GENETICS

Orthologs of the *C. elegans* heterochronic genes have divergent functions in *C. briggsae*

Maria Ivanova and Eric Moss

NOTE: The reviews and decision letters are unedited and appear as submitted by the reviewers.
In extremely rare instances and as determined by a Senior Editor or the EIC, portions of a review may be redacted. If a review is signed, the reviewer has agreed to no longer remain anonymous.
The review history appears in chronological order.

Review Timeline:

Submission Date:	2023-05-22
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June 24, 2023

GENETICS-2023-306207
Orthologs of the *C. elegans* heterochronic genes have divergent functions in *C. briggsae*

Dear Dr. Ivanova:

Experts in the field have reviewed your manuscript, and I have read it as well. We all agree that this is a very impressive study that will be informative to the field and also provide highly useful tools and resources for the community. While your manuscript is not currently acceptable for publication in GENETICS, we would welcome a substantially revised manuscript. Both reviewers have comments and concerns to be addressed in a revised manuscript. You can read their reviews at the end of this email.

The following revisions would make your manuscript acceptable for publication in GENETICS. All reviewers and especially reviewer 2 have many constructive comments about data presentation and these should be addressed by appropriate changes to the text as well as the figures and tables. In addition, a summary figure showing the heterochronic pathway would help the reader. Apart from these changes, which will not require additional experiments, reviewer 3 points out that based on the data presented, it is unclear whether the Cbr mutants are nulls. This should be addressed at least for one of the genes for example Cbr-lin-28. Finally, please provide a Data Availability Statement,

Upon resubmission, please include:
1. A clean version of your manuscript;
2. A marked version of your manuscript in which you highlight significant revisions carried out in response to the major points raised by the editor/reviewers (track changes is acceptable if preferred);
3. A detailed response to the editor's/reviewers' feedback and to the concerns listed above. Please reference line numbers in this response to aid the editor and reviewers.

Your paper will likely be sent back out for review.

Additionally, please ensure that your resubmission is formatted for GENETICS
https://academic.oup.com/genetics/pages/general-instructions

Follow this link to submit the revised manuscript: Link Not Available

Sincerely,

Barbara Conradt
Associate Editor
GENETICS

Approved by:
David Greenstein
Senior Editor
GENETICS

Reviewer #1 (Comments for the Authors (Required)):

Summary:

In this manuscript, Ivanova and Moss sought to shed light on how developmental regulatory systems evolve by comparing C. elegans and C. briggsae, two nematode species that diverged approximately 20 million years ago yet share nearly identical body plans, down to cell lineages. To do so, they studied the heterochronic pathway, which coordinates developmental timing and provides a useful foothold given how extensively it has been studied in C. elegans and the stereotypical phenotypes it involves. Focusing on 6 protein-coding genes (hbl-1, lin-14, lin-28, lin-29, lin-41, lin-46) and 5 microRNAs (let-7, lin-4, mir-48, mir-84, mir-241), the authors characterized and compared phenotypes resulting from perturbations to this system, achieved either through mutant alleles generated by CRISPR or auxin-inducible protein degradation, between the two species. Interestingly, in some cases they observed subtle differences in phenotypes, suggesting that, while the C. briggsae orthologs have mostly conserved functions in regulating developmental timing compared to C. elegans, the stage and tissues that they function in differ. However, the interactions among these components of the regulatory module appear to be highly conserved between both species. These

results provide useful insights into how developmental gene networks can be rewired.

Assessment:

Understanding how gene function and interactions diverge among species is a very interesting question. This work takes advantage of the well-characterized heterochronic pathway to tackle this. The authors should be commended for the amount of work reported in this manuscript, including generation of 35 genetic lesions and 18 double- and triple-mutants, which is not trivial, especially in species such as C. briggsae where fewer tools are readily available. This paper highlights the power of comparative studies between nematode species, and provides a platform for other researchers to study developmental timing in C. briggsae. Once the concerns outlined below are addressed, I feel the data provided in this manuscript will be a valuable resource for those studying developmental timing, gene regulatory networks, and evolution.

Major comments:

I have some concerns about the seam cell counting data, which the authors acknowledge may be susceptible to error (pg. 26, lines 31-33). For example, in Supp. Fig. 4, the second nucleus in the 2nd grouping of seam cells looks morphologically different from the others. First, it might be easier for the reader to interpret by including a panel of the micrograph without the overlaid dotted yellow border. The authors state that there are not fluorescent markers of seam cell fate available in C. briggsae; however, the antibody NE2-1B4 recognizes an antigen expressed only in seam cells (Schnabel, 1991) and could be used to stain fixed worms. Even a transgene ubiquitously expressing a membrane marker might be helpful in increasing confidence of identifying seam cells, which have elongated cell shapes.

In several figures (including Fig. 2, Supp. Fig. 3, Supp. Fig. 11, Supp. Fig. 15, Supp. Fig. 16, etc.) an image of normal development is not provided to compare mutant phenotypes to. It may be difficult for non-C. elegans researchers to interpret the data as currently presented.

Minor comments:

The standard nomenclature for nematodes capitalizes the first letter of the species designation (i.e., Cel-, Cbr-).

It may be helpful to add more schematics showing normal divisions and differentiation of seam, intestinal, and vulval cells over developmental stages to help orient readers (like Supp. fig. 1).

Pg. 4, line 38: I believe you are missing H1 in your list of seam cells that divide symmetrically.

Several figures are missing the sample size. Please also add the standard deviation or confidence interval to Supp. Table 2.

Note that the main text uses the phrase "reiterative" while the figure legends use "retarded". It may be best to be consistent with the terminology used.

The methods are lacking some important details, such as how worm synchronization was performed, concentration of 5-Ph-IAA in auxin plates, and information about imaging (e.g., type of lens, camera, image acquisition software, etc.).

Reviewer #2 (Comments for the Authors (Required)):

This manuscript provides a comprehensive analysis of heterochronic genes in C. briggsae and provides key insights into both functional divergence and overall conservation of pathways and modules. For most genes analyzed, multiple alleles were obtained and analyzed and the authors have performed the key developmental assays including those to monitor the timing of seam cell and intestinal cell divisions, alae formation, and vulva development. This set of mutants will be a great resource for others in the heterochronic field to study the functional divergence of key developmental timing regulators.

The main issue for this manuscript is in the presentation of the data. There are instances throughout the manuscript in which observations/data are discussed in the results section but the data are not included in a figure or table. In this way, it's hard or impossible for the reader to see the full data set with appropriate controls and statistical analysis. It may help to include a complete data table with results from the full phenotypic analysis of all heterochronic features including seam cell number, alae, and vulva defects for all strains analyzed at the different larval stages. Overall, the data presentation could be edited to better and more fully show all of the authors' work. Much of the quantitative data seems absent or included in results (not in a table or figure), while there are repetitive images showing gapped alae, egg laying defects, and abnormal vulvae. Some DIC photomicrographs appear lower quality images compared to others (e.g., Figure 5A, Figure 7B). Comments below list many examples, but a careful review of the data discussed in the results section and presented in figures/tables would greatly strengthen this manuscript.

Minor edits:
--The authors use both specific allele designations and null (0), loss of function (lf), and gain of function (gf) designations. It might be helpful to include "0", "lf", and "gf" terms in a table (Supp Table 1 or a new table) along with the specific allele designations to provide a reference for the reader.
--Page 3, line 28, 30 change microRNA to miRNA
--Supp Fig. 1, could add seam data to show increase in seam cell number in wild type C. briggsae to either Supp fig 1 or Fig. 1A. The seam cell number at the L4/young adult stage is shown in Figure 1A "WT" but there is no data showing seam number in C. briggsae in L1, L2, L3, and L4. These data are discussed on page 4 line 34- page 5 line 4 but the full data set is not included. A figure showing all of the normal developmental landmarks in C. briggsae would strengthen the paper.
--Page 5, line 27. Revise list of what was analyzed to match data organization (seam cell, molts, alae, and intestinal nuclei).
--Figure 1, include cel-lin-4(ae53) seam cell number. Data is discussed on page 5, lines 34-38 but should be included in Figure 1A for an easy comparison to the Cbr-lin-4 data.
--Figure 1, staging information is confusing. L1, L3, L4, and adult stages are shown and specified in some but not all of the X axis labels. For each graph a more consistent display would help (show stage for all groups on the X axis or for all groups in the figure legend).
--Page 5, line 39. Molting data is indicated but not fully provided. This should be more carefully documented or removed.
--Supp fig 3. Revise "retarded phenotype" to "reiterative phenotype" using the term that was introduced on page 3,
--Supp fig 3C. What percentage of worms showed gapped alae?
--Page 6, line 17. Data for the number of intestinal nuclei at 15 degrees is discussed as "not significantly different" but it's unclear what this statement refers to-compared to C. briggsae wildtype at 15? That data is not shown.
--Page 6, line 38. include data for control C. briggsae alae.
--Supp. Fig 7. Image quality for 7A is poor.
--Page 6, line 39. The statement that most seam cells divide symmetrically during the L1 stage is not sufficiently supported by the data. A slightly reduced number of seam cells in the L4 stage is shown, which could reflect any number of developmental changes.
--Page 7, lines 6-25. Authors should include data for lin-4;lin-14 double mutants.
--Page 7, lines 34-36. Authors should include this data.
--Page 7, line 38, add citation to Figure 1A that shows cbr-lin-4(0) data since authors compare cbr-lin-14(gf) data to cbr-lin-4(0)
--Figure 4. Seam cell expression seems quite low in Figure 4B and 4D. It is unclear if this is because of the images chosen for the figure or if this accurately represents expression levels as determined by the presence of the extrachromosomal array.
--Figure 5 and 6. Revise "retarded" to "reiterative" (or introduce "retarded" phenotype when "precocious" is introduced)
--Figure 5. Revise DiC to DIC. The image quality in Figure 5A could be better. A higher magnification image or some annotation to show the retained eggs would be helpful. Figure 5B and C, indicate what stage of worms are being analyzed and include information about statistical analysis performed.
--Figure 6. Indicate what statistical analysis was performed.
--Page 12, line 11. The authors cite Supp Table 1 in a sentence describing the double mutant analysis, instead of Figure 6C.
--Page 12, line 15. Revise "both all three miRNAs"
--Page 13, line 10. Discussion of the specific plasmid seems better in the methods section, with more of a focus on the transgene in the results.
--Page 15, line 32. Delete "or something else" or suggest a possible alternative model.
--Page 16, line 9. Delete "that they were trying to shed"
--Page 17, line 7-8. The use of cbr-spe-8(v142) could be explained in more detail.
--Figure 8, revise figure legend. "DC micrograph" to "DIC micrograph"
--Page 16-19. The authors created cbr-lin-41 and cbr-lin-29 mutants along with double mutants, but include almost no data about these mutants in the paper. Four total micrographs are shown in Figures 8, Supp Fig 22 and Supp Fig 23.

Reviewer #3 (Comments for the Authors (Required)):

In this impressive study, Ivanova and Moss subject the heterochronic gene regulatory network of C. elegans to a comparative analysis by examining a large panel of single and double mutants in C. briggsae. As the pathway is a paradigm for genetic control at various levels of molecular interaction, this is an exciting undertaking. The authors combine cutting-edge tools of gene editing and inducible regulation with standard genetics to create a satisfying portrait of conservation and divergence. Overall, they uncover several interesting examples of developmental system drift, and in the process better define the core functions of the network components. In addition, their C. briggsae TIR-1-expressing strains will be of great use to other labs going forward.

While generally positive about this paper, I do have some concerns and suggestions. Addressing these will substantially

strengthen the paper's claims and increase its appeal for the broad readership of Genetics. I present them below in order of appearance in the MS, not in order of importance. In each case, the location of the text of interest is noted in the form X/N, where X is the page number and N is the line number(s).

5/10-13
This is interesting, especially since the final number in C. elegans can range from 30-34. Variation within species is thus mirrored to some extent by variation between. Is the number of gut nuclei in wild-type adult C. briggsae statistically higher than in C. elegans?

6/1-2
Meaning there were 7 shed cuticles, plus four more still attached to the worms? Or do the four being shed count as part of the 7? Please clarify.

8/14-16 and beyond
This whole section is arguably describing the most important result of the paper (and the authors say as much on 23/9-10). However, it is predicated on the assumption that these C. briggsae lin-28 alleles really are functional nulls. If they are not, the comparative conclusions vis a vis C. elegans fall apart. This is not a crazy idea, as there is another in-frame methionine codon 17 codons down from the mutated target site--initiation of translation there may produce some N-terminally truncated protein that is ~80% complete and may therefore have residual function.
Obviously there are strong phenotypes, but true nulls are needed to be confident that a species difference has been found. In the spirit of C. elegans genetics and the rigor that characterizes it, some additional effort to prove "null-ness" is warranted here. A larger deletion allele or supplementation with injected dsRNA would do that.

12/15
Should read "deleting all three miRNAs".

28/9
There should be a "Data Availability Statement" here. Presumably it would mention strains being sent to the CGC, and perhaps other items.

30/1
Before we get into the data, we could really use a summary diagram of the various genes and their interactions. Otherwise, those not steeped in this mature area of C. elegans research will struggle to follow the motivations for, and interpretations of, the many genetic experiments described. For consistency, such a diagram should highlight the "key regulatory modules" mentioned in the Discussion.

57/21
That this is a CalTech PhD dissertation should be mentioned in the reference.

Associate Editor Comments:

Reviewer #1
Major comments:
I have some concerns about the seam cell counting data, which the authors acknowledge may be susceptible to error (pg. 26, lines 31-33). For example, in Supp. Fig. 4, the second nucleus in the 2nd grouping of seam cells looks morphologically different from the others. First, it might be easier for the reader to interpret by including a panel of the micrograph without the overlaid dotted yellow border.

The yellow dotted lines were removed from Supp. Fig. 4.

The authors state that there are not fluorescent markers of seam cell fate available in C. briggsae; however, the antibody NE2-1B4 recognizes an antigen expressed only in seam cells (Schnabel, 1991) and could be used to stain fixed worms. Even a transgene ubiquitously expressing a membrane marker might be helpful in increasing confidence of identifying seam cells, which have elongated cell shapes.

We mention the difficulty in counting seam cells for the purpose of transparency and believe that it explains some of the variability. We are aware from *C. elegans* heterochronic mutants that seam cells can sometimes have abnormal shapes or positions along the midline. But this level of counting error does not affect our interpretations because we rely only on obvious and significant increases or decreases to infer whether the L2 lineage pattern was repeated or skipped.

We appreciate the suggestion of the antibody. Further tools for *C. briggsae* would definitely allow a more fine-grained analysis that would help us make different statements. We chose to be conservative in this first-level analysis.

In several figures (including Fig. 2, Supp. Fig. 3, Supp. Fig. 11, Supp. Fig. 15, Supp. Fig. 16, etc.) an image of normal development is not provided to compare mutant phenotypes to. It may be difficult for non-C. elegans researchers to interpret the data as currently presented.

Images of wildtype animals have been added to each of these figures, except Supp. Fig. 15, where a reference to Supp. Fig. 11 was added. Wildtype comparison was also added to Fig. 7A.

Minor comments:
The standard nomenclature for nematodes capitalizes the first letter of the species designation (i.e., Cel-, Cbr-).

We apologize for this oversight. We corrected the nomenclature throughout the manuscript, though we did not highlight each change.

It may be helpful to add more schematics showing normal divisions and differentiation of seam, intestinal, and vulval cells over developmental stages to help orient readers (like Supp. fig. 1).

Thank you. We included a seam cell lineage schematic in Fig. S1 because we generated this information which is needed for interpreting other results in the manuscript. We have not included other lineage schematics because adequate descriptions of wildtype development can be provided in the text and interpretation of mutant phenotypes is comparatively simple.

Pg. 4, line 38: I believe you are missing H1 in your list of seam cells that divide symmetrically.

This has been corrected.

Several figures are missing the sample size. Please also add the standard deviation or confidence interval to Supp. Table 2.

Sample size has been added to Figs. 3 and 5 and to Supp. Fig. 9. Standard deviation and sample size have been added to the Supp. Table 2 (in the current version, it is Supp. Table 3).

Note that the main text uses the phrase "reiterative" while the figure legends use "retarded". It may be best to be consistent with the terminology used.

Thank you. We now use “reiterative” everywhere (not highlighted).

The methods are lacking some important details, such as how worm synchronization was performed, concentration of 5-Ph-IAA in auxin plates, and information about imaging (e.g., type of lens, camera, image acquisition software, etc.).

We added to Materials and Methods details about microscopy (objectives, software), 5-Ph-IAA concentration, and a new “Developmental synchronization” section.

Reviewer #2
The main issue for this manuscript is in the presentation of the data. There are instances throughout the manuscript in which observations/data are discussed in the results section but the data are not included in a figure or table. In this way, it's hard or impossible for the reader to see the full data set with appropriate controls and statistical analysis. It may help to include a complete data table with results from the full phenotypic analysis of all heterochronic features including seam cell number, alae, and vulva defects for all strains analyzed at the different larval stages. Overall, the data presentation could be edited to better and more fully show all of the authors' work. Much of the quantitative data seems absent or included in results (not in a table or figure), while there are repetitive images showing gapped alae, egg laying defects, and abnormal vulvae. Some DIC photomicrographs appear lower quality images compared to others (e.g., Figure 5A, Figure 7B). Comments below list many examples, but a careful review of the data discussed in the results section and presented in figures/tables would greatly strengthen this manuscript.

We appreciate this comment. In response to specific issues brought up below, we have incorporated some of the in-text data into one table. Our choices of which observations to list in the text and what to compile in figures and tables were based on two factors: Which of our claims were based on comparisons among multiple strains—presented in figures and tables—and which were merely statements of fact, for example, the data showing that a strain had some precocious characteristics. We appreciate that others may want to look into our data for additional insights, but we make tables for only the data from which we felt comfortable drawing comparative conclusions in the manuscript. Data in the text is there simply to document specific statements. Compiling all such data would be challenging in terms of formatting (grouping apples and oranges) and not fit any narrative we are following in the text, which we feel would offer only diminishing returns for the reader.

We have only included images to document the phenotypes of each of these mutants and see no redundancies. This documentation is primarily for comparison to *C. elegans*, where in most cases the phenotypes' penetrance and expressivity are 100%. One theme of this manuscript is the relative weakness of the heterochronic phenotypes in *C. briggsae*. Showing the reader the extent of the phenotype (without reproducing lots of images of *C. elegans* from other publications) is all that we feel is needed.

We have substituted an improved image for Fig. 5A. Because Fig. 7B shows the general morphology, proportions, and protruding vulva, we believe it is adequate to represent the gross phenotype. Wild type was added to Fig. 2A.

Minor edits:
--The authors use both specific allele designations and null (0), loss of function (lf), and gain of function (gf) designations. It might be helpful to include "0", "lf", and "gf" terms in a table (Supp Table 1 or a new table) along with the specific allele designations to provide a reference for the reader.

We added a column to Table S1 that includes all of these designations.

--Page 3, line 28, 30 change microRNA to miRNA

It has been changed.

--Supp Fig. 1, could add seam data to show increase in seam cell number in wild type C. briggsae to either Supp fig 1 or Fig. 1A. The seam cell number at the L4/young adult stage is shown in Figure 1A "WT" but there is no data showing seam number in C. briggsae in L1, L2, L3, and L4. These data are discussed on page 4 line 34- page 5 line 4 but the full data set is not included. A figure showing all of the normal developmental landmarks in C. briggsae would strengthen the paper.

We modified the first paragraph of the first results section adding data on our observations and indicating the numbers of seam cells during larval development. Larval development of *C. briggsae* is nearly the same as that of *C. elegans*.

--Page 5, line 27. Revise list of what was analyzed to match data organization (seam cell, molts, alae, and intestinal nuclei).

Thank you. This has been changed.

--Figure 1, include cel-lin-4(ae53) seam cell number. Data is discussed on page 5, lines 34-38 but should be included in Figure 1A for an easy comparison to the Cbr-lin-4 data.

The *Cel-lin-4(ae53)* data have been included. The legend now says "Plots show seam cell and intestinal nuclei counts in L4 and young adults (unless otherwise specified)".

--Page 5, line 39. Molting data is indicated but not fully provided. This should be more carefully documented or removed.

We've changed the text to clarify that 7 shed cuticles were found and adult worms had extra molts.

--Supp fig 3. Revise "retarded phenotype" to "reiterative phenotype" using the term that was introduced on page 3,

Thank you. This and other occurrences of "retarded" have been changed to "reiterative."

--Supp fig 3C. What percentage of worms showed gapped alae?

The alae data have been added.

--Page 6, line 17. Data for the number of intestinal nuclei at 15 degrees is discussed as "not significantly different" but it's unclear what this statement refers to-compared to C. briggsae wildtype at 15? That data is not shown.

We’ve added the phrase "compared to the same mutant at 20C".

--Page 6, line 38. include data for control C. briggsae alae.

We left this out because by definition wildtype animals do not develop precocious adult alae.

--Supp. Fig 7. Image quality for 7A is poor.

Thank you. We have increased the magnification on these images and also added a wild-type larva for comparison. We intend only that these show the gross phenotypes of the animals.

--Page 6, line 39. The statement that most seam cells divide symmetrically during the L1 stage is not sufficiently supported by the data. A slightly reduced number of seam cells in the L4 stage is shown, which could reflect any number of developmental changes.

Thank you, you are correct. We’ve revised the paragraph to say that the seam cell counts are consistent with symmetric divisions in the L1, as occurs in *C. elegans*.

--Page 7, lines 6-25. Authors should include data for lin-4;lin-14 double mutants.

Thank you. We have added the data to support the statements about seam cells and intestinal nuclei. We revised the statement about precocious alae and included numbers.

--Page 7, lines 34-36. Authors should include this data.

The alae data has been added.

--Page 7, line 38, add citation to Figure 1A that shows cbr-lin-4(0) data since authors compare cbr-lin-14(gf) data to cbr-lin-4(0)

The citation of Fig. 1A has been added.

--Figure 4. Seam cell expression seems quite low in Figure 4B and 4D. It is unclear if this is because of the images chosen for the figure or if this accurately represents expression levels as determined by the presence of the extrachromosomal array.

The images accurately represent what was observed. The expression in seam cells was weaker than in muscles or neurons.

--Figure 5 and 6. Revise "retarded" to "reiterative" (or introduce "retarded" phenotype when "precocious" is introduced)

Thank you. All occurrences of “retarded” have been replaced with “reiterative.”

--Figure 5. Revise DiC to DIC. The image quality in Figure 5A could be better. A higher magnification image or some annotation to show the retained eggs would be helpful. Figure 5B and C, indicate what stage of worms are being analyzed and include information about statistical analysis performed.

Thank you. "DIC" has been corrected. We have replaced 5A with a better image to show accumulated embryos. We have indicated the stages of the animals examined in the figure legend. The statistical tests, including which types of data they were used on, are described in Materials and Methods.

--Figure 6. Indicate what statistical analysis was performed.

The statistical methods are now stated in the legend. Statistical methods are also described in Materials and Methods.

--Page 12, line 11. The authors cite Supp Table 1 in a sentence describing the double mutant analysis, instead of Figure 6C.

This paragraph has been rewritten to make the points clearer.

--Page 12, line 15. Revise "both all three miRNAs"

Thank you. This has been fixed.

--Page 13, line 10. Discussion of the specific plasmid seems better in the methods section, with more of a focus on the transgene in the results.

Thank you. We've moved that technical information to Materials and Methods.

--Page 15, line 32. Delete "or something else" or suggest a possible alternative model.

We've removed "or something else."

--Page 16, line 9. Delete "that they were trying to shed"

It has been removed.

--Page 17, line 7-8. The use of cbr-spe-8(v142) could be explained in more detail.

The explanation has been expanded.

--Figure 8, revise figure legend. "DC micrograph" to "DIC micrograph"

Thank you. This has been corrected.

--Page 16-19. The authors created cbr-lin-41 and cbr-lin-29 mutants along with double mutants, but include almost no data about these mutants in the paper. Four total micrographs are shown in Figures 8, Supp Fig 22 and Supp Fig 23.

We have added additional numerical data in the text for *Cbr-lin-29(0)* and double mutants, which has been highlighted. We provide all data about these mutants that are relevant to the themes of this paper: whether their mutant phenotypes resemble those of their *C. elegans* counterparts and their epistasis relationships. It should be noted that because *Cbr-lin-41* and *Cbr-lin-29* act later in development than say *Cbr-lin-14* or *Cbr-lin-28*, they affect fewer developmental processes because less happens in late stages. The only tissue to examine for these genes with regard to heterochronic phenotypes is the hypodermis. Neither of these genes affects seam cell number because they act after the L2. Nor do these genes affect the intestinal or vulval divisions per se.

Reviewer #3
5/10-13
This is interesting, especially since the final number in C. elegans can range from 30-34. Variation within species is thus mirrored to some extent by variation between. Is the number of gut nuclei in wild-type adult C. briggsae statistically higher than in C. elegans?

Yes, this difference is statistically significant. See Fig.1C.

6/1-2
Meaning there were 7 shed cuticles, plus four more still attached to the worms? Or do the four being shed count as part of the 7? Please clarify.

Yes, 7 shed cuticles in the bacterial lawn plus 4 cuticles still attached. This has been clarified in the text.

8/14-16 and beyond
This whole section is arguably describing the most important result of the paper (and the authors say as much on 23/9-10). However, it is predicated on the assumption that these C. briggsae lin-28 alleles really are functional nulls. If they are not, the comparative conclusions vis a vis C. elegans fall apart. This is not a crazy idea, as there is another in-frame methionine codon 17 codons down from the mutated target site--initiation of translation there may produce some N-terminally truncated protein that is ~80% complete and may therefore have residual function.
Obviously there are strong phenotypes, but true nulls are needed to be confident that a species difference has been found. In the spirit of C. elegans genetics and the rigor that characterizes it, some additional effort to prove "null-ness" is warranted here. A larger deletion allele or supplementation with injected dsRNA would do that.

Thank you for raising this important issue. After receiving the reviews, we generated a new allele of *Cbr-lin-28*, *ae80*, that deletes 78% of the coding region, including half of the RNA-

binding beta barrel (CSD) and both zinc fingers, leaving no identifiable functional motifs. We describe its phenotype in a new paragraph, finding that it resembles those of *ae35* and *ae39*, the two frameshift alleles from which we derive most of our results. Briefly: *ae80* had approximately the wildtype number of seam cells and precocious alae patches in about half of L4 larvae. These animals arrest in early L4 and have disorganized gonads with a few oocytes and embryos. We are satisfied that *ae35* and *ae39* represent null alleles, although we have qualified this claim in Table S1.

12/15
Should read "deleting all three miRNAs".

Thank you. This has been corrected.

28/9
There should be a "Data Availability Statement" here. Presumably it would mention strains being sent to the CGC, and perhaps other items.

A data availability statement has been added.

30/1
Before we get into the data, we could really use a summary diagram of the various genes and their interactions. Otherwise, those not steeped in this mature area of C. elegans research will struggle to follow the motivations for, and interpretations of, the many genetic experiments described. For consistency, such a diagram should highlight the "key regulatory modules" mentioned in the Discussion.

Thank you. We have added Fig. S24 which depicts the pathway and the regulatory modules and cite it in the “Conservation of key regulatory modules” section of the Discussion.

57/21
That this is a CalTech PhD dissertation should be mentioned in the reference.

Thank you. This has been added.

<end>

September 14, 2023
RE: GENETICS-2023-306486

Ms. Maria Ivanova
Rowan University
Cell and Molecular Biology
115 Wright Ave.
Apt. B9
Stratford, New Jersey 08084

Dear Dr. Ivanova:

Congratulations! We are delighted to inform you that your revised manuscript entitled "Orthologs of the *C. elegans* heterochronic genes have divergent functions in *C. briggsae*" is now acceptable for publication in GENETICS. Many thanks for submitting your research to the journal.

To Proceed to Production:

1. Format your article according to GENETICS style, as discussed at https://academic.oup.com/genetics/pages/general-instructions, and upload your final files at https://genetics.msubmit.net.

2. Your manuscript will be published as-is (unedited-as submitted, reviewed, and accepted) at the GENETICS website as an Advanced Access article and deposited into PubMed shortly after receipt of source files and the completed license to publish. Please notify sourcefiles@thegsajournals.org if you do not wish to publish your article via Advanced Access.

3. We invite you to submit an original color figure related to your paper for consideration as cover art. Please email your submission to the editorial office or upload it with your final files. You can submit a small-sized image for evaluation, and if selected, the final image must be a TIFF file 2513px wide by 3263px high (8.375 by 10.875 inches; resolution of 600ppi). Please avoid graphs and small type.

If you have any questions or encounter any problems while uploading your accepted manuscript files, please email the editorial office at sourcefiles@thegsajournals.org.

Sincerely,

Barbara Conradt
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