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Investigation of SIN-3 Histone Deacetylase Complex in Caenorhabditis elegans

Maria Kontos

Investigation of SIN-3 Histone Deacetylase Complex in
Caenorhabditis elegans

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Maria Kontos
Candidate for BS Degree
and Renée Crown University Honors
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Capstone Project Advisor: _____
Dr. Eleanor Maine

Capstone Project Reader: _____
Dr. John Belote

Honors Director: _____
Stephen Kuusisto, Director

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Abstract

Chromatin is the combination of DNA and the proteins binding to DNA in the cell nucleus. The primary proteins associated with chromatin are histones, which compact the DNA. The DNA strand wraps around the histone octamer core complex forming a structure called the nucleosome. Posttranslational modifications of histones alter chromatin structure. Generally speaking, acetylated histones are associated with active gene expression whereas deacetylated histones are associated with repressed gene expression. Histone deacetylases, which function as a multi protein complex, remove acetyl groups resulting in altered DNA-histone binding affinities. One major histone deacetylase (HDAC) complex is the SIN-3 HDAC complex. SIN-3 acts as a "scaffold," and is specifically thought to interact with certain DNA binding proteins and histone deacetylases to assemble this HDAC complex.

In *C. elegans*, when germ cells enter meiotic prophase I, the chromosomes coil becoming shorter and thicker, and homologous chromosomes pair and synapse. A chromosome that fails to pair with a partner and synapse (for example, the male X chromosome) accumulates a high level of a specific histone modification, histone H3 lysine 9 dimethylation (H3K9me2). This modification is associated with facultative heterochromatin assembly, which may result in transcriptional silencing. It was observed that loss of *sin-3* function disrupts the H3K9me2 accumulation on unpaired X chromosomes in *C. elegans* hermaphrodites. Continuing the studies of a former student, Guang Yu Lee, a genetic approach has been taken to investigate which components of the SIN-3 complex contribute to the accumulation of H3K9me2 on nonsynapsed meiotic chromosomes.

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Acknowledgements

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   I would also like to thank Xia Xu, Yiqing Guo, Matt Snyder, and Bing Yang for their help and great tips to help my lab technique.

   To my parents —I cannot thank you enough for your love, support, and encouragement. I am beyond blessed to have such caring, loving, and hard-working parents.

Introduction

Meiosis is a special type of cell division that produces haploid gametes. Meiosis involves two successive cell divisions, Meiosis I and Meiosis II. Meiosis I and Meiosis II each involves prophase, metaphase, anaphase, and telophase. Prior to the start of meiosis, all chromosomes are duplicated in a process similar to mitosis. The result of meiosis is four haploid (N) progeny cells with one copy of each chromosome, while the result of mitosis is two progeny cells each with two copies of each chromosome.

The chromosome is an organized structure of DNA and protein found in the nucleus of cells. It includes a single piece of coiled DNA containing many genes, regulatory elements, and other nucleotide sequences. Chromosomes also have DNA bound proteins, which help package the DNA and control its function.

Chromatin is the combination of DNA and the proteins binding to DNA (e.g. histones) in the cell nucleus. It functions to package DNA into a smaller volume to fit into the cell nucleus, strengthen DNA to allow mitosis and meiosis to occur, prevent DNA damage, and - one of the most important - to control gene expression. The primary proteins associated with chromatin are histones, which compact the DNA. The DNA wraps around the histone complex forming a structure called the nucleosome. Chromatin structure depends on several factors including: genes encoded by the DNA, the stage of the cell cycle, and posttranslational modifications of histones.

Histones undergo posttranslational modifications that alter their interaction with DNA and other nuclear proteins. The sites of modification are usually in the histone amino-terminal tails. One common modification is acetylation, which loosens DNA binding to histones encouraging transcription. Generally speaking, acetylated histones are associated with active gene expression whereas deacetylated histones are associated with repressed gene expression. Histone deacetylases remove acetyl groups, which increases the positive charge of histone tails resulting in increased DNA-histone binding affinities. Deacetylated histones are associated with repressed gene expression because DNA wraps around the histone more tightly, in turn inhibiting transcription. Histone deacetylases usually function as a multi-protein complex.

There are two major histone deacetylase (HDAC) complexes, which are the NuRD and SIN3 complexes (Ahringer 2000). SIN-3 HDAC is a multi-protein complex that removes acetyl groups from histone proteins, resulting in altered DNA-histone binding affinities. Specifically, SIN-3 protein is thought to bind certain DNA binding proteins and histone deacetylases in order to assemble the SIN-3 HDAC complex. Although the components have not been well described in *C. elegans*, based on work in other organisms, it is thought that this HDAC complex is composed of: SIN-3; the HDA-1 (histone deacetylase), HDA-2, and HDA-3 enzymatic components; and the RBA-1 and/or LIN-53/RBA-2 DNA binding components. For my project, I am interested in histone deacetylation by the SIN-3 HDAC complex in *C. elegans*, specifically which components of the SIN-3 complex contribute to the accumulation of H3K9me2, histone H3 lysine 9

dimethylation, on unpaired meiotic chromosomes. MET-2 (histone methyltransferase) is the enzyme responsible for dimethylating H3K9 in the *C. elegans* germline (Bessler *et al.,* 2010).

The model organism used in the Maine Lab is *Caenorhabditis elegans. C. elegans* is a small, free living soil nematode that lives in many parts of the world feeding on microbes, but primarily bacteria. It is a good model organism for research in biological sciences including genomics, cell biology, neuroscience, aging, and development. This model organism has many advantages such as short life cycle, small size, compact genome, rapid period of embryogenesis, ability to produce large number of progeny, and a transparent body allowing internal cells to be easily visualized without dissection. The anatomy of *C. elegans* is quite simple, with about 1000 somatic cells. Each individual can produce a large number of progeny and can be maintained in a laboratory setting when they are grown on agar plates or liquid cultures with *E. coli* as the food source.

The two sexes of *C. elegans* are hermaphrodites (XX) and males (XO). The hermaphrodites produce both sperm and oocytes and are self-fertile. When homozygous hermaphrodites self-fertilize they generate identical progeny; however, male mating can be useful in isolating and generating certain mutant strains. Males have several key differences in gross morphology, anatomy, and expression of certain behaviors that allow us to distinguish them from hermaphrodites. Males have a slim body, a clear-white ventral gonad, and distinctive tail with a copulatory apparatus. Males arise from the fusion of a nullo-X gamete (a gamete lacking an X chromosome) with an X-bearing gamete. Males

can be generated at a higher frequency through mating because sperm have an equal frequency of being X-bearing or nullo-X. In addition, males can be generated in high numbers using the *him* (high incidence of males) mutations. These mutations increase the frequency of X chromosome nondisjunction in the hermaphrodite germ line, leading to a higher number of nullo-X gametes. This increases the number of males generated through self-fertilization. *him-5* and *him-8* mutations are generally used because they have no obvious deleterious effects on the anatomy or behavior in both *C. elegans* sexes ("Wormatlas homepage," 2003).

When germ cells enter meiotic prophase I, duplicated chromosomes coil becoming shorter and thicker. Synapsis, the pairing of two homologous chromosomes, also occurs during this phase. This allows the matching of homologous chromosomes and possible recombination to occur. In *C. elegans,* a chromosome that fails to pair with a partner and synapse (for example, the male X chromosome) during meiotic prophase I accumulates a high level of histone H3 lysine 9 dimethylation (H3K9me2) (Kelly *et al*., 2002; Bean *et al*., 2004). This modification is associated with facultative heterochromatin assembly, which may result in transcriptional silencing. Also, this modification may mark silenced genes. High levels of H3K9me2 are observed under special and specific conditions in *C. elegans* hermaphrodites (Bean *et al*., 2004). Under normal conditions, hermaphrodite X chromosomes pair and synapse, but this can be disrupted by using mutations in *him-8.* As mentioned earlier, *him-8* mutations increase the frequency of the X chromosome nondisjunction in the hermaphrodite

germ line. This is because the X chromosomes fail to pair and synapse in *him-8*

mutants. During the first meiotic prophase, the *him-8* mutant hermaphrodites

contain a high level of H3K9me2 on their unpaired/nonsynapsed X chromosomes.

Previous studies in the Maine lab have observed that loss of SIN-3

function disrupts H3K9me2 accumulation on unpaired X chromosomes during

meiosis in *C. elegans* hermaphrodites (She *et al*., 2009; Checchi & Engebrecht,

2011). However, H3K9me2 accumulation was not affected on the male X

chromosome. This may be due to differences in the males' chromatin such as the

male X chromosome may not be acetylated. These results suggested that SIN-3

activity is required for H3K9me2 to accumulate on unpaired X chromosomes in

hermaphrodites but not males. One possible model to explain this result is that the

SIN-3 HDAC complex deacetylates H3K9 residues or another residue, which in

turn allows the H3K9 dimethylation to occur. So, when the SIN-3 protein is

disabled, the acetyl groups on H3K9 would not be removed, and H3K9me2 would

not accumulate.

Continuing the studies of a former student, Guang Yu Lee, who

investigated "Meiotic Silencing in *C. elegans* Through SIN3 Histone

Deacetylase," a genetic approach has been taken to investigate which components

of the SIN-3 complex contribute to the accumulation of H3K9me2 on unpaired

meiotic chromosomes. Guang Lee found that the distribution of H3K9me2 was

normal in individual *hda-1, hda-2,* and *hda-3* mutants when compared to controls.

These results suggested that, individually, the HDA-1, HDA-2, and HDA-3

protein components are not essential for H3K9me2 accumulation. To follow up on this result, I investigated whether the HDA proteins were redundant for function. To do so, I constructed double mutant strains containing mutations in different components of the histone deacetylase complex and examined if the distribution of H3K9me2 was altered. In addition, the DNA binding components of SIN3 were investigated by using RNA interference and genetic mutations simultaneously to reduce the gene activity of RBA-1 and LIN-53. The germline development and phenotype in a *sin-3; met-2* double mutant was also observed. By eliminating more than one component at a time in the double mutants, the effects on H3K9me2 accumulation would become clearer.

Methods and Materials

Nematode Maintenance

The worms were maintained on agar plates using *E. coli* strain OP50 as a food source and stored at 20ºC (Brenner, 1974). Mutations that were used in this project were: *hda* (histone deacetylase), *unc* (uncoordinated), *him* (high incidence of males), *lin* (lineage defective), *rba* (RBAp48 related), *sin* (yeast Switch Independent), *met* (histone methyltransferase), *gfp* (green fluorescent protein), and *dpy* (dumpy). Two *gfp*-tagged chromosomal balancers were used: *hT2*[*bli-4(e937) let-?(q782) qIs48*] (I;III), and *mIn1*[*dpy-10(e128) mIs4*] (II). In this thesis, these two balancers are referred to as *hT2g* and *mIn1g*. *lin-53(0)* and *rba-1(0)* are null mutations.

Construction of Mutant Strains

To generate the *hda-3; hda-2; him-8* mutant, an *hda-2;him-8* strain was first constructed, as follows. *unc-75; mIn1/+; him-8* hermaphrodites were first mated with *him-8* males. The cross- progeny (F1) males were then mated with *hda-2* hermaphrodites. The cross-progeny (F2) from the F1 mating were then picked to single plates and screened for production of male (F4) progeny. Production of males indicated that the F3 hermaphrodite was genotype *unc-75; mIn1/hda-2; him-8*. These hermaphrodites were then mated with *hda-3; +; him-8* males. Green, non-Unc males (genotype *unc-75/hda-3; mIn1/+; him8*) were then picked from the cross-progeny and mated with *unc-75; hda-2; him8*

hermaphrodites. *hda-3/unc-75; mIn1/hda-2; him8* hermaphrodites were then cloned. *hda-3; mIn1/hda-2; him8* hermaphrodites were re-cloned in the next generation. The presence of deletion alleles was confirmed via PCR and gel electrophoresis. See Figures 1 and 2**.**

The following strategy was used to generate the *sin-3; met-2* double mutant. *hT2g/om83; hT2g/+; him-8* males were mated with *sin-3* hermaphrodites. Male *sin-3/hT2g; +/hT2g; him-8/+* (F1) progeny were mated with *met-2* hermaphrodites. Non-green male *sin-3/+; +/met-2; him-8/+* and *sin-3/+; +/met-2; + / +* progeny (F2) were then mated with *hT2g/om83; hT2g/+; him-8/+* hermaphrodites. Animals of the genotype *sin-3/hT2g; met-2/hT2g; him-8/+* or *sin-3/hT2g; met-2/hT2g; +/+* were picked to single plates. *sin-3; met-2; him-8* homozygotes were also generated. However, because of the many genotype possibilities, the presence of deletion alleles was confirmed via PCR and gel electrophoresis. See Figures 3 and 4.

RNA interference

To generate *rba-1(RNAi) lin-53(0)* and *rba-1(0) lin-53(RNAi)* animals, *lin-53* mutants were grown on bacteria that produce *rba-1* dsRNA, and *rba-1* mutants were grown on bacteria that produce *lin-53* dsRNA. RNA interference (RNAi) is a biological process in which cells use dsRNA to suppress or silence gene expression and activity. RNAi destroys mRNAs encoded by a specific gene, and therefore prevents production of the protein. Here, dsRNA was introduced into the nematodes by feeding them a strain of bacteria that produces dsRNA via

transcription of a bacterial plasmid containing the gene of interest. The dsRNA

enters the *C. elegans* cells when the bacteria are eaten. Transcription of the

bacterial plasmid produces sense and antisense RNA molecules that then

hybridize to form dsRNA. The dsRNA molecules are cut into small fragments,

which then serve as guides leading the RNAi machinery to mRNAs that are

complementary in sequence to the small fragments. The RNAi machinery slices

the cellular mRNAs, which then prevents their translation (Stiernagle 2006). To

make the RNAi "feeding" plates, a liquid culture of the bacterial strain containing

the plasmid was grown overnight (4mL LB media, 4µL Ampicillin [100mg/mL]

and bacteria) at 37ºC. This culture was seeded onto RNAi plates, which contain

chemicals to induce the expression of the promoters. The bacterial cultures were

then grown at room temperature for 2 days. *lin-53(0)* L1s were picked to an

unseeded to plate to remove the OP50 feeding bacteria. After a few hours, the *lin-53(0)* homozygous and heterozygous mutants were transferred to a *rba-1* RNAi

plate. After three days, *lin-53(0)* homozygous mutants were picked to be used for

indirect immunofluorescence. The same strategy was used to generate *rba-1(0)*

lin-53(RNAi) animals.

Single Worm PCR

2.5µl of lysis buffer was added to the cap of a small PCR tube, and 2-4

worms were placed in the buffer. The cap was shut and labeled according to the

mutant strain. PCR tubes were spun briefly in a centrifuge to move the

buffer/worms to the bottom of the tube and then put at -80ºC for 20 minutes. One

drop of PCR oil was then added to each tube before placing the tubes into the thermocycler. The tubes were incubated at 65ºC for an hour followed by 15 minutes at 95ºC. This process lyses the worms, releasing the DNA, and allowing amplification of the desired region. During incubation, the PCR mixture was made up. This PCR mixture included, per reaction: 16.75 µl distilled water, 2.5 µl of 10X PCR buffer, 1 µl dNTPs, 1 µl of each primer (forward and reverse), and 0.25 µl of TAQ Polymerase (added at the very last minute). 22.5 µl of PCR mixture was added to each tube containing the worm lysate. The tubes were placed back into the thermocycler. The PCR program used was: 94ºC for 1 min; 35 cycles of 94ºC for 30 sec, 50ºC for 30 sec, 72ºC for 1-2 min (depending on the size of the region amplified); 72ºC for 5 minutes; and 4ºC hold. A 1% agarose gel was prepared, and the size of the amplification products was evaluated via gel electrophoresis. Gel electrophoresis is a method used for separation and analysis of macromolecules such as DNA. When the electric current is applied to the gel, the larger molecules move at a slower rate than the smaller molecules. Using a 1 kilobase (kb) ladder marker, wild type and deletion alleles can be distinguished by comparing the size of the amplified DNA product to the marker.

Indirect Immunofluorescence of Gonads for H3K9me2

The H3K9me2 marks were visualized by performing indirect immunofluorescence. The primary antibody used in these studies was Abcam1220 anti-H3K9me2 monoclonal antibody diluted 1:200, and the secondary antibody used was Alexa 488 anti-mouse antibody diluted 1:200.

Details of the H3K9me2 indirect immunofluorescence protocol were as

previously described (Maine *et al.*, 2005; She *et al.*, 2009). Adults were first

washed with M9 buffer in a deep-well slide. The M9 solution was then removed,

and a solution of 1.0 mM levamisole in PBS was added. As soon as the worms

became immobile, the gonads were dissected with needles. The tissue was fixed

in a 2.5% paraformaldehyde (PFA)/ 1X PBS solution for 5 minutes. Next, the

tissue was washed in PBST (1X PBS/ 0.1% Tween-20) for a total of three 15-

minute washes. A drawn capillary was used to remove to solution in order not to

disturb the dissected tissue. The tissue was then blocked for 1-2 hours at room

temperature in a fresh solution of PBST/ 30% GS. The PBST/ 30% GS was

removed, and a 1:200 dilution of primary antibody (in PBST) was added to the

wells. The wells were covered with a slide and parafilm and placed in the

humidifier chamber at 15ºC overnight. The following morning, the tissue was

washed three times for 15 minutes in PBST to remove the antibody solution.

Next, the tissue was incubated in the dark at room temperature for 2 hours with

the secondary antibody. The tissue was then washed in PBST for 15 minutes at

room temperature in the dark, incubated in the dark with 0.2 µg/mL DAPI/1X

PBS for 10 minutes, and washed with 1X PBS for 15 minutes. The gonad arms

and worm carcasses were transferred to flat slides using a drawn capillary pipette.

Excess buffer was removed and 3 µl of VectaShield (Vector Labs) was added to

the slide before a cover slip was gently laid over the gonads and worms. The

slides were stored in the dark overnight at 4ºC before being viewed with a Zeiss

Axioscope compound microscope. Images were captured with a Nikon DS-QiMc

camera using Nikon software.

DAPI Staining of Chromosomes

To view the germline of *sin-3; met-2* hermaphrodites, animals were fixed

in cold methanol and DNA was stained with DAPI, as follows.   Adults were

washed with distilled water in a deep well slide to remove the bacteria. All but of

200 µl of water was removed. -20ºC methanol was added to each well making

sure to mix well with the distilled water. The wells were covered with a slide to

prevent the methanol from evaporating. After 10 minutes, the methanol was

removed and a solution of 0.2 µg/mL DAPI /1X PBS was added for ten minutes

in the dark. The DAPI solution was removed, and 1X PBS was added. Next, the

worms were transferred to a flat slide using a drawn capillary pipette. Excess 1X

PBS was removed and 4 µl of VectaShield was added to the slide before a cover

slip was gently laid over the worms. The slides were stored in the dark overnight

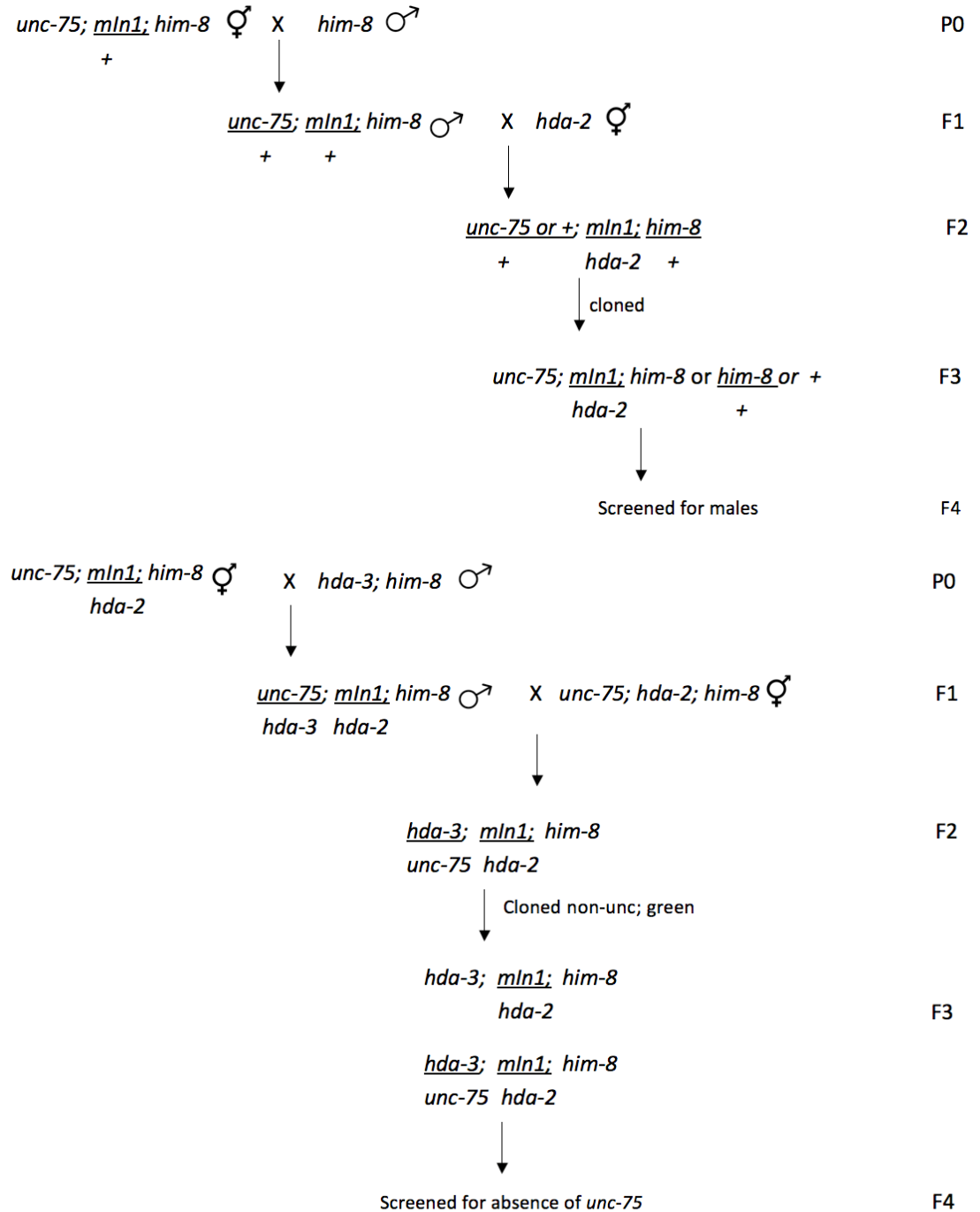at 4ºC before being viewed with the Zeiss Axioscope.

unc-75; mln1; him-8 ♀ X   *him-8* ♂   PO
        +

unc-75; mln1; him-8 ♂ X *hda-2* ♀   F1
    +   +

unc-75 or +; mln1; him-8   F2
    +   *hda-2*   +

| cloned

unc-75; mln1; him-8 or *him-8* or +   F3
       hda-2   +

Screened for males   F4

unc-75; mln1; him-8 ♂   X *hda-3; him-8* ♂   PO
hda-2

unc-75; mln1; him-8 ♂   X *unc-75; hda-2; him-8* ♀   F1
hda-3 hda-2

hda-3; mln1; him-8   F2
unc-75 hda-2

| Cloned non-unc; green

hda-3; mln1; him-8
       hda-2   F3

hda-3; mln1; him-8
unc-75 hda-2

Screened for absence of *unc-75*   F4

Figure 1: This genetic mating scheme was used to construct the *hda-3; hda-2* double mutant strain. It should be noted that the *mIn1* balancer chromosome also carries a *gfp* transgene and *dpy* mutation.
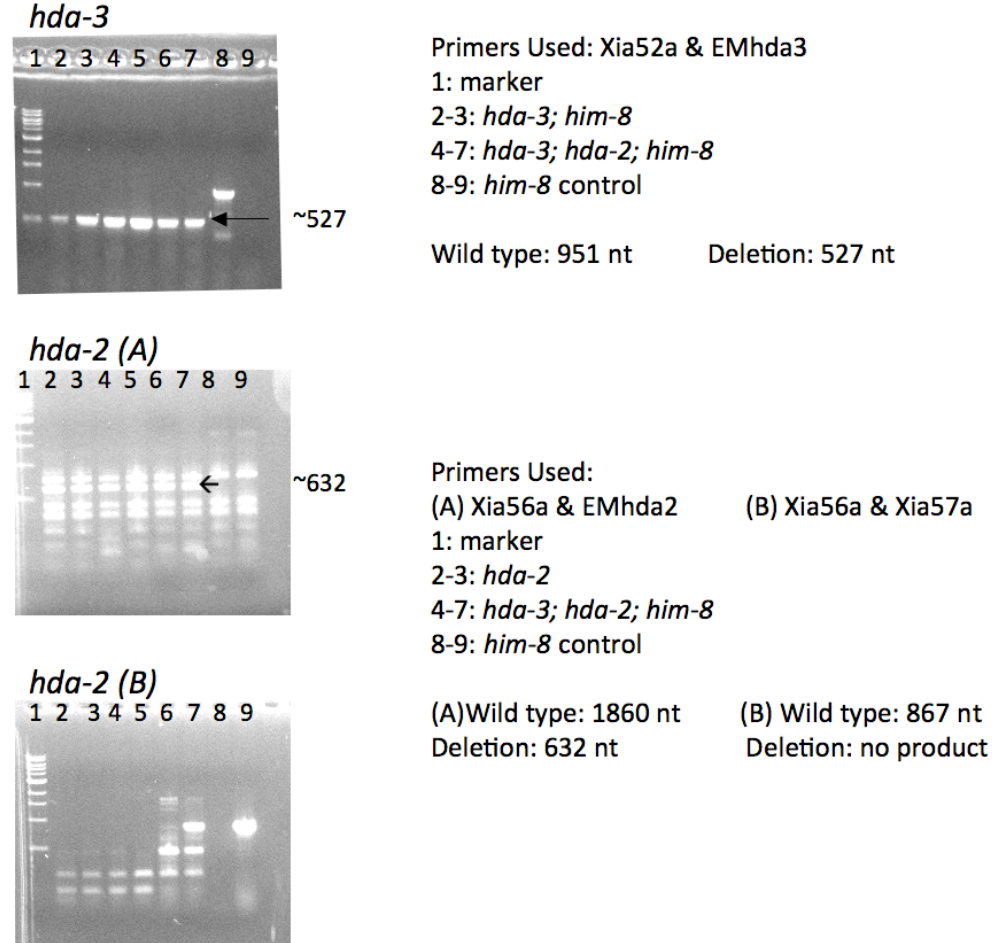
Figure 2: PCR Confirmation of *hda-3; hda-2* deletion alleles

hda-3

Primers Used: Xia52a & EMhda3
1: marker
2-3: *hda-3; him-8*
4-7: *hda-3; hda-2; him-8*
8-9: *him-8* control

~527

Wild type: 951 nt   Deletion: 527 nt

hda-2 (A)

~632

Primers Used:
(A) Xia56a & EMhda2   (B) Xia56a & Xia57a
1: marker
2-3: *hda-2*
4-7: *hda-3; hda-2; him-8*
8-9: *him-8* control

hda-2 (B)

(A)Wild type: 1860 nt   (B) Wild type: 867 nt
Deletion: 632 nt   Deletion: no product

Figure 2: The above panels show the DNA amplification products that were generated to confirm the presence of the *hda-3* and *hda-2* deletion alleles in the *hda-3; hda-2* double mutant, as visualized via gel electrophoresis. The arrows indicate the amplification products from the deletion alleles. For the *hda-2 (B)* photo, note that there is no arrow because there is no amplification product. However, lanes 4 and 5 are homozygous for *hda-2* and lanes 6-7 are heterozygous for *hda-2*.
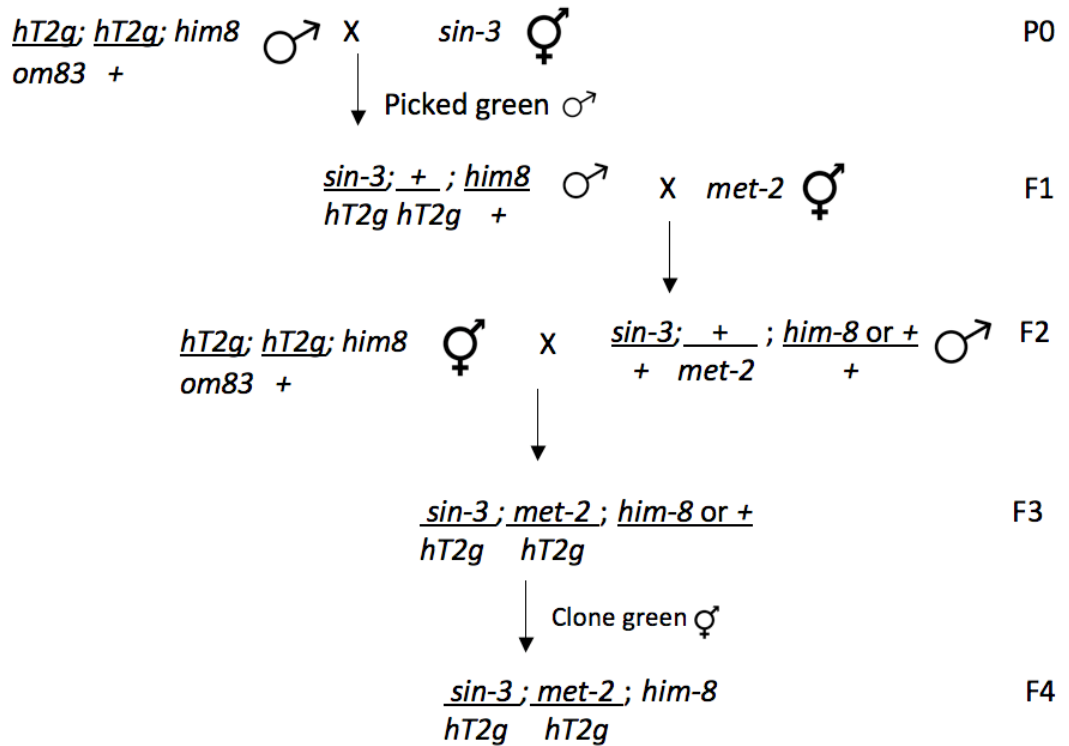
hT2g; hT2g; him8   ♂ X   sin-3 ☿   P0
om83 +

                    | Picked green ♂

                        sin-3; + ; him8   ♂   X   met-2 ☿   F1
                        hT2g hT2g   +

                                        |

    hT2g; hT2g; him8 ☿   X   sin-3;   +   ; him-8 or + ♂   F2
    om83 +   +   met-2   +

                                |

                    sin-3 ; met-2 ; him-8 or +   F3
                    hT2g   hT2g

                        | Clone green ☿

                    sin-3 ; met-2 ; him-8   F4
                    hT2g   hT2g

Figure 3: The mating scheme used to construct the *sin-3; met-2* double mutant strain. Due to the many genotype possibilities in the F3 generation double mutants were distinguished by PCR.
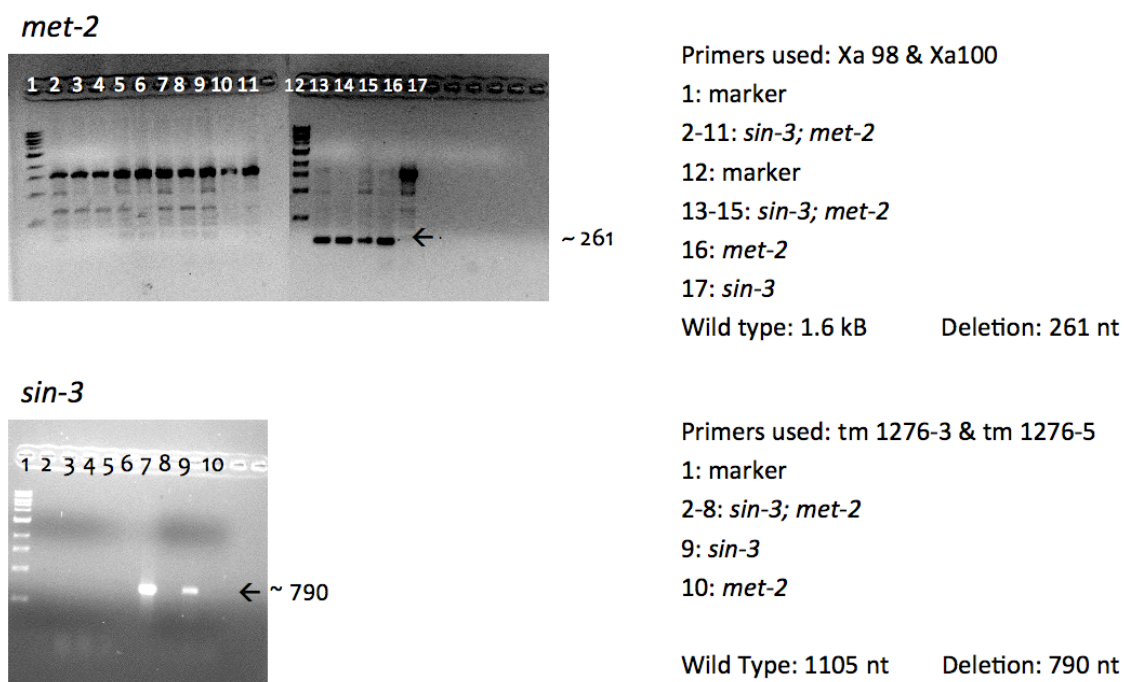
Figure 4: PCR Confirmation of *sin-3; met-2* deletion alleles

met-2

Primers used: Xa 98 & Xa100
1: marker
2-11: *sin-3; met-2*
12: marker
13-15: *sin-3; met-2*
16: *met-2*
17: *sin-3*
Wild type: 1.6 kB   Deletion: 261 nt

~ 261

sin-3

Primers used: tm 1276-3 & tm 1276-5
1: marker
2-8: *sin-3; met-2*
9: *sin-3*
10: *met-2*

Wild Type: 1105 nt   Deletion: 790 nt

← ~ 790

Figure 4: The above panels show DNA amplification products that confirm the presence of the *sin-3* and *met-2* deletion alleles in the *sin-3; met-2* double mutant. Amplification products were visualized via gel electrophoresis. The arrows indicate the size of the amplification product generated from the deletion alleles. For the confirmation of the *met-2* deletion allele, lanes 2-11 and 13-15 were candidate double mutants but only lanes 13-15 contained the *met-2* mutation. *met-2* and *sin-3* were used as controls in both PCR experiments. The image of the *met-2* gel is the photographic negative.

Results

To follow up on previous results from the Maine lab (Lee 2011), we constructed double mutant strains containing mutations in different putative components of the histone deacetylase complex in order to examine if the distribution of H3K9me2 is altered. As mentioned in the Introduction, we made double mutants where possible redundant proteins would be eliminated. By eliminating more than one component at a time in the double mutants, the effects on the H3K9me2 accumulation would become clearer. To determine if the gene products were essential for H3K9me2 accumulation on meiotic unpaired X chromosomes, the mutants were dissected and labeled via indirect immunofluorescence to observe the H3K9me2 pattern in the hermaphrodites' germline. To quantify the results observed, three categories have been created to describe the labeling variations observed from the indirect immunofluorescence experiments. The first category, "with H3K9me2 enrichment," included germlines that had bright and excellent antibody labeling, with defined foci. Two strong foci of anti-H3K9me2 labeling were visible per nucleus in the germline cells (Fig. 5). The second category, "with partial H3K9me2 enrichment," included germlines with reduced anti-H3K9me2 labeling intensity of foci, intermediate and blurry antibody labeling, and some background or non-specific antibody binding where the signal was not in the proper location expected (Fig. 6). The expected signaling in the germline occurs on the foci of the two X chromosomes per nucleus. The last category, "without H3K9me2 enrichment," included germlines where there was no visible antibody labeling on chromosomes, but there was some non-

specific background antibody labeling (Fig. 7). Germlines under this category did

not have two foci of anti-H3K9me2 labeling. There were only faint specks of a

non-specific background labeling that covered the germline. See Table 1.

H3K9me2 enrichment in *hda-3; hda-2* double mutants

As mentioned previously, HDA-2 and HDA-3 are histone deacetylase

enzymes that are likely components of the SIN-3 HDAC complex. The H3K9me

2 distribution was examined in the *hda-3; hda-2* double mutants to investigate if

the HDA-2 and HDA-3 components are redundant in function. Absence of

H3K9me2 enrichment on unpaired hermaphrodite X chromosomes would be an

indication that these factors are essential for the H3K9me2 enrichment process;

however, if there is H3K9me2 enrichment, it would imply that these components

are not crucial for the H3K9me2 modifications. Observations from the indirect

immunofluorescence show that when compared to control *him-8* hermaphrodites,

the *hda-3; hda-2* double mutants did have enrichment. The labeling intensity did

not seem to be reduced, so these components are not essential for the H3K9me2

modification. See Figures 5 and 6.

H3K9me2 enrichment in *rba-1(RNAi) lin-53(0)* and *rba-1(0) lin-53(RNAi)*
animals

We also generated worms with reduced *rba-1* and *lin-53* gene activity. As

stated previously, these genes encode proteins that are thought to be the

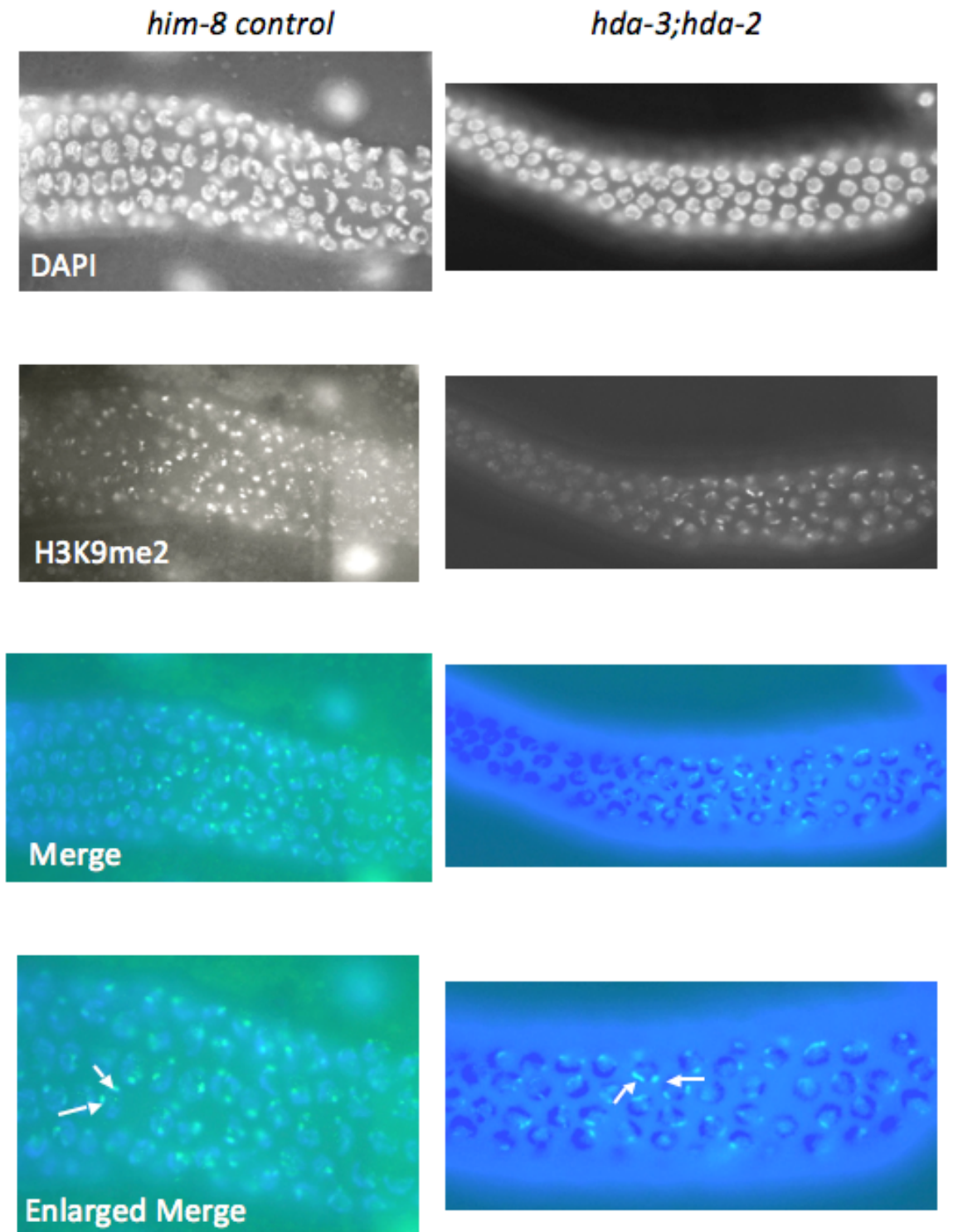components of the SIN-3 HDAC complex responsible for binding to specific

sequences on DNA. If these proteins are redundant for function, then we may find that H3K9me2 is not enriched on unpaired chromosomes when both proteins are knocked out at once. Because these genes are adjacent on the same chromosome we did not make a double mutant. Instead, we used RNA interference to reduce the *rba-1* gene activity in *lin-53(0)* mutants and *lin-53* gene activity in *rba-1(0)* mutants. Observations from the indirect immunofluorescence show that when compared to control *him-8* hermaphrodites, the *rba-1(RNAi) lin-53(0)* and *rba-1(0) lin-53(RNAi)* animals showed variable results (Fig. 5-7). In some nuclei, the H3K9me2 pattern was normal, and in others it was abnormal. There were also variable results and inconsistency within the *him-8* control hermaphrodites where 100% of the germline nuclei are expected to have strong H3K9me2 foci. About a third of the *him-8* controls had partial H3K9me2 enrichment, and two thirds had full H3K9me2 enrichment. The *him-8* controls that had partial enrichment also had non-specific background signaling. This result might be caused by a technical issue when performing indirect immunofluorescence. The data are inconsistent, so it is very difficult to interpret whether loss of LIN-53 and RBA-1 affected the level of H3K9me2 accumulation in the germline. The number of labeled germ lines was also low, so these experiments should be repeated in order to solidify the results. Another important point to mention is that RNAi does not completely eliminate the gene product; therefore, there is a possibility that some residual RBA-1 or LIN-53 activity exists. This may also explain the variable results. If a double knock-out mutant were made, the results might be more consistent. However, *him-8* controls were also variable, which suggests there was an

unknown technical issue encountered during the performance of Indirect

Immunofluorescence.

Table 1: H3K9me2 Distribution in HDA, RBA-1, and LIN-53 Mutants

Genotype	No. of Germlines with H3K9me2 enrichment	No. of Germlines with partial H3K9me2 enrichment	No. of Germlines without H3K9me2 enrichment
hda-3; hda-2	21 (87.5%)	3 (12.5%)	0
him-8 control	36 (92.3%)	3 (7.7%)	0
rba-1(RNAi) lin-53(0)	13 (59.1%)	6 (27.3%)	3 (13.6%)
him-8 control	12 (57.1%)	9 (42.9%)	0
rba-1(0) lin-53(RNAi)	5 (26.3%)	10 (52.6%)	4 (21.1%)
him-8 control	20 (69%)	9 (31%)	0

Germlines were scored as having H3K9me2 enrichment, partial H3K9me2 enrichment, or no H3K9me2 enrichment. Germlines with H3K9me2 enrichment included nuclei that had bright and excellent antibody labeling, with defined foci. Two strong foci of anti-H3K9me2 labeling were visible per nucleus in the germ cells. Germlines with partial H3K9me2 enrichment included nuclei with reduced anti-H3K9me2 labeling intensity of foci, intermediate and blurry antibody labeling, and/or some background or non-specific antibody binding. Germlines categorized as without H3K9me2 enrichment included germlines where there was no visible antibody labeling on chromosomes; nuclei did not have two foci of anti-H3K9me2 labeling. However there were faint specks of non-specific background antibody labeling that covered the germline including the cytoplasm. As a control, *him-8* animals were analyzed in parallel in each individual experiment. Data are arranged with the corresponding *him-8* data listed below each experimental data.
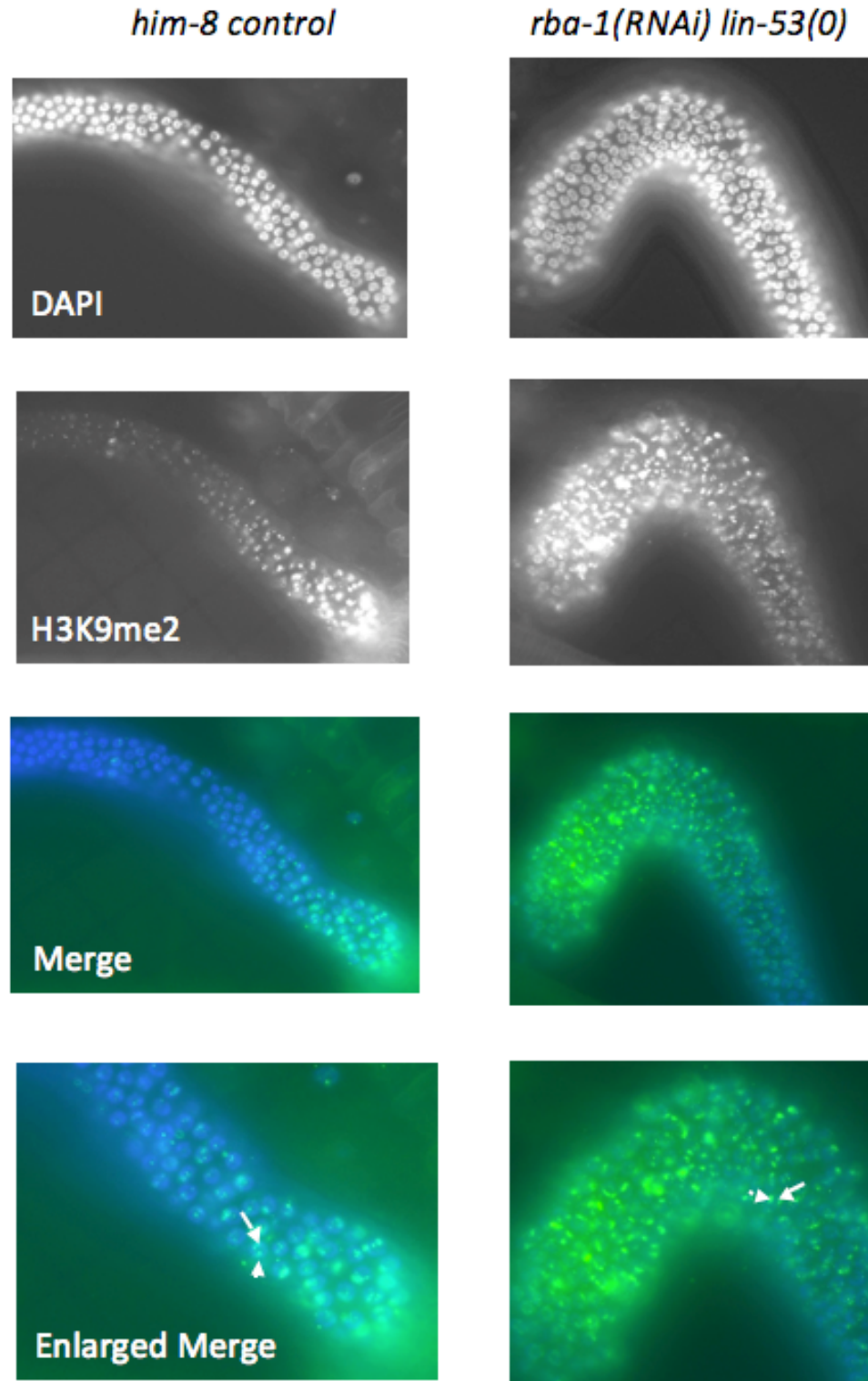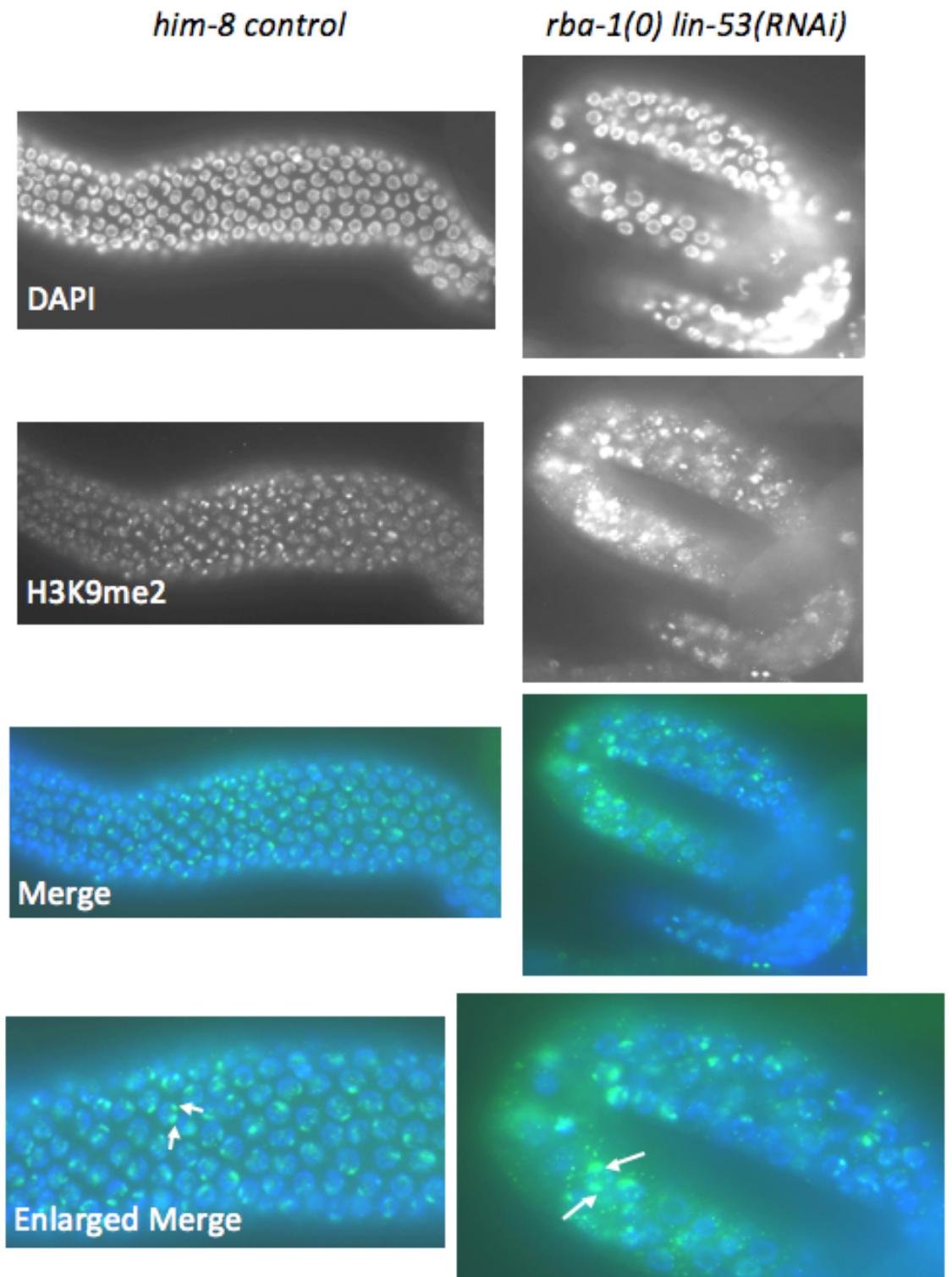
Figure 5

him-8 control · *rba-1(RNAi) lin-53(0)*

DAPI

H3K9me2

Merge

Enlarged Merge

Figure 5

him-8 control   *rba-1(0) lin-53(RNAi)*

DAPI

H3K9me2

Merge

Enlarged Merge

Figure 5

Figure 5: H3K9me2 distribution in *hda-3; hda-2, rba-1(RNAi) lin-53(0)* and *rba-1(0) lin-53(RNAi)*.

Each photo shows nuclei stained with DAPI to visualize DNA and labeled with antibody against H3K9me2. These nuclei are categorized as having "H3K9me2 enrichment" because they had bright and excellent antibody labeling, with defined foci. Two strong foci of anti-H3K9me2 labeling were visible per nucleus. Arrows indicate H3K9me2 enrichment on unpaired X chromosomes. All DAPI images were taken at the same exposure. All H3K9me2 images were taken at same exposure.
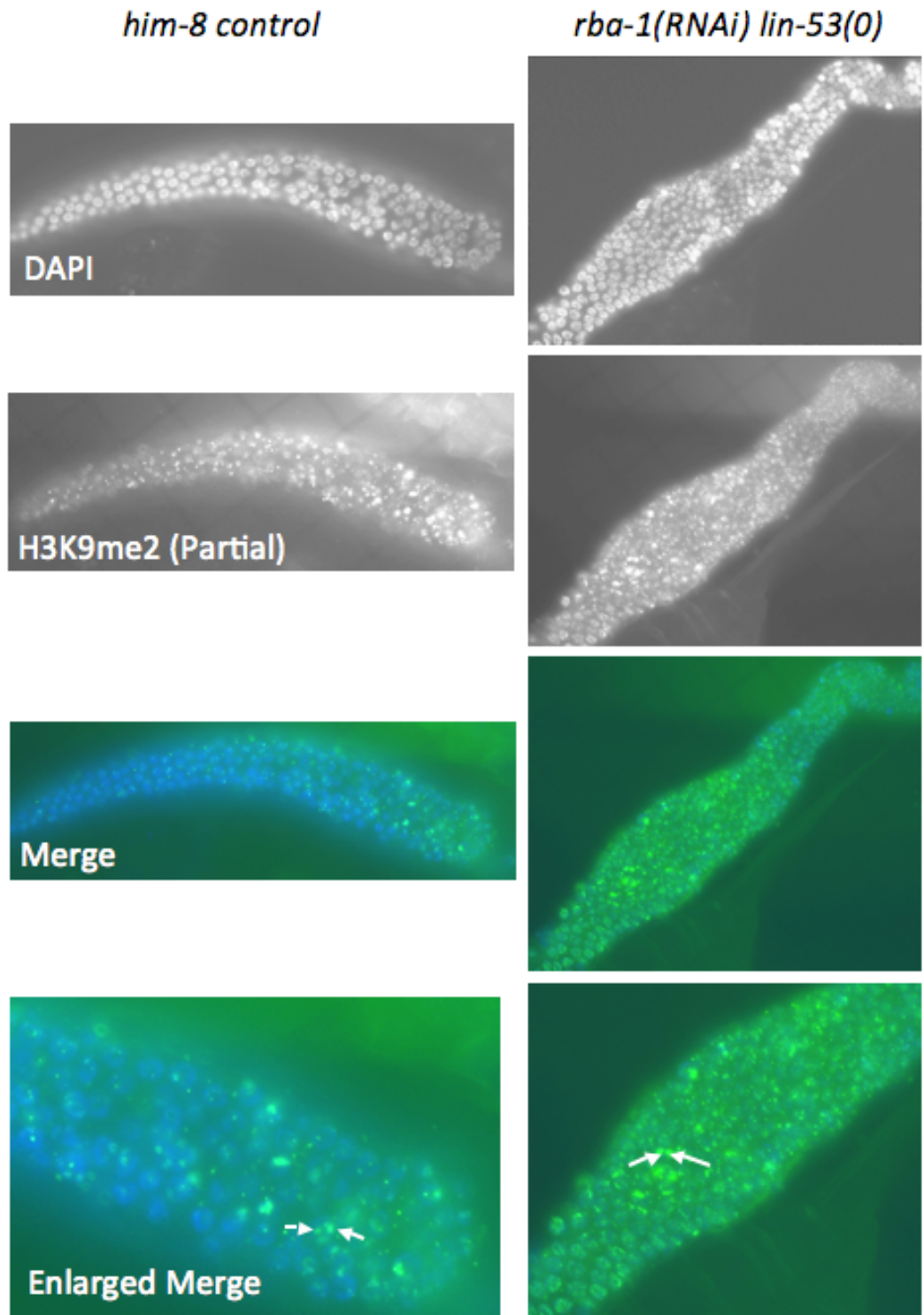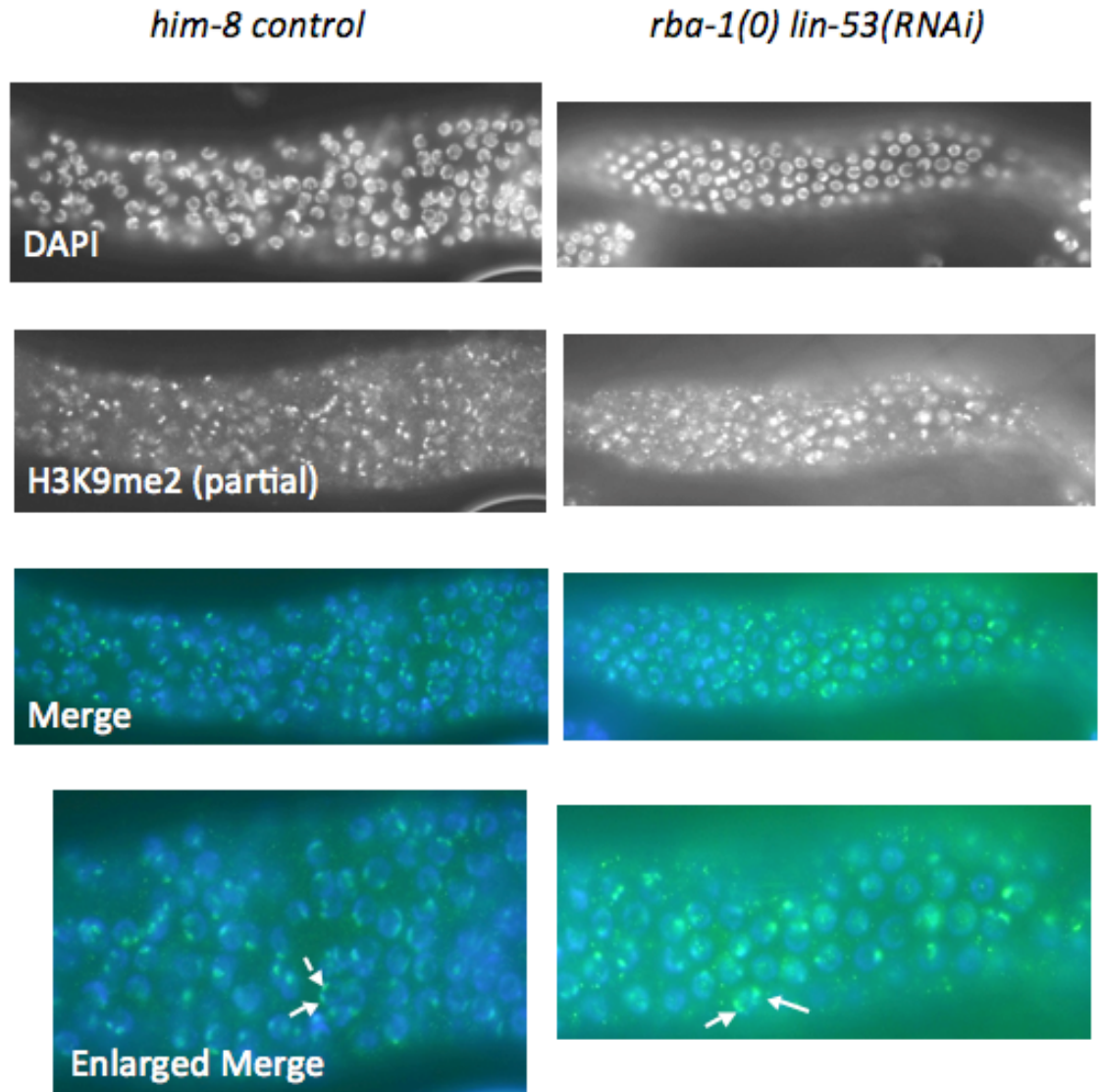
Figure 6

him-8 control   rba-1(RNAi) lin-53(0)

DAPI

H3K9me2 (Partial)

Merge

Enlarged Merge
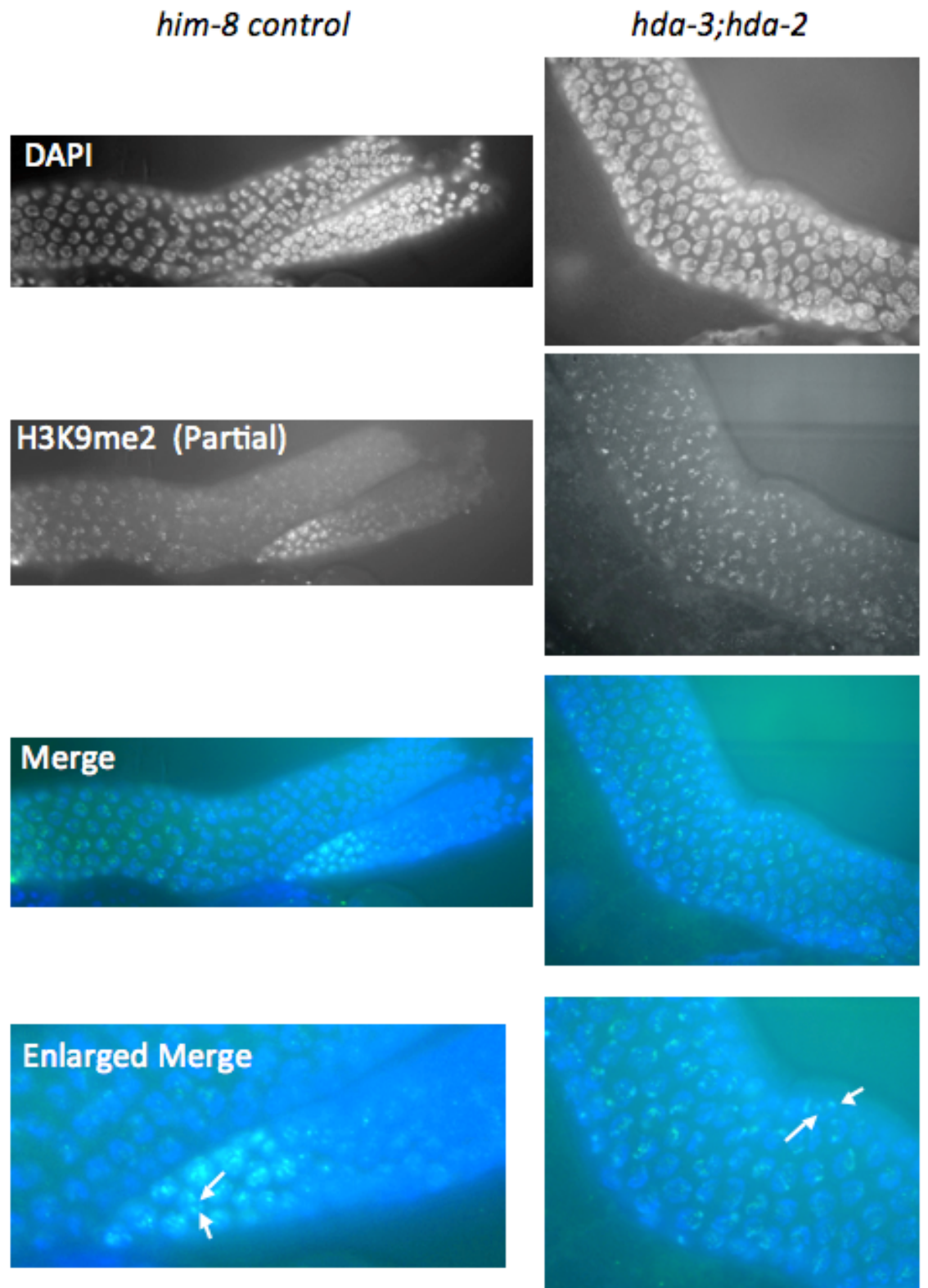
Figure 6

Figure 6

Figure 6: H3K9me2 distribution in *hda-3; hda-2, rba-1(RNAi) lin-53(0)* and *rba-1(0) lin-53(RNAi)* germ cells.

Each photo shows meiotic nuclei stained with DAPI to visualize DNA and labeled with antibody against H3K9me2. These nuclei are categorized as having "partial H3K9me2 enrichment" because they have reduced anti-H3K9me2 labeling intensity of foci, intermediate and blurry antibody labeling, and some background or non-specific antibody binding. Arrows indicate "partial H3K9me2 enrichment" on unpaired X chromosomes. All DAPI images were taken at the same exposure. All H3K9me2 images were taken at same exposure.
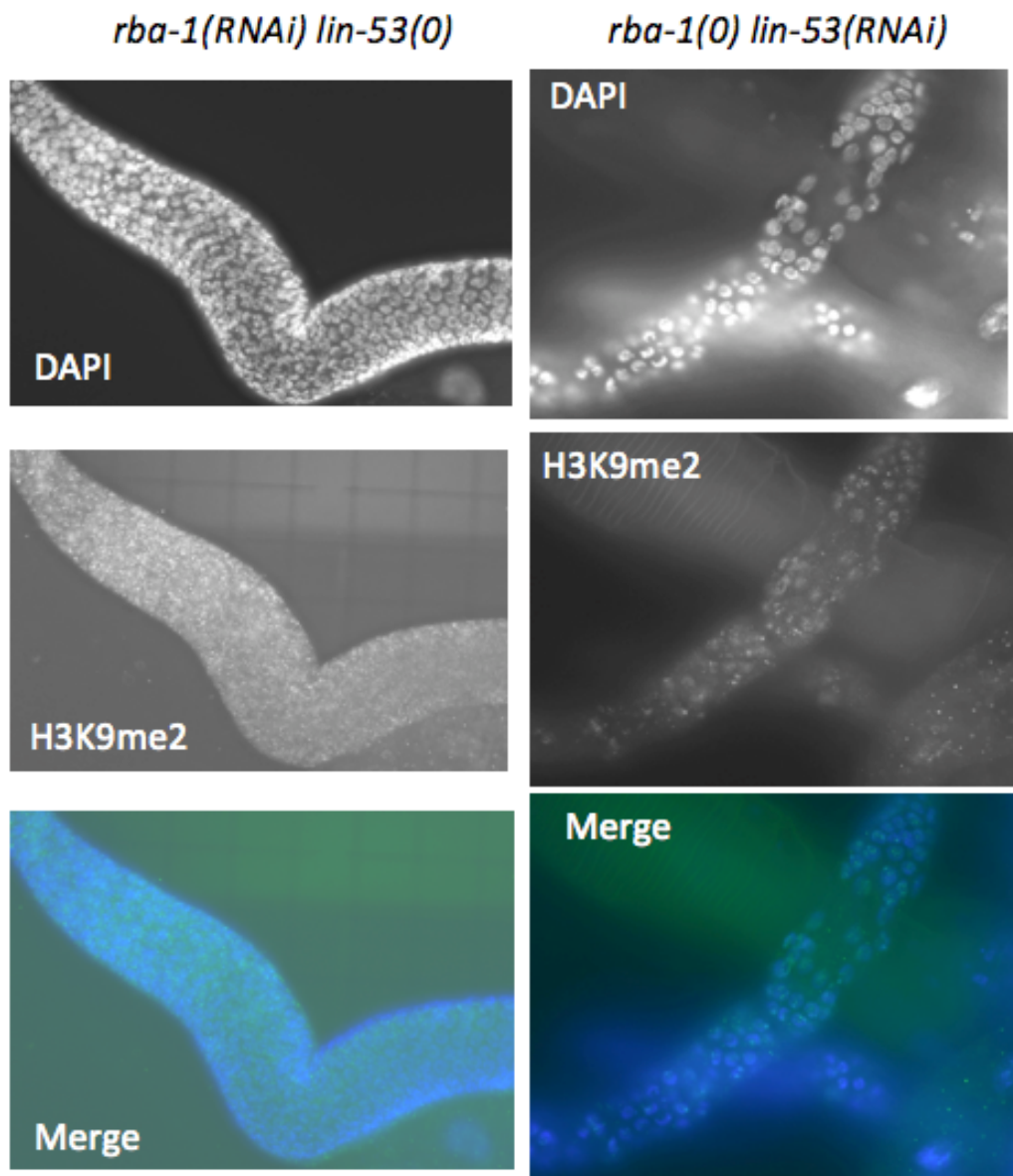
rba-1(RNAi) lin-53(0)   *rba-1(0) lin-53(RNAi)*

Figure 7

Figure 7: H3K9me2 distribution in *rba-1(RNAi) lin-53(0)* and *rba-1(0) lin-53(RNAi)* germ cells.

These images show examples of nuclei that failed to label well with anti-H3K9me3 antibody. DNA is stained with DAPI. These nuclei are categorized as "without H3K9me2 enrichment" because there was no visible antibody labeling on chromosomes; nuclei did not have two foci of anti-H3K9me2 labeling. However there were faint specks of non-specific background antibody labeling that covered the germline. The *him-8* control is not shown because all *him-8* controls either had full or partial H3K9me2 enrichment. All DAPI images were taken at the same exposure. All H3K9me2 images were taken at same exposure.

Germline Development in *sin-3; met-2* double mutants

We constructed a *sin-3; met-2* double mutant to observe its germline phenotype. The question is whether SIN-3 activity primarily removes H3K9ac, which would allow MET-2 to generate H3K9me2. If it does, then the *met-2; sin-3* double mutant should resemble the *met-2* single mutant. This would also suggest that the SIN-3 and MET-2 proteins work together by interacting in a common biochemical pathway that allows this dimethylation to occur. In contrast, if SIN-3 has other or additional functions, then we would notice that the phenotype of the *met-2; sin-3* mutant would be more severe than the *met-2* phenotype. After analyzing and comparing germlines from all three mutants, the *sin-3; met-2* double mutant seems normal when compared to *sin-3* and *met-2* germlines (Fig. 8). The *sin-3; met-2* double mutants are fertile and viable.
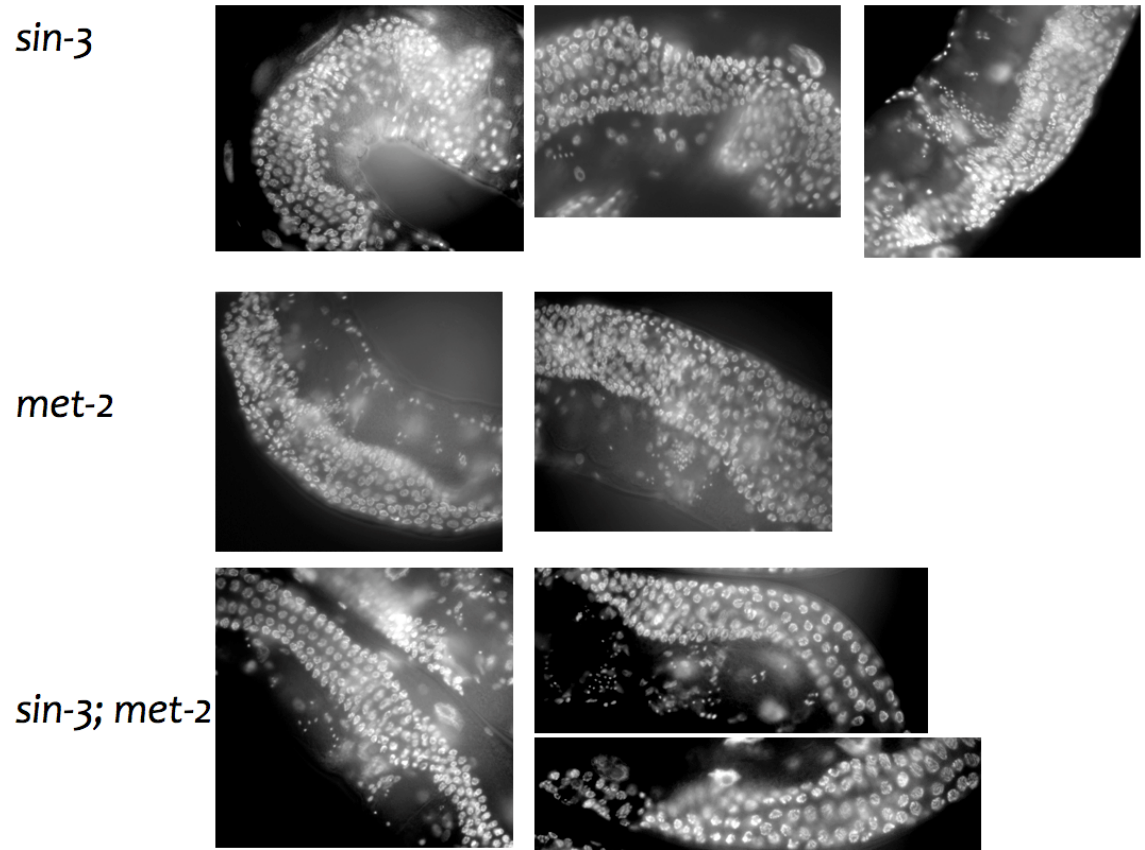
Figure 8: DAPI Staining of *sin-3*, *met-2*, and *sin-3; met-2* mutants

Each photo shows a region of the germline. DAPI staining was used to visualize the DNA. The *sin-3; met-2* double mutant does not have a more severe phenotype when compared to the *sin-3* and *met-2* single mutants. The germline of the *sin-3; met-2* double mutant seems normal, and the animals are viable and fertile, suggesting that SIN-3 may work in a common biochemical pathway with MET-2 to generate H3K9me2.

Discussion

The experiments and data mentioned previously demonstrate that together HDA-2 and HDA-3, and LIN-53 and RBA-1 activities are not essential for the accumulation of H3K9me2 on nonsynapsed chromosomes during meiosis. The data showed that *hda-3; hda-2, rba-1(RNAi) lin-53(0)* and *rba-1(0) lin-53(RNAi)* germlines all accumulated H3K9me2 on unpaired meiotic X chromosomes. The mechanism involved in this chromatin regulation was not completely inhibited by the reduced protein activity of HDA-2 and HDA-3, and LIN-53 and RBA-1 components.

Reduced activity of some components (i.e., *rba-1(0) lin-53(RNAi)*) did show some effect while removal of other components (*hda-3; hda-2*) showed little effect when compared to the *him-8* control. Some of the collected data were extremely variable and inconsistent. It is unknown why the results from the *rba-1(RNAi) lin-53(0)* and *rba-1(0) lin-53(RNAi)* mutants and *him-8* controls were inconsistent and variable. However, based on the data, we can consider which of the genes seem to be of least importance and which seem to have some role in determining the H3K9me2 pattern in the germline.

From the results, *hda-3; hda-2* germlines showed the same level of labeling as the *him-8* controls, so these components may not be of importance in H3K9me2 accumulation. However, results from past experiments performed by Guang Lee suggest that when these two components were individually removed, labeling intensity was decreased. The result was not completely solidified due to low number of animals used, but constructing a mutant lacking all three HDAs

would remove possible overlapping and redundant functions performed by the HDA components.

H3K9me2 labeling in the *rba-1(RNAi) lin-53(0)* and *rba-1(0) lin-53(RNAi)* mutants and *him-8* controls was diffuse. When viewed, some germline nuclei contained strong labeling on the X chromosomes while others had only faint specks of non-specific background signaling. RNAi may not completely eliminate the gene product; therefore, there is a possibility that some residual RBA-1 or LIN-53 activity exists. This may also explain the variable results. If a double knock-out mutant were made, the results might be more consistent. The same variation and inconsistency was also seen in the *him-8* controls. This would suggest that there was an unknown technical issue encountered during the performance of Indirect Immunofluorescence protocol because 100% of the *him-8* germline nuclei are expected to have strong H3K9me2 foci. Another possible explanation for this inconsistency is that during meiosis the X chromosome were not properly compacted which could explain the low level of H3K9me2 labeling and non-specific labeling in the germline cells.

As mentioned previously, we constructed a *sin-3; met-2* double mutant to observe its germline phenotype. The question we asked is whether SIN-3 activity primarily removes H3K9ac, which would allow MET-2 to generate H3K9me2. If it does, then the *met-2; sin-3* double mutant should resemble the *met-2* single mutant. This would also suggest that the SIN-3 and MET-2 proteins work together by interacting in a common biochemical pathway that allows this dimethylation to occur. After analyzing and comparing germlines from all three

mutants, the *sin-3; met-2* double mutant seems normal when compared to *sin-3* and *met-2* germlines. The *sin-3; met-2* animals are fertile and viable. These results suggest that SIN-3 may interact with MET-2 in a common biochemical pathway that allows this dimethylation.

How these four gene products interact along with the other gene products of the SIN-3 HDAC complex to allow for the H3K9me2 chromatin modification is unclear. However, as more components are systematically eliminated at a time, the question of which components of the SIN-3 HDAC contribute to the H3K9me2 accumulation will become answered. Future studies will include constructing other different mutant combinations of the SIN-3 components.

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Summary

Chromatin is the combination of DNA and the proteins binding to DNA in the cell nucleus. The primary proteins associated with chromatin are histones, which compact the DNA. The DNA strand wraps around the histone octamer core complex forming a structure called the nucleosome. Histones undergo posttranslational modifications that alter their interaction with DNA and other nuclear proteins. The sites of modification are usually in the histone amino-terminal tails. One common modification is acetylation, which loosens DNA binding to histones and promotes transcription. Generally speaking, acetylated histones are associated with active gene expression whereas deacetylated histones are associated with repressed gene expression.

Histone deacetylases remove acetyl groups, which increases the positive charge of histone tails resulting in increased DNA-histone binding affinities. Deacetylated histones are associated with repressed gene expression because DNA wraps around the histone more tightly, in turn inhibiting transcription. Histone deacetylases usually function as a multi-protein complex. One major histone deacetylase (HDAC) complex is the SIN-3 HDAC complex. SIN-3 acts as a "scaffold," and is specifically thought to interact with certain DNA binding proteins and histone deacetylases to assemble this HDAC complex. Although the components have not been well described in *C. elegans*, based on work in other organisms, it is thought that this HDAC complex is composed of: SIN-3; the HDA-1 (histone deacetylase), HDA-2, and HDA-3 enzymatic components; and the RBA-1 and/or LIN-53/RBA-2 DNA binding components. For my project, I

am interested in understanding which components of the SIN-3 HDAC complex contribute to the accumulation of H3K9me2, histone H3 lysine 9 dimethylation, on unpaired meiotic chromosomes.

The model organism used in the Maine Lab is *Caenorhabditis elegans. C. elegans* is a small, free living soil nematode that lives in many parts of the world feeding on microbes, but primarily bacteria. It is a good model organism for research in biological sciences including genomics, cell biology, neuroscience, aging, and development. This model organism has many advantages: short life cycle, small size, compact genome, rapid period of embryogenesis, ability to produce large number of progeny, and a transparent body allowing internal cells to be easily visualized without dissection.

When germ cells enter meiotic prophase I, duplicated chromosomes coil becoming shorter and thicker. In *C. elegans,* a chromosome that fails to pair with a partner and synapse (for example, the male X chromosome) during meiotic prophase I accumulates a high level of histone H3 lysine 9 dimethylation (H3K9me2) (Kelly *et al*., 2002; Bean *et al*., 2004). This modification is associated with facultative heterochromatin assembly, which may result in transcriptional silencing. Also, this modification may mark silenced genes. High levels of H3K9me2 are observed under special and specific conditions in *C. elegans* hermaphrodites (Bean *et al*., 2004).

Previous studies in the Maine Lab have observed that loss of SIN-3 function disrupts H3K9me2 accumulation on unpaired X chromosomes during meiosis in *C. elegans* hermaphrodites (She et al., 2009). However, the H3K9me2

accumulation was not affected on the male X-chromosome. These results suggested that SIN-3 activity is required for H3K9me2 to accumulate on unpaired X-chromosomes in hermaphrodites but not males.

Continuing the studies of a former student, Guang Yu Lee, who investigated "Meiotic Silencing in *C. elegans* Through SIN-3 Histone Deacetylase," a genetic approach has been taken to investigate which components of the SIN-3 complex contribute to the accumulation of H3K9me2 on unpaired meiotic chromosomes. Guang Lee found that the distribution of H3K9me2 was normal in individual *hda-1, hda-2,* and *hda-3* mutants when compared to controls. These results suggested that, individually, the HDA-1, HDA-2, and HDA-3 protein components are not essential for H3K9me2 accumulation. To follow up on this result, I investigated whether the HDA proteins were redundant for function. To do so, I constructed double mutant strains containing mutations in different components of the histone deacetylase complex and examined if the distribution of H3K9me2 was altered. By eliminating more than one component at a time in the double mutants, the effects on H3K9me2 accumulation would become clearer. I also generated mutant worms with reduced gene activity by growing mutant worms on bacteria that produce double stranded RNA of the gene of interest to be suppressed.

The H3K9me2 marks in the mutant worms were visualized by performing indirect immunofluorescence. This protocol involves the dissection of hermaphrodites' gonad arm to view the germ cells and the use of an antibody that binds to the histone modification, H3K9me2. Slides were prepared after the

protocol, and the gonads were viewed using a Zeiss Axioscope compound microscope.

Germlines were scored as having H3K9me2 enrichment, partial H3K9me2 enrichment, or no H3K9me2 enrichment on the (nonsynapsed) X chromosomes in the meiotic nuclei. Germlines with H3K9me2 enrichment included nuclei that had bright and excellent antibody labeling, with defined foci. Two strong foci of anti-H3K9me2 labeling were visible per nucleus. Germlines with partial H3K9me2 enrichment included nuclei with some defined foci, intermediate and blurry antibody labeling, and some background or non-specific antibody binding. Germlines categorized as without H3K9me2 enrichment included nuclei where there was no visible antibody labeling on chromosomes; nuclei did not have two foci of anti-H3 K9me2 labeling. However there were faint specks of non-specific background antibody labeling that covered the germline. As a control, *him-8* animals were analyzed in parallel in each individual experiment.

The experiments and data demonstrate that together HDA-2 and HDA-3, and LIN-53 and RBA-1, activities are not essential in the accumulation of H3K9me2 on nonsynapsed chromosomes during meiosis. The data showed that *hda-3; hda-2*, *rba-1(RNAi) lin-53(0)* and *rba-1(0) lin-53(RNAi)* meiotic nuclei all accumulated H3K9me2 on unpaired meiotic X chromosomes. The mutations and RNAi both reduce protein activity. Therefore, the mechanism involved in this chromatin regulation was not completely inhibited by the reduced protein activity of HDA-2 and HDA-3, and LIN-53 and RBA-1 components.

The role of these four gene products in the SIN-3 HDAC is still unclear. However, as more components are systematically eliminated, the question of which components of the SIN-3 HDAC contribute to the H3K9me2 accumulation will be answered. Future studies will include constructing other different mutant combinations of the SIN-3 components.