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# Characterizing a role for CoREST (SPR-1) in regulating the function of LSD1 (SPR-5)

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# Introduction

During fertilization and the embryogenesis which follows, cells face the fundamental problem of navigating the precise genomic expression which will give rise to proper zygotic development. Organisms complete this difficult task through a complex interplay of numerous pathways which involve structures both inside and outside the genome itself. Until recent advances in scientific thinking brought about a greater understanding of organismal development, gene expression and cell fate were believed to be determined solely by enzymes located within the genome. These include regulatory elements such as promoters, enhancers, silencers, and transcription factors. Now, however, epigenetic elements found "above" the genome have emerged as major players in regulating gene expression during development (Weaver et al., 2004). Epigenetics, simply put, is the study of heritable changes in phenotype that result from modifications in access to the DNA by mechanisms outside the genome. These "epigenetic" mechanisms include DNA methylation and histone modifications, both of which alter transcription to facilitate proper gene expression during development.

DNA methylation acts on the genome itself to regulate how accessible particular genes are to transcriptional machinery such as tissue-specific transcription factors and RNA polymerases. To prevent improper transcriptional initiation and the misexpression that would result, various DNA methyltransferases add methyl groups ($-CH_3$) to the CpG islands of promoter regions (Weaver et al., 2004). As methyl groups accumulate, these regions physically close, blocking the binding of transcriptional machinery through steric hindrance. In other words, the methyl groups repress gene expression and turn genes "off" by marking regions of the genome which are to be silenced. Following this logic, to turn a gene "on," promotor regions must be rid of methylation to allow transcriptional machinery to regain access to the DNA. This presence or absence of DNA methylation, thus, acts as a "switch" during development to regulate transcription and the later formation of gene products.

Interestingly, methylation can also appear on histones, the unique proteins around which DNA wraps when chromatin is packed into the nucleus of every cell. Histones possess exposed "tails" which can be modified through the addition of a number of chemical groups (Ooi and Henikoff, 2007). These reversible modifications can reorganize chromatin packing into either open (euchromatin) or closed (heterochromatin) conformations. Euchromatin represents active regions, areas where chromatin is open and transcription can occur as machinery is able to access genes. Here, modifications such as the dimethylation of histone 3 at lysine residue 4 (H3K4me2) alter the physical properties of the previously compacted chromatin and cause it to unravel accordingly. H3K4me2 thus acts as an activate mark, opening chromatin and highlighting regions of the genome which are to be transcribed. If H3K4me2 is removed or dimethylation of histone 3 at lysine residue 9 (H3K9me2) is added, for example, chromatin may be modulated into its closed conformation. With this, heterochromatin is formed as chromatin becomes so condensed that transcriptional machinery are physically incapable of accessing

and binding with the genome. As such, H3K9me2 is a repressive mark, serving to create regions of chromatin which display an overall repressive environment and are said to be transcriptionally inactive (Ahringer and Gasser, 2018). Together with DNA methylation, dynamic histone modifications such as these function to epigenetically regulate gene expression and have been implicated to play a major role in mediating development, disease, and inheritance (Greer and Shi, 2012). In fact, several of these modifications have been shown to persist through fertilization events and alter gene expression in subsequent generations (Greer et al., 2011) (Greer et al., 2014).

With this in mind, the mechanism underlying inheritance of epigenetic marks has been the topic of much scientific debate in recent years. At fertilization, sperm and egg fuse and this newly formed genome acquires the epigenetic landscape of its mother. To erase this epigenetic memory and restore totipotency in the zygote, epigenetic factors, which have been deposited maternally, must reprogram the chromatin environment (Wasson et al., 2016). In other words, to transition from the highly specialized sperm or egg cells to an undifferentiated zygote able to form any cell in the body, the parental genome must be inactivated. Two such factors are LSD1 and SETDB1, both of which work together to remodel chromatin and prepare the zygotic genome for proper development by creating a transcriptional "ground state." LSD1, an H3K4me2 demethylase, first removes these active marks to halt transcription and silence germ specific genes (Shi et al., 2004). SETDB1, an H3K9me2 methyltransferase, then adds a repressive mark to form heterochromatin and further inhibit transcription (Eymery et al., 2016). The chromatin landscape and resulting gene expression which previously formed sperm or egg is, thus, erased and totipotency is regained in the developing zygote. Unsurprisingly, this epigenetic reprogramming is vital to development. If this event is altered, however, such that reprogramming during fertilization is unsuccessful, developmental defects may arise due to the subsequent misexpression of genes. These defects and the significance of such reprogramming events have been extensively studied in *Caenorhabditis elegans*.

Remarkably, worms share much homologous DNA with humans and orthologs of *Lsd1* and *Setdb1*, in the form of *spr-5* and *met-2*, have provided great insight into the role these epigenetic factors play during fertilization. In fact, *spr-5* mutants have been shown to display progressive sterility along with decreasing brood size over many generations (Katz et al., 2009). Unable to remove H3K4me2 in the absence of SPR-5, chromatin immunoprecipitation (ChIP) assays showed that these animals accumulated H3K4me2 with each successive generation (Katz et al., 2009). A similar result was found in *met-2* mutants, which phenocopied *spr-5* animals and demonstrated increased sterility/decreased brood size (Kerr et al., 2014). Here, ChIP experiments revealed that, without MET-2 to add H3K9me2 and form heterochromatin, *met-2* mutants showed decreased levels of H3K9me2 genome wide (Kerr et al., 2014). In both cases, mutants were unable to properly silence genes and quantitative reverse transcription PCR (qRT-PCR) indicated this misregulation resulted in the overexpression of spermatogenesis genes (Katz et al., 2009) (Kerr et al., 2014). Upon seeing that *spr-5* and *met-2*

mutants exhibited similar aberrant phenotypes and comparable misexpression, *spr-5; met-2* double mutants were constructed to test for possible synergistic effects (Kerr et al., 2014). Interestingly, *spr-5; met-2* mutants are completely sterile after a single generation and display severe developmental delay, often arresting at the first larval (L1) stage (Kerr et al., 2014). Knowing that epigenetic modifiers such as SPR-5 and MET-2 require the activity of other proteins to bind with histone tails, these observations led us to investigate the presence of an additional gene or complex of genes which regulate their enzymatic function.

To the further understand the role of *spr-5* during epigenetic reprogramming, we examined structures which have been suggested to interact with SPR-5 or its ortholog, LSD1. In mammals, LSD1 has been shown to physically associate with CoREST, among other proteins, and together they function to demethylate specific histone residues (Shi et al., 2005). In fact, CoREST appears, as a known repressor whose activity maintains nonneural cell identity in neural sodium channels, to collaborate with LSD1 in generating an overall repressive chromatin environment (Andres et al., 1999). Fascinatingly, depletion of CoREST in cell culture reduces the stability of LSD1 and impairs its function, suggesting LSD1 is dependent on CoREST (Shi et al., 2005). Much of this interaction remains unknown, however, even in the simple neuronal network that makes up *C. elegans*. In worms, the ortholog of human CoREST, SPR-1, has been found to co-immunoprecipitate with SPR-5, demonstrating the proteins physically interact *in vitro* (Eimer et al., 2002). Perhaps most striking is the finding that worms lacking *spr-1*, much like their *spr-5* mutant counterparts, are able to rescue the egg-laying defect associated with *sel-12* mutants (Jarriault et al. 2002).

In humans, *sel-12* is connected with the presenilin genes which regulate Notch signaling and mutation has been implicated in the progression of Alzheimer's disease (Eimer et al., 2002). In *C. elegans*, on the other hand, mutations in *sel-12* produce transgenerational sterility defects and obvious vulva deformations. Previous screens for repressors of *sel-12* identified *spr-5* and *spr-1* as being able to rescue the mutant phenotype, suggesting both may function as part of a co-repressor complex (Wen et al., 2000). On top of that, SPR-5 resembles the polyamine oxidase (PAO) component of human CoREST, further pointing to the idea that a convserved regulatory pathway exists where SPR-1 interacts with SPR-5 to regulate its function (Jarriault et al. 2002). We hypothesize that, given the role LSD1 (SPR-5) plays in epigenetic reprogramming during fertilization, this interaction is required to ensure proper development. Despite the plausibility of this claim, however, the relationship between *spr-1* and *spr-5* has never been fully characterized. Here, we seek to investigate their potential interaction in hopes of determining whether LSD1 is dependent on CoREST to function.

If LSD1 (SPR-5) requires CoREST (SPR-1) to function properly, loss of *spr-1* should result in transgenerational epigenetic defects that resemble those of *spr-5* mutants. As such, we ask whether mutants in *spr-1* demonstrate

abnormalities that phenocopy *spr-5* mutants. These phenotypes include progressive sterility and a lower brood size at around generation 25 (Katz et al., 2009). Our germline mortality data show *spr-1* animals produce progeny at levels intermediate to that of *spr-5* and wildtype but never show evidence of increased sterility. As such, research is still ongoing as we work to further tease apart these inconclusive results. Experiments with *met-2; spr-1* double mutants were far more promising, however. In the *met-2* mutant background, *spr-1* animals display many of the characteristic defects associated with *spr-5; met-2* mutants, such as disorganized germlines, developmental delay, and maternal effect sterility. Interestingly, these phenotypes do not fully manifest themselves after just one generation. Instead, the deformations worsen over time and eventually reach a penetrance which phenocopies the defects seen in *spr-5; met-2* mutants. This build up has proved difficult to quantify but these data suggest the absence of SPR-1 lowers the activity of SPR-5. Unable to fully remove H3K4me2, active marks accumulate over generations, leading to the misexpression of disease-causing genes. While this idea is promising, we must use RNAseq to rule out the activity of other complexes and confirm the overexpression of similar genes is found in both *spr-5; met-2* and *met-2; spr-1* mutants. These findings will highlight the role of CoREST (SPR-1) in regulating epigenetic reprogramming and ensuring proper development, in addition to informing the future development of drug therapeutics which target epigenetic abnormalities.

## Results

*Germline Mortality of spr-1*

Based on the hypothesis that SPR-5 function is dependent on the activity of CoREST (SPR-1), we believe *spr-1* mutants should phenocopy *spr-5* and, as such, the same characters should be seen in both worms. These phenotypes include progressive sterility due to misregulation of H3K4me along with an ability to rescue the *sel-12* egl defect. As Eimer et al (2002) previously demonstrated, *spr-1* mutants can rescue the egl phenotype, we turned to the germline morality of *spr-1* to determine if lower brood sizes are observed over many generations. Given that Katz et al (2009) reported increased sterility in *spr-5* mutants at generation 25 with the misexpression of germline germs, we sought to test whether *spr-1* also showed similar defects. Using the spr-1 (ar200) allele from Jarriault et al (2002), we found our mutant displays a “hypermorphic” or intermediate phenotype. In other words, we see an incomplete phenocopy, with average progeny counts across generations hovering somewhere in between those of wildtype and *spr-5* (Figure 1). In fact, despite continuing the experiment well beyond 50 generations, no evidence of apparent sterility was found in *spr-1* mutants. Instead of rapidly declining as with *spr-5* mutants, the brood size of *spr-1* animals remained relatively stable throughout. In spite of several noticeable dips and an overall downward trend, the length of this experiment made reaching a definitive conclusion on the potential transgenerational accumulation of H3K4me difficult. With that in mind, we turned to another tool in the laboratory in hopes of creating a more easily scoreable phenotype.

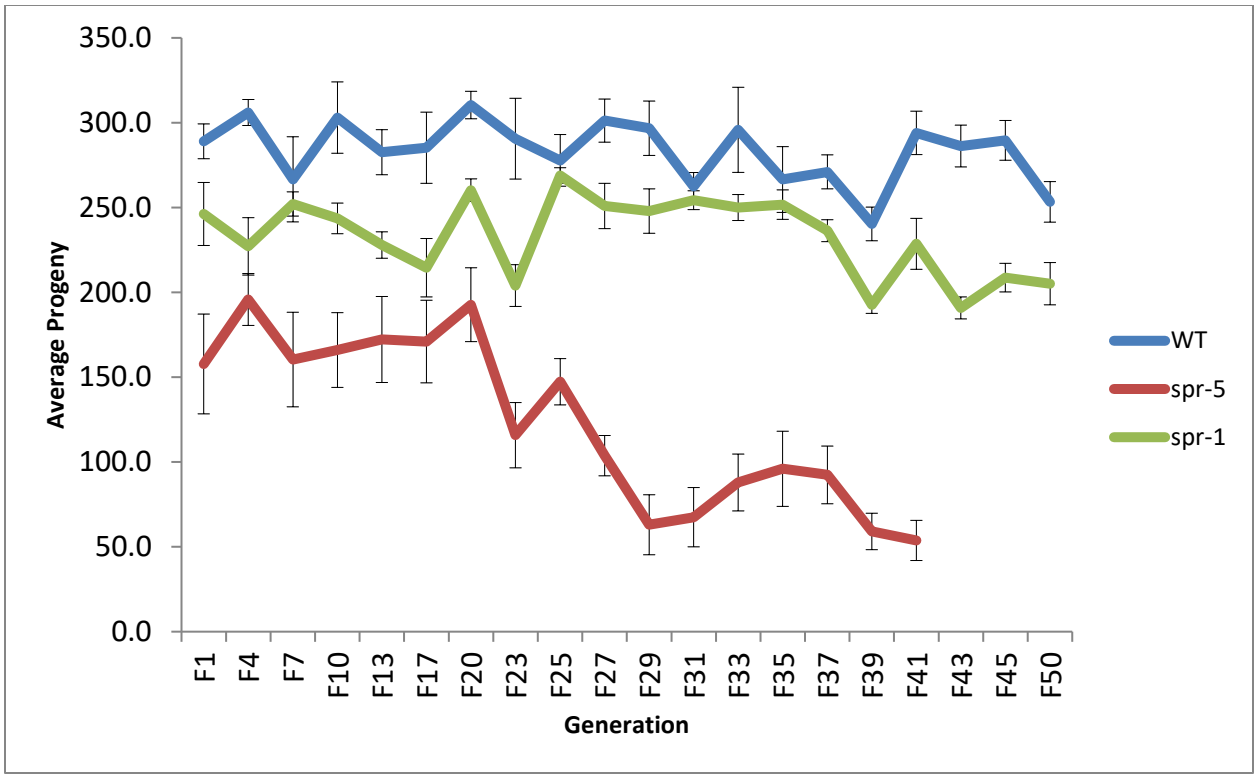



**Figure 1** – Germline mortality of *spr-1* mutant worms against *spr-5* and wildtype (N2) with average progeny counts plotted over generations

*RNAi Knockdown of spr-1 in met-2 mutants*

Using our knowledge of *spr-5; met-2* double mutants and their severe sterility after a single generation (Kerr et al. 2014), we set out to construct *met-2; spr-1* double mutants and observe if any phenotype whatsoever was present in this improved system. A preliminary RNAi experiment was first used to search for evidence of a phenotype comparable to that of *spr-5; met-2* double mutants. This opportunity was also taken to verify many of the constructs from the Ahringer RNAi library (*data not shown*) and, as a result, the assay was not entirely optimized for following worms on a variety of RNAi. Nevertheless, *met-2* worms were placed on L4440, *spr-5*, and *spr-1* backgrounds and kept at 16ºC. After nine days, the experiment was scored simply by counting the number of embryos present on each plate (Figure 2). This scheme was chosen given the fact that, after nine days under these conditions, the second generation (which is sterile in *spr-5; met-2* mutants) would be depositing embryos. Compared to the wildtype control, a decreased number of embryos were laid by adults on SPR-1 RNAi, a finding which suggested a fertility defect. As expected, worms on SPR-5 were almost completely sterile, demonstrating that the constructs and procedure worked well (see *Materials and Methods*). Thus, in a fashion similar to the *spr-1* single mutants, a complete phenocopy of *spr-5; met-2* was not seen and, instead, a sort of intermediate phenotype became evident. Perhaps the RNAi was inconsistent or

this is simply how the biology manifests itself but, in either case, the data gave us reason to construct the *met-2; spr-1* double mutant genetically.

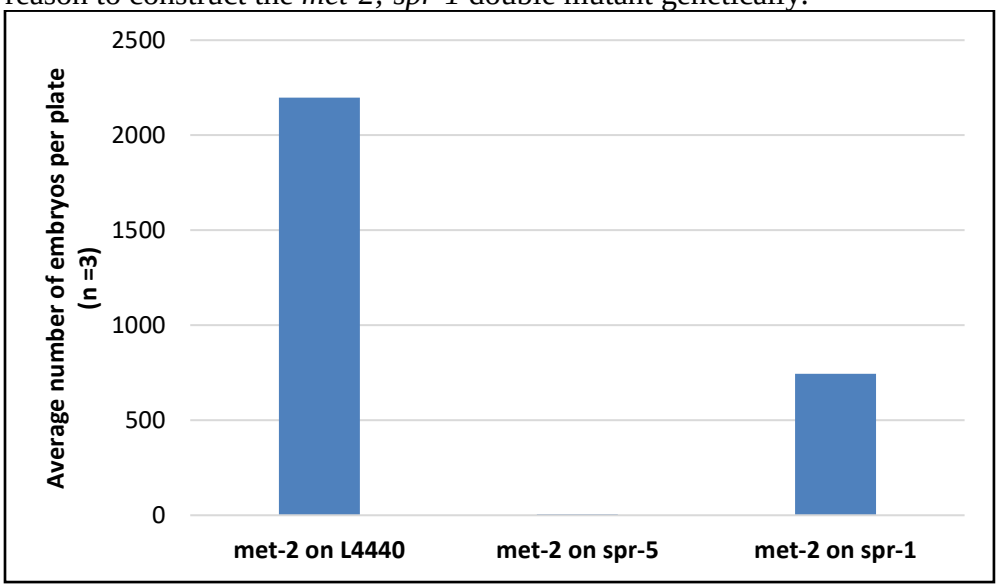



**Figure 2** – RNAi knockdown of *spr-1* vs *spr-5* in *met-2* mutant worms

*Developmental Delay of first generation met-2; spr-1 double mutants*

Upon building the *met-2; spr-1* double mutant strain (*see Materials and Methods*), we asked whether these organisms phenocopied those of *spr-5; met-2*. Again, if SPR-5 is truly dependent on SPR-1 for its function, the same characters should be seen in both double mutants. These include phenotypes such as severe developmental delay, complete sterility after one generation, and malformed germlines due to upregulation of gametes in the soma. Using a four hour synchronized lay, we first investigated developmental delay (Figure 3). At 48 hours after embryos arose, N2 worms all appeared in the L4 stage with characteristic crescent moon mark in the vulval region while *spr-5; met-2* mutants arrested at the L1/L2 stage with reduced proliferation of germ cells. The *met-2; spr-1* mutants, on the other hand, again display an intermediate phenotype and appear in the L3 stage. At 72 hours, N2 could be found in the adult stage and *met-2; spr-1* lagged slightly behind at L4, with incomplete germlines. As such, a delay is apparent when compared with wildtype, though certainly not as severe as that seen in *spr-5; met-2* double mutants. This finding, despite not being a complete phenocopy, supported our previous RNAi results and further pointed toward the presence of a defect-causing genetic interaction.


**Figure 3** – Developmental time course of *met-2; spr-1* double mutants alongside N2 and *spr-5; met-2* at 24, 48, and 72 hour time points. Hashed areas indicate germ cells

*Sterility of first generation met-2; spr-1 double mutants*

After confirming the developmental abnormality of first generation *spr-5; met-2* double mutants during both our RNAi and time course experiments, we turned to the sterility of *met-2; spr-1* double mutants. Among first generation (F2) organisms, approximately 25 percent of worms were sterile, with another 26 percent dying before reaching the adult stage (Figure 4). Interestingly, many of these double mutants appear to "bag" before releasing larvae, suggesting a vulva deformation. This bagging phenotype is associated with egl, an egg laying defect which occurs during vulva formation and prevents worms from depositing embryos. Embryos then hatch inside the parent, killing the organism. Despite the widespread bagging, sterility in *met-2; spr-1* animals was much less prominent than that of *spr-5; met-2* as nearly 50 percent of our mutants were able to lay embryos, with progeny counts ranging from <20 to well over 100. Seeing that many *met-2; spr-1* worms were fertile, however, we maintained the strain for several generations in an effort to monitor whether sterility counts varied over time. Theoretically, if H3K4 methylation accumulates with each generation, the aberrant chromatin should result in misexpression of the germline and increased sterility.

| | **Fertile** | **Sterile** | **Dead** |
|---|---|---|---|
| **met-2; spr-1 DM** | 122/253 | 64/253 | 67/253 |
| *GEN 1* | 48.22% | 25.30% | 26.48% |

**Figure 4** – Sterility of first generation *met-2; spr-1* double mutants

*Capturing the germline deformations of late generation met-2; spr-1 double mutants*

Given the "hypomorphic" phenotype seen in each assay and the idea that misregulation of H3K4me would build up with each successive generation, we asked whether sterility in *met-2; spr-1* double mutants would worsen over time. As the strain was passaged, we noted that, at first glance, the number of progeny on each plate decreased and, by generation 10, extreme sterility could be observed. In fact, differential interference contrast (DIC) microscopy captured squat, under developed germlines which appear very similar to those seen in *spr-5; met-2* double mutants (Figure 5). With disorganized sperm/oocytes and obvious protruding vulva, it appears as if the misregulation of germline genes that we see immediately in *spr-5; met-2* accumulates in *met-2; spr-1* double mutants to where they become increasingly sterile with time. To investigate this idea and follow this increase more closely, a germline mortality assay was needed to document progeny size at every generation.

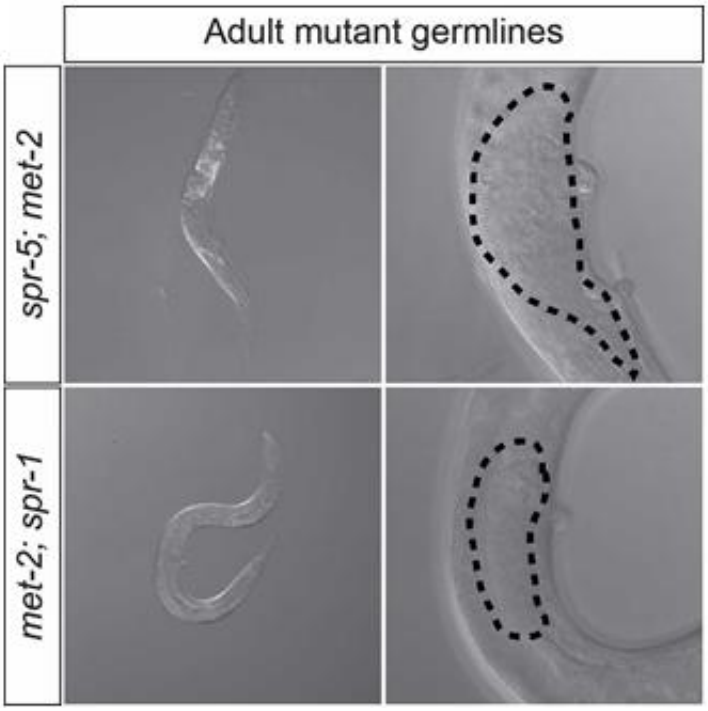



**Figure 5** – Differential interference contrast (DIC) microscopy of generation 10 *met-2; spr-1* double mutants in comparison to generation 1 *spr-5; met-2*. Hashed areas indicate germ cells

*Germline mortality of met-2; spr-1 double mutant*

Seeing that a transgenerational sterility phenotype became more prominent in late generation *met-2; spr-1* double mutants, we used this initial observation as rationale to complete a germline mortality experiment. Following a similar procedure to that used with *spr-1* single mutants (see *Materials and Methods*), the average brood size of *met-2; spr-1* double mutants appeared to progressively

decrease starting at generation 6, while that of wildtype and *met-2* remained at previously established levels (Figure 6). Given that roughly 25 percent of double mutants initially displayed a sterility defect after just one generation, we simultaneously worked to monitor sterility over time by recording the number of sterile worms at each generation. Unsurprisingly, *met-2; spr-1* progeny counts declined as the amount of sterile worms increased, with this percentage increase beginning at generation 6 (Figure 7). Thus, it was at this generation (GEN 6) that we began isolating L1 material to run RNAseq analysis or other molecular biology on each strain at a later date (see *Materials and Methods*). We continued to isolate larvae through to generation 8, where it was noted that sterility had reached a maximum and it became difficult to takedown adequate numbers of L1s. In fact, percent sterility plateaued upon reaching generation 8 and remained at a relatively stable level throughout the end of the assay (Figure 7). Average brood size, on the other hand, was found to continually decrease until we were no longer able to clone out adequate numbers of *met-2; spr-1* at generation 10 (Figure 6). With nearly sixty percent of worms completely sterile and progeny counts well below 30 at this point, the experiment further supported the idea of a "hypermorphic" phenotype, with sterility due to germline misregulation building up over several generations.

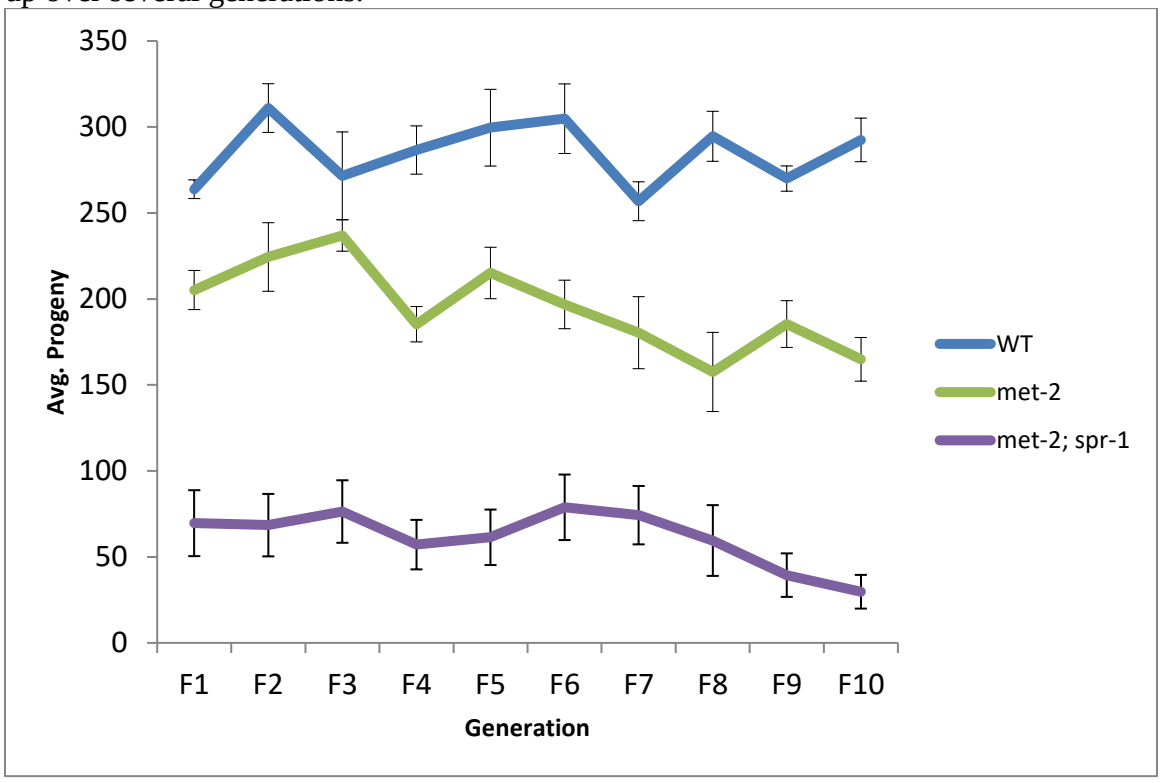



**Figure 6** – Germline mortality of *met-2; spr-1* double mutant worms against *met-2* and wildtype (N2) with average progeny counts plotted over generations

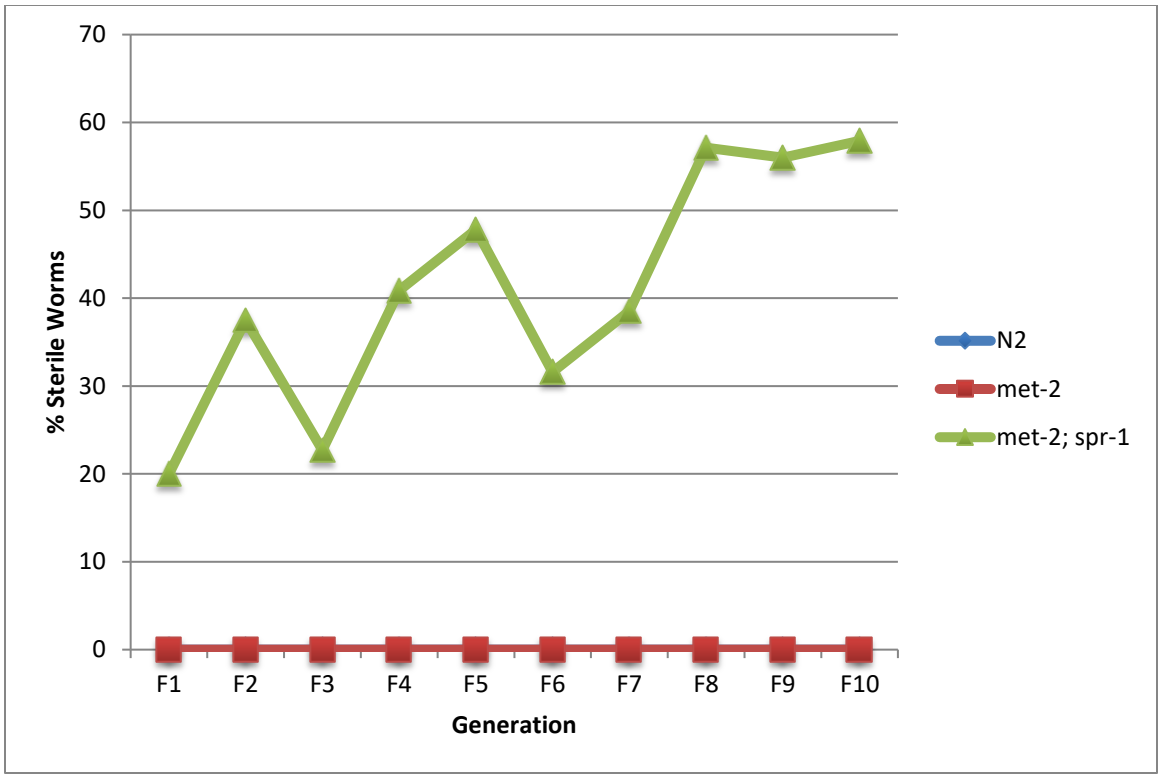



**Figure 7** – Percent sterility of *met-2; spr-1* double mutant worms over many generations in comparison with wildtype (N2) and *met-2*

*Developmental Delay of late generation met-2; spr-1 double mutants*

In addition to this obvious progressive sterility, we sought to quantify something slightly less evident; we hoped to capture whether the developmental delay seen in first generation *met-2; spr-1* worms also increased after the strain had been maintained for many generations. Using the same synchronized lay protocol as previously mentioned (see *Materials and Methods*), seventh generation double mutants were cloned out alongside that of wildtype (N2), *spr-1*, and *met-2* and all were allowed to lay embryos for four hours. Twenty-four hours after progenitor worms were removed from the plates and embryos began to appear, little variance could be seen between the genotypes as all appeared to be in the L1/L2 stage (data not shown). At the 48 hour timestamp, however, differences became increasingly evident. Both wildtype and *spr-1* worms looked remarkably similar and are L3/L4 across the board. *met-2* and *met-2; spr-1*, on the other hand, displayed much more variability, with most organisms delayed at L2/L3. This pattern continued at 72 hours, when all visible N2 worms and about 90% of *spr-1* had reached the adult stage. In other words, roughly one in ten mutants picked for imaging could be found at L4, with the rest being young adults. For *met-2*, approximately 30% of all worms were still L4, and this estimate swelled to nearly 50% for *met-2; spr-1*. In fact, despite being difficult to detect from the representative images, very few embryos had been deposited on the double mutant plate and several L3s were also present. As such, *met-2; spr-1*

again displayed an intermediate phenotype that worsened over a number of generations to bring about a developmental delay somewhere between that of *met-2* and the L1 arrest of *spr-5; met-2*. While variability could be seen throughout, some overlap between genotypes did exist, a phenomenon we will work to quantify more concretely in future experiments.

## Discussion and Future Directions

With our research question in mind, we expected to see *spr-1* mutant *Caenorhabditis elegans* phenocopy those of *spr-5* over the course of each experimental manipulation. In other words, if SPR-5 is truly dependent on CoREST (SPR-1) to function, we expected to observe the same characters in both mutants, with *spr-1* organisms displaying increased sterility and lower brood sizes around generation 25. Building on that, we anticipated *met-2; spr-1* double mutants would phenocopy *spr-5; met-2* and show malformed germlines, developmental delay, and extreme sterility after just one generation. Given the fact that SPR-5 is similar to the polyamine oxidase component of CoREST and has been implicated to physically associate with two DNA binding sites that can be found within the complex (Eimer et al 2002), this idea was entirely plausible. However, despite being able to rescue *sel-12* in a mode similar to *spr-5*, each experiment uncovered an incomplete phenocopy as *spr-1* organisms displayed a type of intermediate, almost "hypermorphic" phenotype.

In single mutants, for example, *spr-1* showed an average brood size in between that of wildtype and *spr-5*. Counts remained as such throughout the entirety of the germline mortality experiment and, instead of finding extreme sterility at generation 25 as in *spr-5*, our mutant of interest did not go germline mortal until after generation 55 (Figure 1). Thus, the progressive sterility phenotype seen in *spr-5* mutants was much less severe and took longer to manifest in *spr-1*. For *met-2; spr-1* double mutants, we observed a developmental delay which increased over time, but never quite reached the L1 arrest of *spr-5; met-2*. Initially, *met-2; spr-1* was delayed one larval stage behind N2 (Figure 3) and, by generation 8, the delay was even more evident (data not shown). A similar trend could be seen in terms of double mutant sterility. Again, *met-2; spr-1* displayed a type of intermediate phenotype, with sterility that worsened over every generation yet never reached the level of *spr-5; met-2* (Figure 7). Though the severity was difficult to quantify, we were able capture images of squat, sterile germlines in *spr-1; met-2* double mutants at generation 10 (Figure 5). With disorganized germlines and unrecognizable sperm/egg, it appears as if the misexpression of germline genes present in first generation *spr-5; met-2* double mutants builds up over time in *met-2; spr-1*.

To tease this apart further, we isolated *met-2; spr-1* double mutants at generation 6, 7, and 8 to complete molecular biology. Using L1 material, we can run RT-PCR/ChIP-seq on selected candidate targets or RNAseq/ChIP-seq on genome-wide H3K4me2 levels to determine SPR-5 activity in *spr-1* mutants.

RNAseq in particular may demonstrate that SPR-5 is less active in the absence of SPR-1 and will allow us to evaluate the potential expression of germline genes in the soma of *spr-1* mutants. Given the hypermorphic phenotype seen across all *spr-1* mutants, our data supports the idea that SPR-5 function is negatively affected by the complex and organisms are no longer able to properly remove K4me, allowing it the accumulate over time. Upregulated genes in both *spr-5* and *spr-1* mutants should overlap as such, ruling out any genetic interaction. If this is not the case, RNAseq would help point to something outside of CoREST; the NURD complex, for example, could also play a role in regulating SPR-5 function and may be a logical alternative. In sum, along with our unconfirmed data, these steps would answer our research question and establish whether there exists an interaction between SPR-1 and SPR-5 which affects protein stability by way of H3K4me2 demethylase activity.

## Materials and Methods

*Strain Maintenance*

Throughout experimentation, N2 (wild-type), *spr-1* (ar200v), *spr-5* (by101), *met-2* (n4256) III, and *et1* III ; *et1* (umnls 81) V *C. elegans* strains were provided by the *Caenorhabditis* Genetics Center and used to construct *spr-1* (ar200v) / *et1* [umnls 81 (myo-2p :: GFP + NeoR, III: 9421936) V] and *met-2* (n4256) III / *et1* [umnls 81 (myo-2p :: GFP + NeoR, III: 9421936) V] ; *spr-1* (ar200v) / *et1* [umnls 81 (myo-2p :: GFP + NeoR, III: 9421936) V] (see mechanism below). Each stock was maintained on Nematode Growth Media (NGM) 6 cm petri plates. OP50 was utilized as a nutrition source and all plates were spotted with three equidistant drops of liquid culture in a lily pad arrangement. Plates were allowed to dry overnight at room temperature following spotting with OP50. L4 larvae were then transferred to these spotted plates, placed in the 20$^{o}$C incubator, and left to self-fertilize. After four days, three L4 worms from the subsequent generation were transferred to fresh NGM plates and the line was allowed to continue. For both the *spr-1* and *met-2; spr-1* double mutant strains, the stocks were maintain as balanced heterozygous organisms.

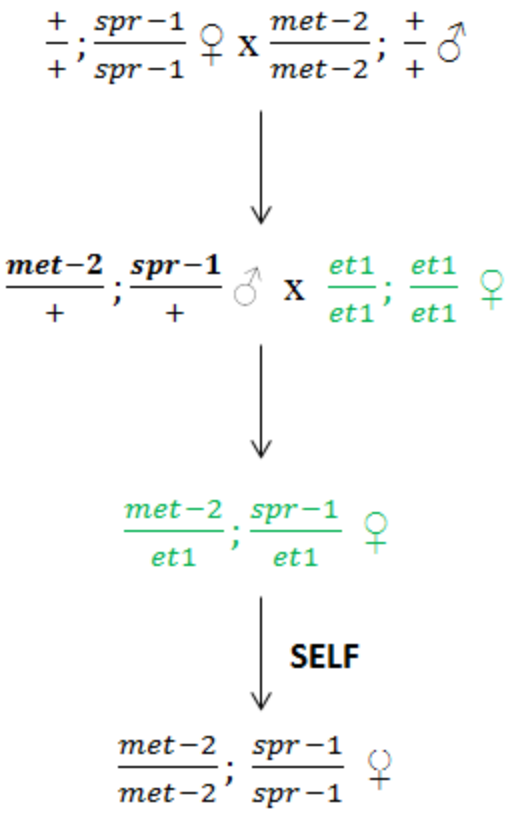
In these strains, the *et1* balancer was incorporated into the mutant background in order to eliminate homologous recombination events involving the mutation-bearing alleles on chromosome 3 (*met-2*) and 5 (*spr-1*). Because the balancer chromosome completely spanned both mutant loci, the *met-2* and *spr-1* alleles could be maintained with certainty across many generations. However, due to the nonlethal nature of *et1*, some green worms would remain wildtype (*et1*/*et1*) even after attempting the incorporate the *spr-1* or *met-2*; *spr-1* mutant background. As such, the longevity of the balanced lines was ensured by cloning out eight green worms from plates which showed evidence of non-green progeny. Always conscious to monitor for the presence of mutant (non-green) worms, this process served to verify that heterozygosity was successfully being passed on with each generation.

*RNAi for F2 sterility of met-2; spr-1 mutants*

RNAi feeding was used to evaluate, in a preliminary manner, if *spr-1* and *met-2* interacted such that sterility results in a manner similar to that seen in *spr-5*; *met-2* double mutants. Genes were inactivated by placing *met-2* (n4256) III worms on *spr-1* RNAi. Constructs were taken from the Ahringer RNAi library (Kamath and Ahringer, 2003) and transformed into bacterial *Escherichia coli*. This bacteria was then seeded onto NGM plates containing 50 ug/ml ampicillin and 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and the dsRNA was allowed to induce at room temperature for two days as detailed previously (Kamath et al., 2000). After two days, the plates were "super-induced" by placing at 37$^{o}$C for 1 hour immediately prior to transferring L2/L3 larvae onto the lawn and moving to a 16$^{o}$C incubator. There, the progenitor worms remained for two days before being picked to fresh plates. This procedure was repeated an additional two instances as organisms were followed through to the second generation, screening for sterility and developmental arrest along the way. After 192 hours, F2 progeny were scored for numbers of embryos present.

*Building the met-2; spr-1 double mutant strain*

To compare the phenotype of *spr-1* to that which has been heavily documented in spr-5; met-2 double mutants, it was necessary to build a similar strain incorporating *spr-1* (ar200v). As such, 6 *met-2* (n4256) III males were crossed with two *spr-1* hermaphrodites. When male progeny began crawling out, several males were plucked to genotype by Polymerase Chain Reaction (PCR) and confirm their predicted *met-2/+; spr-1/+* genotype. When PCR confirmed this double heterozygous genotype, showing both mutant and wild-type bands for *met-2*, 6 males were crossed with 2 *et1* III ; *et1* (umnls 81) V hermaphrodites. As progeny became increasingly visible on these plates, 22 green L4 hermaphrodites were cloned out and allowed to lay before being plucked off to genotype. Candidates which showed evidence of mutant bands were prepped with a Qiagen PCR Purification Kit and the PCR products were sent to sequencing with *spr-1* forward and reverse primers. Analysis of sequencing data proved that several green worms were both mutant and wild-type for *spr-1*, demonstrating the expected *met-2/et1; spr-1/et1* balanced double heterozygous phenotype. The non-green progeny which could be seen consistently arising from such worms were, in turn, the *met-2/met-2; spr-1/spr-1* double mutant.




*Resetting the spr-1 strain*

Upon receiving the *spr-1* (ar200v) allele from Caenorhabditis Genetics Center, we sought to reset and balance the strain such that both balanced heterozygotes and true 1st generation spr-1 mutants could be maintained with confidence. As such, 6 *spr-1* (ar200v) males, generated by heat shock, were crossed with two *et1* III ; *et1* (umnls 81) V hermaphrodites and male progeny was allowed to arise. From there, 8 green L3/L4 hermaphrodites were cloned out and plates were monitored for evidence of non-green progeny. Plates which displayed such non-green offspring were selected and 8 green L3/L4 hermaphrodites were again cloned out from these plates. This process was repeated for five generations, after which both green, balanced heterozygotes (*spr-1*/*et1*) and non-green, 1st generation spr-1 mutants (*spr-1*/*spr-1*) were cloned out and the progeny of which was frozen down. Additionally, the progenitor worms were plucked for PCR in order to genotype and confirm the mutants had been successfully balanced. To freeze down, the plates, once starved, were washed with M9 buffer, pipetting up and down to create a suspension of worms. 0.5 mL of this solution was deposited in a number of 1.5 mL conical and each was then placed in the -80ºC freezer.

*Confirmation of Genotypes by Polymerase Chain Reaction*

In order to confirm the genotype of worms after each cross in the strain building process,
L4 larvae were cloned out onto fresh NGM plates and these progenitor worms were allowed to lay embryos for at least 48 hours. After sufficient numbers of eggs could be seen, worms were plucked into PCR tubes filled with worm lysis buffer. For every 2 mL of lysis buffer, 100 µL of 1 M KCL, 20 µL of 1 M Tris at pH 8.3, 5 µL of 1 M $MgCl_2$, 90 µL of 10% NP-40, 90 µL of 10% Tween-20, 10 µL of 2% gelatin, and 1685 µL of molecular grade water were mixed. 5 µL of 20 mg/mL Proteinase K was then added to each 100 uL aliquot of buffer and 10 µL of this mixture was added to each PCR tube. After picking worms into the lysis buffer and ensuring excess bacteria was avoided, PCR tubes were placed in a -80$^o$C freezer overnight. Upon freezing, tubes were moved into the PCR machine and lysis reaction was performed at 60$^o$C for 1 hour, 95$^o$C for 30 minutes, and 10$^o$C forever.

Whilst the lysis reaction ran, PCR Master Mix was prepared with the amount of reagents varying based on the number of reactions. For each 25 µL reaction, 2.5 µL of 10x PCR buffer, 1.5 µL of 25 mM $MgCl_2$, 0.4 µL of 10 mM dNTP, 1 µL of 5 µM forward and reverse primer, 5 µL of 5x loading dye, 11.52 µL of molecular grade water, and 0.08 µL of Taq polymerase were mixed. Forward and reverse primers for *met-2* reaction 1 were 5'-GTCACATCACCTGCATCA GC-3' and 5'-ATTTCATTACGGCTGCCAAC-3' respectively. To run *met-2* reaction 2, the forward primer 5'-ATTCGAAAAATGGACCGTTG-3' and reverse primer 5'-TCTATTCCCAG GAGCCAATG-3' were used. Immediately after completion of lysis, 23 µL of the above mix was pipetted into labeled PCR tubes, to which 2 µL of lysate was added as template for PCR. Tubes were returned to the PCR machine and amplification conditions for both *met-2* reaction 1 and 2 were programmed to 94$^o$C for 5 minutes. This initial denaturation was followed by 44 cycles of 94$^o$C for 30 seconds of denaturation, 59.5$^o$C for 30 seconds of annealing, and 72$^o$C for 90 seconds of extension. Final extension was run at 72$^o$C for 5 minutes and the PCR products were maintained at 12$^o$C until results could be read by gel electrophoresis.

While PCR occurred, 2% agarose gel in 1x TAE was poured by mixing 7.5 grams of agar with 450 mL of TAE into the mold and allowed to cool. 16-18 µL of ethidium bromide was also added to this mixture to aid in the visualization of bands. 1.5 µL of 100 bp DNA ladder was then measured into the first lane. Reactions were removed from the PCR machine and 10 µL of each sample was pipetted into the lanes. Gel electrophoresis was allowed to run for 35 minutes at 110 V, after which the gel was imaged under UV light.

*Preliminary sterility and progeny counts of met-2; spr-1 mutants*

To quantify the sterility of first generation *met-2; spr-1* mutants, non-green L4 worms where plucked off the balancer and allowed to lay embryos. The progeny were then cloned out onto one spot NGM and followed into adulthood. As many of these progenitor worms deposited embryos, those which died or appeared sterile were recorded as a percentage of the total number of clones. To

further investigate the fertility of these double mutants, the above protocol was followed, but N2 and *met-2* (n4256) III worms were cloned out alongside the first generation *met-2; spr-1* mutants. To prevent overcrowding and increase the ease of counting progeny, progenitor worms were moved to fresh NGM plates two days later. After an additional two days, worms were transferred again to a third plate. In the meantime, the initial plates were counted twice to ensure all progeny had been recorded and sucked off with a suction apparatus. Once the progenitor worm ceased depositing embryos, the total number of progeny from each plate was recorded and the average brood for each strain was calculated.

*Germline mortality assay of spr-1 and met-2; spr-1 mutants*

Following a procedure similar to that outlined by Katz et al. (2009), worms were maintained at 20$^{o}$C and three fertile young adults with visible embryos were transferred to new NGM plates every four days. Given the translucent nature of C. elegans, only those worms which displayed healthy, ordered embryos within the germline were selected. Before beginning the *spr-1* experiment, each strain was thawed from -80$^{o}$C in M9 buffer and homozygoused for one generation, with progeny of the thaw being deemed $P_o$, the progenitor generation. These worms gave rise to the first experimental generation when brood counts began. Brood sizes of wild-type (N2), *spr-1* (ar200v), and *spr-5* (by101) were counted every third generation until generation 17, after which counts were completed every other generation. Average brood size for *spr-5* was calculated from the progeny of 10 worms until counts were stopped at generation 41. For N2, average brood size was determined from the progeny of 5 worms until generation 41 when it was increased to 6 through the end of the experiment. Average brood counts for *spr-1* were maintained at 10 worms throughout the entirety of the experiment. In order to complete RNAseq or CHIPseq analysis, mixed stage worms of each experimental line were collected from early, middle, and late generations.

This protocol was adapted to evaluate the germline mortality of the double mutants by using wild-type (N2), spr-1 (ar200v), met-2 (n4256) III, and met-2; spr-1. Here, brood sizes were counted every generation through the end of the experiment, save for *spr-1* which was counted every fourth generation. Average brood size for *spr-1* and *met-2* was calculated using the progeny of 10 worms, while that of *met-2; spr-1* and wild-type was calculated from 30 and 10 worms respectively. Starting at generation 6 until completion of the experiment, L1 larvae from each strain were isolated to complete RNAseq analysis at a later date.

*L1 isolation of late stage met-2; spr-1 Double Mutants*

To gather the material necessary for RNAseq analysis, *met-2; spr-1* L1 larvae were isolated at generation 6, 7, and 8. Approximately 40 N2, 50 *spr-1*, 60 *met-2*, and 120 *met-2; spr-1* L4 worms were first moved to new plates and kept at 20$^{o}$C overnight. The next day, adults were plucked and washed in 300 μL of M9 buffer three times before being transferred into 2 mL conical microcentrifuge tubes in preparation for the overnight lay. Specifically, 3-4 gravid N2, 4-5 *spr-1*, and 5-6 *met-2* adults were placed in ten individual tubes while 10-12 *met-2; spr-1*

double mutants were pipetted into 12-14 appropriately labeled tubes. Each genotype was allowed to shake overnight at room temperature. Following the lay, L1s were taken down by sequentially pipetting the contents of each individual tube into a separate glass well plate and removing progenitor worms with a scoop. L1s were then pipetted into a fresh tube, 25 μL at a time, and the process was repeated until all larvae had been consolidated into the new 2 mL conical. This tube was spun down for 5 minutes at 3,000 rcf before aspirating the excess M9 buffer down to 0.25 ml. The conical was then spun again and submerged in liquid nitrogen to snap freeze the material. From there, tubes were placed in a -80$^{o}$C freezer for storage until completion of RNAseq or other biochemistry.

*Developmental time course of late generation met-2; spr-1 DMs*

A developmental time course experiment was used to document the progressive developmental delay of late generation *met-2; spr-1* double mutant worms. Here, 6 GNE 7 gravid N2, 8-10 *spr-1*, 8-10 *met-2*, and 12-15 *met-2; spr-1* adult worms with visible embryos were transferred onto separate plates and allowed to lay for 3-4 hours. Following this synchronized lay, progenitor worms were plucked off and the time was noted. We then returned 24 hours later to sample several progeny from each plate and capture representative larval stage images of each population using MetaMorph Microscopy Automation and Image Analysis Software. This procedure was repeated at the 48 and 72 hour time stamps in order to track the relative development of each genotype.

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