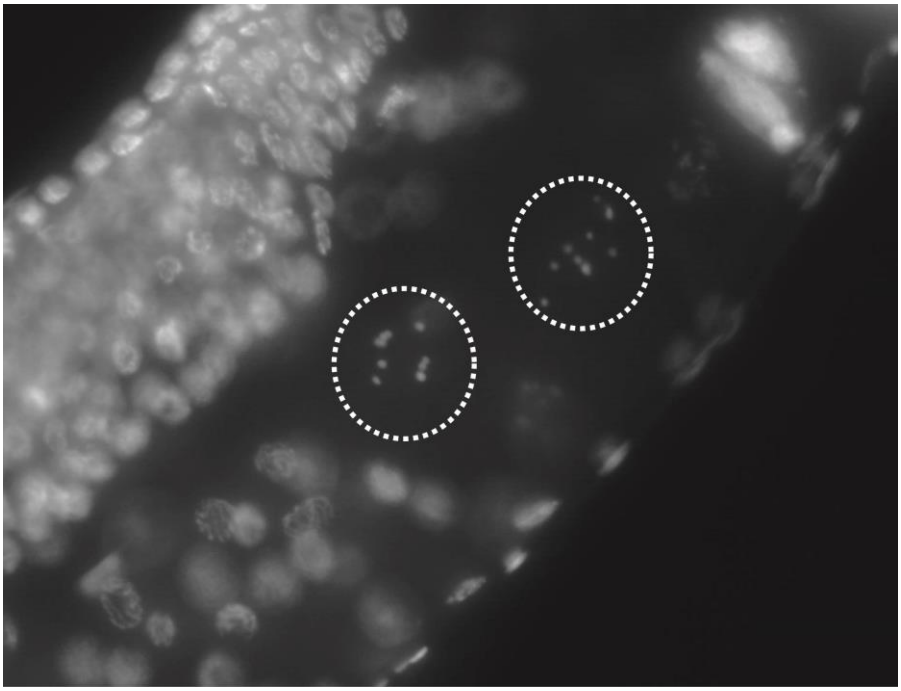
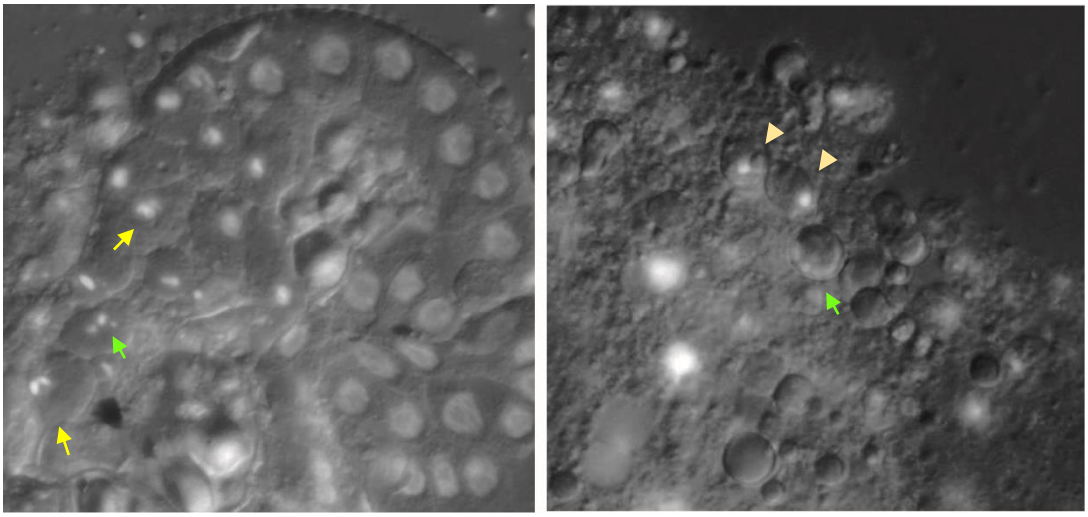


Figure S3.2 Chromosome quantification in late generation *nrde-1*. (A) Chromosome counts in late generation *nrde-1* compared to wild type animals. The average chromosome number between late generation *nrde-1* and wild type animals are the same. Bars represent standard deviation. (B) *nrde-1* animal showing abnormal number of chromosomes (within dotted circle) in the proximal portion of the germline. *C. elegans* has six chromosomes which will appear as six bivalents in oocytes. Each circle represents chromosomes of individual oocytes.

A



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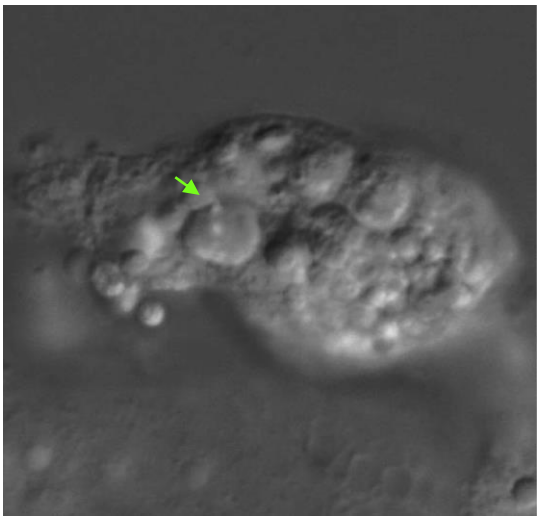

Figure S3.3. Late generation *nrde-1* show chromosome defects in adult sperm. (A) Sperm isolated from late generation *nrde-1* adults stained via Hoechst. (B) Whole spermathecae from sterile *nrde-1* adults stained with Hoechst. Arrowhead indicate normal sperm, green arrow indicate multinucleated sperm, and yellow arrow indicate chromosomes bound by anaphase bridges.

CHAPTER 4: Discussion

Delineating the stress or stresses that accumulate in Mortal Germline mutants may offer a view into many known and unknown damaging agents in the cell and studying mechanisms of how normal germline defend against these agents can outline ameliorative or even rejuvenating processes. We've shown Germline Mortality in the *nrde-1* mutant results in the increased expression of a number of genes and that upregulation of transposable elements does not explicitly correlate with *nrde* mediated Germline Mortality. We can also see the byproduct of loss of NRDE-1 function is the accumulation of stress that results ultimately in aberrant developmental and functional behavior that includes increased embryo lethality, germline degeneration, and sperm and oocyte defects in the hermaphrodite.

Maintaining silent regions of the genome in a heterochromatic state is imperative to proper migration of chromosomes. Determining whether NRDE-1 plays an auxiliary role in chromosome segregation may help define phenotypes that occur in late generation populations. Late generation *nrde-1* oocytes infrequently show an increase in the number of chromosomes counted via DAPI DNA stain. The number of chromosomes in late generation animals may range from the normal 6 bivalents up to 7 or 8 (Figure S3.2). Determining whether this is a direct or indirect byproduct of *nrde-1* Germline Mortality could define an additional role for the NRDE-1 protein. Analyzing FISH stained wild type animals probed for NRDE-1 and *nrde-1* controls could lend an idea to where endogenous NRDE-1 localizes. Additionally, fluorescently tagged NRDE-1 transgenic animals counterstained for chromatin could allow for visualization of NRDE-1 protein acting at sites such as the centromere during cell division. This could be done in early stage embryos where the cells are large and different cells will allow for the capture of different stages in cell cycle.

The transgenerational lack of gene function leading to Germline Mortality taxes the germlines ability to maintain genome stability. This stress is inherited in the subsequent offspring and can accrue over generations. Stress can function as advantageous to the cell. The theory of hormesis predicts that continued exposure of low doses of toxicity may yield beneficial effects for the cell (Cypser et al. 2006; Vaiserman, 2011). To test whether stress accumulated in *nrde-1* animals may have hormetic effects on the soma; post-mitotic lifespan analysis can be conducted on mid-generation, late generation and sterile generation *nrde-1* animals and compared to freshly outcross early generation *nrde-1* and wild type controls. If the consequence of NRDE-1 inactivity causes upregulation of stress resistance pathways in the soma, it could be reasoned that an extension of lifespan will occur. As sterility is known to cause lifespan extension, it is possible that sterile generation animals will appear long lived as well. Alternatively, accumulated stress in sterile generation *nrde-1* animals may result in a general sickness causing the worms to have shortened lifespans. If sterile generation *nrde-1* experience shortened lifespans then possibly mid-generation *nrde-1* experiencing more moderate amounts of stress will show longevity phenotypes. Additionally, lifespan extensions may occur in the penultimate late generation *nrde-1* prior to the cessation of germline lifespan. Outlining the behavior of the soma will add to the body of evidence defining the stress that accumulates which include embryo lethality and progressive sterility.

Mining the mechanisms of germline immortality for use in fortifying somatic tissues holds great potential for the field of aging. Some of the more interesting pieces of information to evolve from the work on germline immortality is the evidence showing that time to Mortality can be manipulated and in some cases reversed. For example, rescue of the PIWI and piRNA mutant *prg-1* by *daf-2* offers some proof that there are mechanisms that intersect with germline immortality that can be beneficial when applied to the soma. There already exists much in the literature illustrating the health effects of reduced

insulin signaling and these ameliorative effects have been shown to be applicable to many model organisms.

The absence of DAF-2 in a *nrde-1* background results in an extension of the time to Mortality. Although this contrasts to the rescue seen in *prg-1; daf-2* animals, the extension of time to sterility in the *nrde-1, daf-2* animal's means that the damage accumulated over time in absence of *nrde-1* can be modulated by the actions of decreased insulin signaling or potentially by DAF-16 in a fashion that's similar to *prg-1*. Modulation of time to sterility is further evident in the absence of piRNAs and DAF-2 regulation in a *nrde-1* background as *prg-1; nrde-1, daf-2* triple mutants show even further extension in the time to sterility. The extension seen in the triple mutant surpasses that of either the *nrde-1, daf-2* or the *prg-1; nrde-1* double. The synthetic extension of *nrde-1* Germline Mortality point towards a stress that may be the result of unresolved silencing that is triggered by the initiation of silencing via piRNAs and PIWI proteins. This may explain why in the literature *prg-1* time to Germline Mortality is longer than *nrde-1* mutants as the lack of an initial trigger for silencing doesn't exist nor goes unresolved. Somehow reduced insulin or potentially DAF-16 can take on a secondary role that makes germline lifespan more robust even if for only a limited time.

In comparison to the suppression of the time to Mortality seen with the downregulation of DAF-2 an EMS mutagenesis screen of late generation *nrde-1* animals was conducted and four suppressor mutations of *nrde-1* Germline Mortality were isolated. Each of the suppressors were backcrossed to *nrde-1* and strains where the suppressor had been removed were isolated and assayed for time to Mortality. In the presence of the second site mutation designated J#12, late generation *nrde-1* animals remain fertile enough to maintain genome stability. Once the J#12 suppressor is outcrossed sterility begins to occur in 2-3 generations. Two other suppressors, designated #50 and #45, when outcross show a delay in the onset of sterility in comparison to sibling non-mutagenized late generation *nrde-1* siblings. This argues that #45 and #50 suppressor reset what causes the Mortality phenotype. Once identified it

will be interesting to see what pathways are responsible for suppression Germline Mortality and how they may affect somatic tissues and aging.

Of noticeable interest are the findings surrounding *daf-2* mutants. In many organisms piRNA species are crucial for the development of the germline in a single generation yet alone germline that subsequently require development during germline immortality. The identification that *daf-2* mutants don't require piRNAs in *C. elegans* is profound, but requirement for *nrde-1* transcriptional silencing in the *daf-2* mutant germline immortality displays a new pathway downstream of silencing initiation with a possible restriction at the process of silencing. It would be fascinating to see whether other genes hypothesized to be required for germline gene silencing are required for *daf-2* germline immortality. Confirming the requirements for other components of the Nrde pathway would prove useful in defining whether *daf-2* Germline Mortality is a *nrde-1* specific response or a byproduct of loss of germline transcriptional gene silencing. Additionally, other mutants defective for germline gene regulation that show Mortal Germline phenotypes could be tested. Mutations such as *spr-5* or *met-2* have increased spermatogenesis gene expression and work cooperatively to maintain germline immortality. SPR-5 has a known role as a H3K4 demethylase while MET-2 is predicted to function as a H3K9 methyltransferase. Investigating whether these genes play any role in germline immorality in *daf-2* mutant animals, both in the presence and absence of piRNAs, would help construct a pathway that identifies the mechanism of rescue that downregulation of DAF-2 in *nrde-1* and *prg-1* mutants contributes to robust Germline lifespan.

Thus far many of the mechanisms governing Germline Mortality encompass physical alterations to the genetic material such as telomere erosions or processes that lead to alterations of chromatin states such as silencing of foreign genetic elements. NRDE-1 will be a good tool in further discovering the role small RNA mediated silencing plays in this process and the interactions with the insulin signaling factor DAF-2 could outline a new mechanism of cellular stress that in its absence could extend cellular

lifespan. The future of germline immortality will be in discovering new ways of manipulating these pathways and discovering unforeseen pathways responsible for the maintenance of germline immortality.

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