Caenorhabditis elegans establishes germline versus soma by balancing inherited histone methylation

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DOI: 10.1242/dev.196600

Editor: Swathi Arur

Review timeline
Transfer from Review Common received: 31 August 2020
Editorial decision: 3 September 2020
First revision received: 3 November 2020
Editorial decision: 15 December 2020
Second revision received: 3 January 2021
Accepted: 4 January 2021

Submission to Review Commons

Reviewer 1

Evidence, reproducibility and clarity

The Katz lab has contributed greatly to the field of epigenetic reprogramming over the years, and this is another excellent paper on the subject. I enjoyed reviewing this manuscript and don't have any major comments/suggestions for improving it. The findings presented are novel and important, the results are clear cut, and the writing is clear.

It's important to stress the novelty of the findings, which build upon previous studies from the same lab (upon a shallow look one might think that some of the conclusions were described before, but this is not the case). Despite the fact that this system has been studied in depth before, it remained unclear why and how germline genes are bookmarked by H3K36 in the embryo, and it wasn't known why germline genes are not expressed in the soma.

To study these questions Carpenter et al. examine multiple phenotypes (developmental aberrations, sterility), that they combine with analysis of multiple genetic backgrounds, RNA- seq, CHIP-seq, single molecule FISH, and fluorescent transgenes.

Previous observations from the Katz lab suggested that progeny derived from spr-5;met-2 double mutants can develop abnormally. They show here that the progeny of these double mutants (unlike spr-5 and met-2 single mutants) develop severe and highly penetrate developmental delays, a Pvl phenotype, and sterility. They show also that spr-5; met-2 maternal reprogramming prevents developmental delay by restricting ectopic MES-4 bookmarking, and that developmental delay of spr-5;met-2 progeny is the result of ectopic expression of MES-4 germline genes. The bottom line is that they shed light on how SPR-5, MET-2 and MES-4 balance inter-generational inheritance of H3K4, H3K9, and H3K36 methylation, to allow correct specification of germline and somatic cells. This is all very important and relevant also to other organisms.

(very) Minor comments:

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-Since the word "heritable" is used in different contexts, it could be helpful to elaborate, perhaps in the introduction, on the distinction between cellular memory and transgenerational inheritance.

-It might be interesting in the Discussion to expand further about the links between heritable chromatin marks and heritable small RNAs. The do hint that the result regarding the silencing of the somatic transgene are especially intriguing

Significance

This is an exciting paper which build upon years of important work in the Katz lab. The novelty of the paper is in pinpointing the mechanisms that bookmark germline genes by H3K36 in the embryo, and explaining why and how germline genes are prevented from being expressed in the soma.

Reviewer 2

Evidence, reproducibility and clarity

Katz and colleagues examine the interaction between the methyltransferase MES-4 and spr-5; met-2 double mutants. Their prior analysis (PNAS, 2014) showed the dramatic enhancement in sterility and development for spr-5; met-2; this paper extends that finding by showing these effects depend on MES-4. The results are interesting and the genetic interactions dramatic. The examination by RNAseq and ChIP helps move the phenotypes into a more molecular analysis. The authors hypothesize that SPR-5 and MET-2 modify chromatin of germline genes (MES-4 targets) in somatic cells, and this is required to silence germline genes in the soma. A few issues need to be resolved to test these ideas and rule out others.

Main comments:
The authors' hypothesis is that SPR-5 and MET-2 act directly, to modify chromatin of germline genes (MES-4 targets), but alternate hypothesis is that the key regulated genes are i) MES-4 itself and/or ii) known regulators of germline gene expression e.g. the piwi pathway.Mis regulation of these factors in the soma could be responsible for the phenotypes. Therefore, the authors should analyze expression (smFISH and where possible protein stains) for MES-4 and PIWI components in the embryo and larvae of wildtype, double and triple mutant strains. These experiments are essential and not difficult to perform.

A second aspect of the hypothesis is that spr-5 and met-2 act before mes-4 and that while these genes are maternally expressed, they act in the embryo. There really aren't data to support these ideas - the timing and location of the factors' activities have not been pinned down. One way to begin to address this question would be to perform smFISH on the target genes and on mes-4 in embryos and determine when and where changes first appear. smFISH in embryos is critical - relying on L1 data is too late. If timing data cannot be obtained, then I suggest that the authors back off of the timing ideas or at least explain the caveats. Certainly, figure 8 should be simplified and timing removed. (note: Typical maternal effect tests probably won't work because if the genes' RNAs are germline deposited, then a maternal effect test will reflect when the RNA is expressed but not when the protein is active. A TS allele would be needed, and that may not be available.)

Writing/clarity:

-It would be helpful to include a table that lists the specific genes studied in the paper and how they behaved in the different assays e.g. RNAseq 1, RNAseq 2, MES-4 target, ChIP. That way, readers will understand each of the genes better.

-At the end of each experiment, it would be helpful to explain the conclusion and not wait until the Discussion. For readers not in the field, the logic of the Results section is hard to follow.

-The model is explained over three pages in the Discussion. It would be great to begin with a single paragraph that summarizes the model/point of the paper simply and clearly.

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Specific comments:
-Figure 1 has been published previously and should be moved to the supplement.
-Cite their prior paper for the vulval defects e.g. page 6 or show in supplement.
-The second RNAseq data should be shown in the Results since it is much stronger. The first RNAseq, which is less robust, should be moved to supplement.
-Figure 3 is very nice. Please explain why the RNAs were picked (+ the table, see comment above), and please add here or in a new figure mes-4 and piwi pathway expression data in wildtype vs double/triple mutants.
-Figure 3 here or later, please show if mes-4 RNAi removes somatic expression of target genes.

-Is embryogenesis delayed?

-Figure 4 since htp-1 smFISH is so dramatic, it would be helpful to include htp-1 in the lower panels.

-Figure 4, please add an extra 2 upper panels showing all the genes in N2 vs spr-5;met-2, for comparison to the mes-4 cohort.

-Figure 6. Please show a control that met-1 RNAi is working.

-To quantify histone marks more clearly, it would be wonderful to have a graph of the mean log across the gene. showing the mean numbers would help clarify the degree of the effect. we had an image as an example but it does not paste into the reviewer box. Instead, see figure 2 or figure 4 here: https://www.nature.com/articles/ng.322

Significance

Katz and colleagues examine the interaction between the methyltransferase MES-4 and spr- 5; met-2 double mutants. Their prior analysis (PNAS, 2014) showed the dramatic enhancement in sterility and development for spr-5; met-2; this paper extends that finding by showing these effects depend on MES-4. The results are interesting and the genetic interactions dramatic. The examination by RNAseq and ChIP helps move the phenotypes into a more molecular analysis.

This work will be of interest to people following transgenerational inheritance, generally in the C. elegans field. People using other organisms may read it also, although some of the worm genetics may be complicated. Some of the writing suggestions could make a difference.

I study C. elegans embryogenesis, chromatin and inheritance.

Reviewer 3

Evidence, reproducibility and clarity

In the paper entitled "C. elegans establishes germline versus soma by balancing inherited histone methylation" Carpenter BS et al examined a double mutant worm strain they had previously produced of the H3K4me1/2 demethylase spr-5 and the predicted H3K9me1/me2 methylase met-2. These mutant worms have a developmental delay that arises by the L2 larval stage. They performed an analysis of what genes get misexpressed in these double mutants by performing RNAseq and compare this to datasets generated from other labs on an H3K36me2/me3 methylase MES-4 where they see a high degree of overlap. They validate the misexpression of some germline specific genes in the soma by in situ and validate that there is a dysregulation of H3K36me3 in their double mutant worms. They further find that knocking down mes-4 reverts the developmental delay.

I think that the authors need to make more of an effort to be a bit more scholarly in terms of placing their work in the context of the field as a whole and also need to add a few additional experiments as well as reorganize a bit before this is ready for publication. Remember that the average reader is not necessarily an expert in C. elegans or this particular field and you really want to try and make the manuscript as accessible to everyone as possible.

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Major Points
1)It would be good to see western blots or quantitative mass spec examining H3K36me3 in the WT and spr-5;met-2 double mutant worms. I believe this was also previously reported by Greer EL et al Cell Rep 2014 in the single spr-5 mutant worm so that work should be cited here in addition to the identification of JMJD-2 as an enzyme involved in the inheritance of H3K4me2 phenotype.

2)Missing from Fig.5 is mes-4 KD by itself. This is needed to determine whether these effects are specific to the spr-5;met-2 double mutants or more general effects that KD of mes-4 would decrease the expression of all these genes to a similar extent. Then statistics should be done to see if the decrease in the WT context is the same or greater than the decrease in the double mutants.

Minor Points
1)A greater attempt needs to be made to be more scholarly for citing previously published literature. This includes work on the inheritance of H3K27 and H3K36 methylation in C. elegans and other species as well. A few papers which seem germane to this story which should be cited in the intro are (Nottke AC et al PNAS 2011, Gaydos LJ et al Science 2014, Ost A et al Cell 2014, Greer EL et al Cell Rep 2014, Siklenka K et al Science 2015, Tabuchi TM et al Nat Comm 2018, Kaneshiro KR et al Nat Comm 2019). This problem is not restricted to the intro.

2)I think that the authors need to be a little less definitive with your language. Theories should be introduced as possibilities rather than conclusions. Should remove "comprehensive" from intro as there are many other methods which could be done to test this.

3) The authors should describe what PIE-1 is. Is this a transcription factor?

4) The language needs clarification about MES-4 germline genes and bookmark genes. Are these bound by MES-4 or marked with K36me2/3?

5)I think Fig S1 E+F should be in the main figure 1 so readers can see the extent of the phenotype.

6) For Fig S2 it would be good to do the same statistics that is done in Fig 2 and mention them in the text so the readers can see that the overlap is statistically significant.

7) Fig S2.2 should be yellow blue rather than red green for the colorblind out there.

8) When saying "Many of these genes involved in these processes..." the authors need to include numbers and statistics.

9) Should use WT instead of N2 and specify what wildtype is in methods.

10) Fig. 2A + B could be displayed in a single figure. And Fig 2D seems superfluous and could be combined with 2C or alternatively it could be put in supplementary.

11) Non-C. elegans experts won't understand what balancers are. An effort should be made to make this accessible to all. Explaining when genes are heterozygous or homozygous mutants seems relevant here.

12) The GO categories (Fig. S2) should be in the main figure and need to be made to look more scientific rather than copied and pasted from a program.

13) Fig. 7 seems a bit out of place. If the authors were to KD mes-4 and similarly show that the phenotype reverts that would help justify its inclusion in this paper. Without it seems like a bit of an add on that belongs elsewhere.

Significance

I think this is an interesting and timely piece of work. A little more effort needs to be put in to make sure it is accessible to the average reader and has sufficient inclusion of more of the large

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body of work on inheritance of histone modifications. I think C. elegans researchers as well as people interested in inheritance and the setup of the germline will be interested in this work.

REFEREES CROSS COMMENTING

I agree with Reviewer #2's comments on experiments to include or exclude alternative models. I also agree about their statement about rewriting to make it more accessible to others who aren't experts in this specialized portion of C. elegans research. All in all it seems like the experiments which are required by reviewer #2 and myself as well as the rewriting should be quite feasible.

Author response to reviewers' comments

We thank the reviewers for their close reading and constructive comments on our manuscript. We believe that their insight has substantially strengthened our manuscript. Please find our responses to each comment below (in blue).

Reviewer #1 (Evidence, reproducibility and clarity (Required)):

The Katz lab has contributed greatly to the field of epigenetic reprogramming over the years, and this is another excellent paper on the subject. I enjoyed reviewing this manuscript and don't have any major comments/suggestions for improving it. The findings presented are novel and important, the results are clear cut, and the writing is clear.

It's important to stress the novelty of the findings, which build upon previous studies from the same lab (upon a shallow look one might think that some of the conclusions were described before, but this is not the case). Despite the fact that this system has been studied in depth before, it remained unclear why and how germline genes are bookmarked by H3K36 in the embryo, and it wasn't known why germline genes are not expressed in the soma.

To study these questions Carpenter et al. examine multiple phenotypes (developmental aberrations, sterility), that they combine with analysis of multiple genetic backgrounds, RNA-seq, CHIP-seq, single molecule FISH, and fluorescent transgenes.

Previous observations from the Katz lab suggested that progeny derived from spr-5;met-2 double mutants can develop abnormally. They show here that the progeny of these double mutants (unlike spr-5 and met-2 single mutants) develop severe and highly penetrate developmental delays, a Pvl phenotype, and sterility. They show also that spr-5; met-2 maternal reprogramming prevents developmental delay by restricting ectopic MES-4 bookmarking, and that developmental delay of spr-5;met-2 progeny is the result of ectopic expression of MES-4 germline genes. The bottom line is that they shed light on how SPR-5, MET-2 and MES-4 balance inter-generational inheritance of H3K4, H3K9, and H3K36 methylation, to allow correct specification of germline and somatic cells. This is all very important and relevant also to other organisms.

(very) Minor comments:

-Since the word "heritable" is used in different contexts, it could be helpful to elaborate, perhaps in the introduction, on the distinction between cellular memory and transgenerational inheritance. We added text clarifying what heritable is referring to in the introduction (lines 23-27)

-It might be interesting in the Discussion to expand further about the links between heritable chromatin marks and heritable small RNAs. The do hint that the result regarding the silencing of the somatic transgene are especially intriguing. We have added further discussion of the potential links between heritable chromatin marks and heritable small

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RNAs (lines 381-392, 442-447).

Reviewer #1 (Significance (Required)):

This is an exciting paper which build upon years of important work in the Katz lab. The novelty of the paper is in pinpointing the mechanisms that bookmark germline genes by H3K36 in the embryo, and explaining why and how germline genes are prevented from being expressed in the soma.

Reviewer #2 (Evidence, reproducibility and clarity (Required)):

Katz and colleagues examine the interaction between the methyltransferase MES-4 and spr-5; met-2 double mutants. Their prior analysis (PNAS, 2014) showed the dramatic enhancement in sterility and development for spr-5; met-2; this paper extends that finding by showing these effects depend on MES-4. The results are interesting and the genetic interactions dramatic. The examination by RNAseq and ChIP helps move the phenotypes into a more molecular analysis. The authors hypothesize that SPR-5 and MET-2 modify chromatin of germline genes (MES-4 targets) in somatic cells, and this is required to silence germline genes in the soma. A few issues need to be resolved to test these ideas and rule out others.

Main comments:

The authors' hypothesis is that SPR-5 and MET-2 act directly, to modify chromatin of germline genes (MES- 4 targets), but alternate hypothesis is that the key regulated genes are i) MES-4 itself and/or ii) known regulators of germline gene expression e.g. the piwi pathway. Mis regulation of these factors in the soma could be responsible for the phenotypes. Therefore, the authors should analyze expression (smFISH and where possible protein stains) for MES-4 and PIWI components in the embryo and larvae of wildtype, double and triple mutant strains. These experiments are essential and not difficult to perform. To determine if MES- 4 is being ectopically expressed in *spr-5; met-2* double mutants, we obtained a GFP tagged version of MES- 4 from Dr. Susan Strome and examined the expression of MES-4. We do not detect any ectopic MES-4 protein in *spr-5;met-2* progeny, suggesting that SPR-5 and MET-2 do not function indirectly by repressing MES-4 (Fig S12, lines 276-283).

We are definitely interested in the potential interaction between PIWI components and the histone modifying enzymes that we have explored in this study. However, since RNAi of MES-4 is sufficient to rescue the developmental delay of *spr-5; met-2* mutants, we have chosen to focus on that interaction in this paper. Nevertheless, we have added further discussion of the potential links between chromatin marks and small RNAs to the paper (lines 381-392, 442-447). We have also added a table specifically listing the expression changes that we observed in piRNA related genes (Supplementary file 8, line 386).

A second aspect of the hypothesis is that spr-5 and met-2 act before mes-4 and that while these genes are maternally expressed, they act in the embryo. There really aren't data to support these ideas - the timing and location of the factors' activities have not been pinned down. One way to begin to address this question would be to perform smFISH on the target genes and on mes-4 in embryos and determine when and where changes first appear. smFISH in embryos is critical - relying on L1 data is too late. If timing data cannot be obtained, then I suggest that the authors back off of the timing ideas or at least explain the caveats.
Certainly, figure 8 should be simplified and timing removed. (note: Typical maternal effect tests probably won't work because if the genes' RNAs are germline deposited, then a maternal effect test will reflect when the RNA is expressed but not when the protein is active. A TS allele would be needed, and that may not be available.) We really appreciate the reviewers insight into a potential caveat to our model. To determine the timing of the ectopic expression of MES-4 targets, we have performed smFISH on the MES-4 target htp-1

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in embryos. These experiments show that the MES-4 target htp-1 is ectopically expressed in the embryo at the 200+ cell stage, after the maternal to zygotic transition (Fig. S8, Lines 206-211). In the early embryo, prior to zygotic genome activation, it is impossible to determine if htp-1 is ectopically expressed because maternal htp-1 is distributed throughout the embryo. Our finding that htp-1 is ectopically expressed in the embryo after zygotic genome activation, is consistent with our proposed model. In addition, our model is predicated on the known embryonic protein localization of SPR-5 and MES-4. Maternal SPR-5 protein is present in the early embryo up to around the 8-cell stage, but absent in later embryos (Katz et al., 2009). In addition, in mice, the SPR-5 ortholog LSD1 is required maternally prior to the 2-cell stage (Wasson et al., 2016 and Ancelin et al., 2016). In contrast, MES-4 continues to be expressed in the embryo until later embryonic stages where it is concentrated into the germline precursors Z2 and Z3 (Fong et al., 2002). This is consistent with SPR-5 establishing a chromatin state that continues to be antagonized by MES-4. There is evidence that MET-2 is expressed both in early embryos and later embryos. However, since the phenotype of MET-2 so closely resembles the phenotype of SPR-5 (Kerr et al., 2014), we have included it in our model as working with SPR-5. We believe the model is consistent with all of the current data. Nevertheless, further experimentation will be required to substantiate the timing of the model. We have added a new section in the discussion that describes this and notes the caveat that further experimentation will be needed to substantiate the timing of our proposed model (lines 496-505).

Writing/clarity:

-It would be helpful to include a table that lists the specific genes studied in the paper and how they behaved in the different assays e.g. RNAseq 1, RNAseq 2, MES-4 target, ChIP. That way, readers will understand each of the genes better. We have now included this table (Supplementary file 7).

-At the end of each experiment, it would be helpful to explain the conclusion and not wait until the Discussion. For readers not in the field, the logic of the Results section is hard to follow. This seems like a stylistic choice. Traditionally, papers did not include any conclusions in the results section, and it is our preference to keep our paper organized this way.

-The model is explained over three pages in the Discussion. It would be great to begin with a single paragraph that summarizes the model/point of the paper simply and clearly. The discussion has been revised to include a short paragraph summarizing the model, before elaborating on the evidence on which the model is based (lines 408-414).

Specific comments:

-Figure 1 has been published previously and should be moved to the supplement. In our original paper (Kerr et al.) we reported in the text that *spr-5; met-2* mutants have a developmental delay. However, we did not characterize this developmental delay. Nor did we include any images of the double mutants, except for one image of the adult germline phenotype. As a result, we believe that the inclusion of the developmental delay in the main body of this manuscript is warranted.

-Cite their prior paper for the vulval defects e.g. page 6 or show in supplement. We have included a citation of our previous paper when we mention the vulval defects (line 125).

-The second RNAseq data should be shown in the Results since it is much stronger. The first RNAseq, which is less robust, should be moved to supplement. We have made this suggested change to the manuscript.

-Figure 3 is very nice. Please explain why the RNAs were picked (+ the table, see comment above), and please add here or in a new figure mes-4 and piwi pathway expression data in wildtype vs double/triple mutants. We performed RT-PCR on 9 MES-4 targets. These 9 targets were picked because they had the highest ectopic expression in *spr-5; met-2* mutants and largest change in H3K36me3 in *spr-5; met-2* mutants versus Wild Type.

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Amongst these 9 genes, we performed smFISH on *htp-1* and *cpb-1* because they are relatively well characterized as germline genes.

In the revised manuscript, we have included tables that contain the expression changes in mes-4, as well as the changes in piRNA related genes (Supplementary file S7 and S8).

-Figure 3 here or later, please show if mes-4 RNAi removes somatic expression of target genes. We thank the reviewer for suggesting this important control. We have now added new data showing that mes-4 RNAi eliminates the somatic expression of *htp-1* and *cpb-1* (Fig. S11, lines 268-274).

-Is embryogenesis delayed? We have added a new figure showing that embryogenesis is sped up in *spr-5; met-2* progeny (Fig. S2, lines 120-123). This indicates that the larval delay is not just due to a general delay in all cell divisions.

-Figure 4 since htp-1 smFISH is so dramatic, it would be helpful to include htp-1 in the lower panels. htp-1 has been added to Fig. 4 and Fig. S9 (lines 219-221).

-Figure 4, please add an extra 2 upper panels showing all the genes in N2 vs spr-5;met-2, for comparison to the mes-4 cohort. As a control, we have now examined H3K36me3 across all germline genes. This data indicates that the enrichment of H3K36me3 that we observe in MES-4 germline genes is substantially reduced when we evaluate all germline genes, suggesting that the enrichment in H3K36me3 is confined to the subset of germline genes that are MES-4 targets. This data has been added to Fig. 4 and Fig. S9 (lines 229-232).

-Figure 6. Please show a control that met-1 RNAi is working. We performed RT-PCR to try and confirm that *met-1* RNAi was working. Despite controls repeating the MES-4 suppression and verifying that RNAi was working, we were unable to demonstrate that *met-1* was knocked down. As a result, we have removed this result from the paper. Importantly, this does not affect the conclusion of the paper.

-To quantify histone marks more clearly, it would be wonderful to have a graph of the mean log across the gene. showing the mean numbers would help clarify the degree of the effect. we had an image as an example but it does not paste into the reviewer box. Instead, see figure 2 or figure 4 here: https://www.nature.com/articles/ng.322 We have now added this analysis to Fig. 4 and Fig. S9.

Reviewer #2 (Significance (Required)):

Katz and colleagues examine the interaction between the methyltransferase MES-4 and spr-5; met-2 double mutants. Their prior analysis (PNAS, 2014) showed the dramatic enhancement in sterility and development for spr-5; met-2; this paper extends that finding by showing these effects depend on MES-4. The results are interesting and the genetic interactions dramatic. The examination by RNAseq and ChIP helps move the phenotypes into a more molecular analysis.

This work will be of interest to people following transgenerational inheritance, generally in the C. elegans field. People using other organisms may read it also, although some of the worm genetics may be complicated. Some of the writing suggestions could make a difference.

I study C. elegans embryogenesis, chromatin and inheritance.

Reviewer #3 (Evidence, reproducibility and clarity (Required)):

In the paper entitled "C. elegans establishes germline versus soma by balancing inherited histone methylation" Carpenter BS et al examined a double mutant worm strain they had previously produced of the H3K4me1/2 demethylase spr-5 and the predicted H3K9me1/me2 methylase met-2. These mutant worms have a developmental delay that arises by the L2

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larval stage. They performed an analysis of what genes get misexpressed in these double mutants by performing RNAseq and compare this to datasets generated from other labs on an H3K36me2/me3 methylase MES-4 where they see a high degree of overlap. They validate the misexpression of some germline specific genes in the soma by in situ and validate that there is a dysregulation of H3K36me3 in their double mutant worms. They further find that knocking down mes-4 reverts the developmental delay.

I think that the authors need to make more of an effort to be a bit more scholarly in terms of placing their work in the context of the field as a whole and also need to add a few additional experiments as well as reorganize a bit before this is ready for publication. Remember that the average reader is not necessarily an expert in C. elegans or this particular field and you really want to try and make the manuscript as accessible to everyone as possible.

****Major Points****

1) It would be good to see western blots or quantitative mass spec examining H3K36me3 in the WT and spr- 5;met-2 double mutant worms. I believe this was also previously reported by Greer EL et al Cell Rep 2014 in the single spr-5 mutant worm so that work should be cited here in addition to the identification of JMJD-2 as an enzyme involved in the inheritance of H3K4me2 phenotype. We sincerely thank the reviewer for their insight into this previous result. The ectopic H3K36me3 that we observe is confined to a small set of MES-4 targets. For example, we don't see ectopic H3K36me3 at non-MES-4 germline genes (see Fig. 4, Fig. S9 and above response). Therefore, we don't expect to see any global differences in bulk H3K36me3. Greer et al reported that there are elevated H3K36me3 levels in *spr-5* mutants. This discrepancy may be due to different stages (embryos, germline) present in their bulk preparation. Alternatively, the *met-2* mutant may counteract the effect of the *spr-5* mutation on H3K36me3. Regardless, we believe that the genome-wide ChIP-seq is more informative than bulk H3K36me3 levels. We have added text to the discussion noting the Greer et al. result (lines 359-360).

2) Missing from Fig.5 is mes-4 KD by itself. This is needed to determine whether these effects are specific to the spr-5;met-2 double mutants or more general effects that KD of mes-4 would decrease the expression of all these genes to a similar extent. Then statistics should be done to see if the decrease in the WT context is the same or greater than the decrease in the double mutants. The MES-4 targets are generally expressed only in the germline and defined by having *mes-4* dependent H3K36me3. Knocking down *mes-4* would be expected to prevent the expression of these genes in the germline, but this is difficult to test because *mes-4* mutants basically don't make a germline. Regardless, knocking down *mes-4* by itself would only assess the role of MES-4 in germline transcription, not the ectopic expression that is being assayed in *spr-5; met-2* mutants in Fig 5. Importantly, it remains possible that *spr-5; met-2* mutants might also result in an increase in the expression of MES-4 targets in the germline. However, the experiments performed in this manuscript were conducted on L1 larvae, which do not have any germline expression, to eliminate this potential confounding contribution.

Minor Points

1)A greater attempt needs to be made to be more scholarly for citing previously published literature. This includes work on the inheritance of H3K27 and H3K36 methylation in C. elegans and other species as well. A few papers which seem germane to this story which should be cited in the intro are (Nottke AC et al PNAS 2011, Gaydos LJ et al Science 2014, Ost A et al Cell 2014, Greer EL et al Cell Rep 2014, Siklenka K et al Science 2015, Tabuchi TM et al Nat Comm 2018, Kaneshiro KR et al Nat Comm 2019). This problem is not restricted to the intro. Although many of these excellent papers are broadly relevant to this current work, they are not necessarily directly relevant to this paper. For this reason, they were not originally cited.
Nevertheless, we have added citations to most of these papers in the revised version of the manuscript.

2)I think that the authors need to be a little less definitive with your language. Theories

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should be introduced as possibilities rather than conclusions. Should remove "comprehensive" from intro as there are many other methods which could be done to test this. Throughout the manuscript, we have tried to be clear what the data suggests versus what is model based on the data. Nevertheless, to further clarify this, we are happy to remove "comprehensive" from the intro (line 98).

3) The authors should describe what PIE-1 is. Is this a transcription factor? PIE-1 is a transcriptional inhibitor that is thought to block RNA polII elongation by mimicking the CTD of RNA polII and competing for phosphorylation. We have added text referencing this function in the revised manuscript (lines 50-54).

4) The language needs clarification about MES-4 germline genes and bookmark genes. Are these bound by MES-4 or marked with K36me2/3? The revised manuscript has been modified to make this definition more clear (line 62-67, 152-155).

5)I think Fig S1 E+F should be in the main figure 1 so readers can see the extent of the phenotype. The original single image of the *spr-5; met-2* adult germline phenotype (including the protruding vulva) was included in our previous publication. In this manuscript, we have now quantified this phenotype, which is why it is included in the supplement here. However, because the original picture was included in our original publication, we prefer to leave it as supplemental.

6) For Fig S2 it would be good to do the same statistics that is done in Fig 2 and mention them in the text so the readers can see that the overlap is statistically significant. These statistics have been added to what is now Fig. 2.

7) Fig S2.2 should be yellow blue rather than red green for the colorblind out there. We thank the reviewer for pointing this out. The red/ green heat maps have been eliminated from the manuscript. This is something that really should be done on all papers.

8) When saying "Many of these genes involved in these processes..." the authors need to include numbers and statistics. The text has been amended to make the definition of the MES-4 genes more clear (line 62-67, 152-155).

9) Should use WT instead of N2 and specify what wildtype is in methods. The text has been revised to use WT instead of N2.

10) Fig. 2A + B could be displayed in a single figure. And Fig 2D seems superfluous and could be combined with 2C or alternatively it could be put in supplementary. Figure 2A and 2B were purposely separated to make it clear how many of the overlapped changes are up versus down. The comparison of the two repeat data sets has been moved to Fig. S6.

11) Non-C. elegans experts won't understand what balancers are. An effort should be made to make this accessible to all. Explaining when genes are heterozygous or homozygous mutants seems relevant here. The text of the revised manuscript has been amended to define what a balancer chromosome is. In all cases the mutants analyzed were homozygous for the mutation (lines 171-173).

12) The GO categories (Fig. S2) should be in the main figure and need to be made to look more scientific rather than copied and pasted from a program. The GO categories were included to be comprehensive and do not contribute substantially to the main conclusion of the paper. This is why they are supplemental. The GO categories were already revised from the original program. It is not clear what would make them look more scientific, but we are happy to further revise them if there is a specific suggestion.

13) Fig. 7 seems a bit out of place. If the authors were to KD mes-4 and similarly show that the phenotype reverts that would help justify its inclusion in this paper. Without it seems like a bit of an add on that belongs elsewhere. We believe that the somatic expression of a transgene in *spr-5; met-2* mutants adds to our potential understanding of how this double mutant may lead to developmental delay. This is true, regardless of whether of whether the somatic transgene

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expression is *mes-4* dependent or not.

Reviewer #3 (Significance (Required)):

I think this is an interesting and timely piece of work. A little more effort needs to be put in to make sure it is accessible to the average reader and has sufficient inclusion of more of the large body of work on inheritance of histone modifications. I think C. elegans researchers as well as people interested in inheritance and the setup of the germline will be interested in this work.

REFEREES CROSS COMMENTING

I agree with Reviewer #2's comments on experiments to include or exclude alternative models. I also agree about their statement about rewriting to make it more accessible to others who aren't experts in this specialized portion of C. elegans research. All in all it seems like the experiments which are required by reviewer #2 and myself as well as the rewriting should be quite feasible.

Submission to Development

First decision letter

MS ID#: DEVELOP/2020/196600

MS TITLE: C. elegans establishes germline versus soma by balancing inherited histone methylation

AUTHORS: Brandon S Carpenter, Teresa W Lee, Caroline F Plott, Jovan S Brockett, Dexter A Myrick, and David Katz

Thank you submitting your manuscript to Development with the assessment from Review Commons and a potential revision plan. I have reviewed the reviewer comments and your response. Development would be interested in inviting a revision of your manuscript following the revision plan that you have outlined. In addition, I ask that you pay special attention to reviewer 2's comment on the Figure 8 of the current draft with regards to the timing of the genes under question. I read your revision plan and the manuscript and I agree with the reviewer that the data currently presented is correlative rather than definitive to support the timing model. Thus, careful caveating of this model and softening the claims to reflect the results will be fruitful. The reviewers also ask that the manuscript be written with a broader audience in mind, which I recommend. I realize that accessing the lab may be slow at this time, thus we ask that you return the revision in 90 days. We may need to send your revised manuscript back to one or more of the Review Commons referees, and acceptance will depend on your satisfactorily addressing the reviewers' comments. Please do let us know if you will need more time.

Please attend to all of the reviewers' comments and ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion. I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

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Author response to reviewers' comments

First revision

We have revised the manuscript according to our previous response letter and feedback from the editor.

Second decision letter

MS ID#: DEVELOP/2020/196600

MS TITLE: C. elegans establishes germline versus soma by balancing inherited histone methylation

AUTHORS: Brandon S Carpenter, Teresa W Lee, Caroline F Plott, Juan D Rodriguez, Jovan S Brockett, Dexter A Myrick, and David J Katz

I have now obtained the referees reports on the above manuscript, and have reached a decision. The referees' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

Overall the reviewers are positive on the manuscript and Development remains interested in this work. However, Reviewer 1 makes critical points which need to be addressed prior to any final decisions are reached. In particular, Development is very serious on accurate attribution of prior work and appropriate citations. Reviewer 1 points out lack of scholarliness due to absence of appropriate discussion and citation of prior published work in the field. The points raised by the reviewer are important to consider in the revision and need to be incorporated into the revised manuscript. Please provide a point by point response to the reviewer suggestions and<u fr-original-style=3D"font-size: 12px;" style=3D"font-size: 12px; margin: 0px; padding: 0px; border: 0px; outline: 0px; vertical-align: baseline; background: transparent;">highlight </u>the sections which have been revised.

Reviewer 1

Advance summary and potential significance to field

I think the authors did make some effort to address the critiques and the manuscript has significantly improved but more is still required before publication. My suggestions are not really that onerous but will improve the accessibility of the study to others and place it better in the context of the field. Also the mes-4 KD experiment I still think should be performed even if it has zero affect on expression because of the stage.

Comments for the author

Major Points
1. My initial critique about a lack of scholarliness and placing your work in the context of the field as a whole still stands. Instead of taking a little extra time to add context in the opening paragraph the authors simply included additional citations without really expanding on the information given to the readers. You need to make a serious effort to make this manuscript accessible to the average reader who is not someone who is familiar with your labs whole body of research or thinking about the subject. All that is really required is a couple of additional sentences. I think the more detailed description in the subsequent paragraphs of the intro does a better job (although see the paragraph below for some glaring omissions) but the first paragraph needs to be expanded a bit to get everyone on board from the 30,000 foot view.

2. Additionally the authors seem determined to cite only work from their own lab while ignoring the work from other groups who work on histone methylation in C. elegans, even on SPR-5 itself! This

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body of work is not that extensive but the context is required for the reader to understand what the state of the current literature is when you began your work. It seems appropriate to include the work of the labs of Ahmed, Colaiacovo, Shi, and Wicky in your introduction. It seems like the place where the authors discuss LET-418 in the intro would be a perfect place to cite Kaser-Pebernard S et al Stem Cell Rep 2014 which discusses the synergism between SPR-5 and LET-418. Additionally the work from the Shi lab (Greer EL et al Cell Rep 2014) showed met-2 was also involved with spr-5 and should be cited when Kerr S et al is cited. They additionally demonstrated a dysregulation of H3K36me3 in the spr-5 single mutants and demonstrated that jmjd-2 deletion partially suppressed this. They also demonstrated that JMJD-2 was an H3K9 and an H3K36 demethylase which directly connects with the main thrust of this paper. As the authors point out in their rebuttal this effect in the single mutant might predominantly be due to its role as a H3K9 demethylase rather than a K36 demethylase but to not cite it in your introduction shows a lack of scholarliness. This context bolsters your work and does not detract from it and it is always good when independent labs have the same findings. These papers have to be cited in the introduction.

3. Even if mes-4 KD has no effect on gene expression (as the authors suggest in their rebuttal) it is still necessary to include this control in Figure 5 as there could be off-target effects of the mes-4 KD. Simple epistasis experiments need to examine all 4 conditions not just 3.

Minor points
For the GO analysis presentation in Figure S3 to make the data look presentable:
-Change the font so it is consistent with the rest of the figure in terms of size and style.
-Similarly the font sizes seem to be randomly chosen and sometimes all capital and sometimes not. Be consistent.
-Justify the title to the left most portion of the text
-The gray background of the box should be eliminated.
-It would be good to justify it so that the text isn't going different distances to the left border of the figure. You could switch the text line to the right side of the figure and justify them all left so that the overflowing text is on the right side of the figure.
A few simple changes to make it look like it wasn't copied and pasted.

Second revision

Author response to reviewers' comments

We thank the reviewer for the helpful comments. We believe that the manuscript is now substantially improved thanks to their efforts. Please see individual responses to comments below in blue.

Reviewer 1 Advance Summary and Potential Significance to Field:
I think the authors did make some effort to address the critiques and the manuscript has significantly improved but more is still required before publication. My suggestions are not really that onerous but will improve the accessibility of the study to others and place it better in the context of the field. Also the mes-4 KD experiment I still think should be performed even if it has zero affect on expression because of the stage.

Reviewer 1 Comments for the Author:
Major Points
1. My initial critique about a lack of scholarliness and placing your work in the context of the field as a whole still stands. Instead of taking a little extra time to add context in the opening paragraph the authors simply included additional citations without really expanding on the information given to the readers. You need to make a serious effort to make this manuscript accessible to the average reader who is not someone who is familiar with your labs whole body of research or thinking about the subject. All that is really required is a couple of additional sentences. I think the more detailed description in the subsequent paragraphs of the intro does a better job (although see the paragraph below for some glaring omissions) but the first paragraph needs to be expanded a bit to get everyone on board from the 30,000 foot view.

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We have now substantially revised the introduction. Hopefully, this will enable the manuscript to be more accessible to all readers. We have also provided some additional details in the discussion.
2. Additionally the authors seem determined to cite only work from their own lab while ignoring the work from other groups who work on histone methylation in C. elegans, even on SPR-5 itself! This body of work is not that extensive but the context is required for the reader to understand what the state of the current literature is when you began your work. It seems appropriate to include the work of the labs of Ahmed, Colaiacovo, Shi, and Wicky in your introduction.
We have included work from all of these labs.

It seems like the place where the authors discuss LET-418 in the intro would be a perfect place to cite Kaser-Pebernard S et al Stem Cell Rep 2014 which discusses the synergism between SPR-5 and LET-418.
We have included this work in the discussion.

Additionally the work from the Shi lab (Greer EL et al Cell Rep 2014) showed met-2 was also involved with spr-5 and should be cited when Kerr S et al is cited.
They additionally demonstrated a dysregulation of H3K36me3 in the spr-5 single mutants and demonstrated that jmjd-2 deletion partially suppressed this. They also demonstrated that JMJD-2 was an H3K9 and an H3K36 demethylase which directly connects with the main thrust of this paper. As the authors point out in their rebuttal this effect in the single mutant might predominantly be due to its role as a H3K9 demethylase rather than a K36 demethylase but to not cite it in your introduction shows a lack of scholarliness. This context bolsters your work and does not detract from it and it is always good when independent labs have the same findings. These papers have to be cited in the introduction.
We have added this work and cited the reference when we initially describe Kerr et al. in the introduction. This work is also discussed in the discussion.

3. Even if mes-4 KD has no effect on gene expression (as the authors suggest in their rebuttal) it is still necessary to include this control in Figure 5 as there could be off-target effects of the mes-4 KD. Simple epistasis experiments need to examine all 4 conditions not just 3.
We respectively disagree. While we absolutely agree that in typical epistasis, all 4 genotypes are required to interpret the results, Figure 5 is not epistasis analysis. Figure 5 shows the suppression of an ectopic result. This is the reason why in WT, there is hardly any expression of these genes at all. If mes-4 RNAi decreased the slight expression of these mes-4 targets that we see in WT, it would suggest that the expression of these genes is dependent on mes-4. If mes-4 RNAi does not affect the slight expression of these genes, it would suggest that part of their expression is mes-4 independent. If mes-4 RNAi increased the expression of these mes-4 targets, it might imply some type of secondary effect. Regardless, none of these outcomes tells us anything about the ectopic expression, because there is only ectopic expression of these mes-4 targets in the spr-5;met-2 double mutant. As a result, the only condition where mes-4 RNAi is informative about the ectopic expression we observe in *spr-5; met-2* mutants, is in the context of the double mutants. If we were to perform mes-4 RNAi on WT, it would only tell us about the normal expression of these genes.
In addition, to rule out global off target effects, we examined the expression of Ama-1, the large subunit of RNA polymerase. We do not see any effect. We have added a statement about this in the results.

Minor points
For the GO analysis presentation in Figure S3 to make the data look presentable:
-Change the font so it is consistent with the rest of the figure in terms of size and style.
-Similarly the font sizes seem to be randomly chosen and sometimes all capital and sometimes not. Be consistent.
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A few simple changes to make it look like it wasn't copied and pasted.

The formatting of the figure has been changed as requested.

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Third decision letter

MS ID#: DEVELOP/2020/196600

MS TITLE: C. elegans establishes germline versus soma by balancing inherited histone methylation

AUTHORS: Brandon S Carpenter, Teresa W Lee, Caroline F Plott, Juan D Rodriguez, Jovan S Brockett, Dexter A Myrick, and David J Katz
ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Development, pending our standard ethics checks.

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