The conserved molting/circadian rhythm regulator NHR-23/NR1F1 serves as an essential co-regulator of *C. elegans* spermatogenesis

James Matthew Ragle, Abigail L. Aita, Kayleigh N. Morrison, Raquel Martinez-Mendez, Hannah N. Saeger, Guinevere A. Ashley, Londen C. Johnson, Katherine A. Schubert, Diane C. Shakes and Jordan D. Ward
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Original submission: 11 June 2020
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First decision letter

MS ID#: DEVELOP/2020/193862

MS TITLE: The conserved molting/circadian rhythm regulator NHR-23/NR1F1 serves as an essential co-regulator of *C. elegans* spermatogenesis

AUTHORS: James Matthew Ragle, Abigail L. Aita, Kayleigh N. Morrison, Raquel Martinez-Mendez, Hannah N. Saeger, Guinevere A. Ashley, Londen C. Johnson, Katherine A. Schubert, Diane C. Shakes, and Jordan D. Ward

I have now received all 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.

As you will see, the referees express considerable interest in your work, but have some significant criticisms and recommend a substantial revision of your manuscript before we can consider publication. If you are able to revise the manuscript along the lines suggested, which may involve further experiments, I will be happy receive a revised version of the manuscript. Your revised paper will be re-reviewed by one or more of the original referees, and acceptance of your manuscript will depend on your addressing satisfactorily the reviewers' major concerns. Please also note that Development will normally permit only one round of major revision.

We are aware that you may currently be unable to access the lab to undertake experimental revisions. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

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.

Reviewer 1

Advance summary and potential significance to field

In this paper, Ragle et al. describe their discovery of an unexpected pleiotropy of the nuclear hormone receptor NHR-23 in C. elegans. This transcription factor has been known to be essential for embryonic development for over twenty years. However, two recent advances in genetic technology revealed a previously unappreciated role in spermatogenesis. First, the endogenous nhr-23 gene was edited to encode a GFP moiety, which revealed spermatocyte expression in both males and larval hermaphrodites. Second, an auxin-inducible degron (AID) moiety also present in the edited allele allowed conditional inactivation after the initial essential embryonic period. By waiting until late larval stages to induce degradation, a spermatogenesis phenotype was discovered that results in complete male sterility. The authors then use these tools, in combination with beautiful immunofluorescence assays and other mutations, to place NHR-23 into the broader contexts of meiosis, sex determination, and spermatogenesis regulation.

Comments for the author

Overall, this is a technically impressive study that provides new insights. I have two suggestions, however:

1. Spermatocytes are actually unusual among germ cells of the worm in that cell type-specific mRNA transcription seems to be the main driver of their gene expression, as is often the case in the soma. In contrast, germline stem cells and oocytes appear to primarily localize/time protein production via translational control and RNA-binding proteins acting via 3' UTRs. Mentioning this provides further incentive for the experiments described. Citing the work of Geraldine Seydoux's lab, by Merritt et al. (2008), would be appropriate.
2. It would be really cool to compare the transcriptomes of the NHR-23 AID strain with and without auxin. This could provide positive data about genes that depend upon NHR-23 for activation or repression, and would complement the SPE-44 study of Kulkarni et al. nicely. It's not a hard experiment, but would add a great deal to the impact of the study.
3. As a nuclear hormone receptor, there is at least a chance that NHR-23 actually has a hormonal ligand. Meanwhile, the timing of germline development in relation to the soma is precisely coordinated in both sexes, yet the mechanism for that remains unknown. Are there any candidates that could be discussed, even at the level of broad biochemical category? To me this is a clear future direction.

Reviewer 2

Advance summary and potential significance to field

"The conserved molting/1 circadian rhythm regulator NHR-23/NR1F1 serves as an essential co-regulator of C. elegans spermatogenesis" by James Matthew Ragle et al. is a manuscript that describes roles for the NHR-23 transcription factor, which is required somatically for C. elegans molting, during spermatogenesis. The authors use state of the art degron inducible tissue-specific degradation to deplete this widely expressed transcription factor in the germline. They convincingly show that NHR-23 depletion in the germline causes sperm-specific defects that mimic aspects of multiple other previously characterized spermatogenesis-defective mutants. This includes previously identified genes that participate in cell cycle control during spermatogenesis and other genes that encode structural components of vesicular structures that are specific to C. elegans spermatogenesis. A thorough genetic analysis indicates that NHR-23 plays a role during spermatogenesis that is downstream from the canonical germline sex determination pathway and functions in parallel to the only other known spermatogenesis-specific transcription factor encoded by the spe-44 gene. Unlike nhr-23, spe-44 is testis-specific in its expression. While nhr-23 expression in somatic tissue oscillates, it is constitutively expressed during C. elegans

spermatogenesis, indicating that it is regulated in a fundamentally different (still unknown) way. This is the first example of a somatically expressed transcription factor that plays a critical somatic role also being utilized in the germline and, not generally for both oogenesis and spermatogenesis, but specifically for spermatogenesis. The NHR-23 ortholog is widely expressed mammals, including the testis, and its depletion in Sertoli cells reduces sperm count in rats, suggesting an interesting evolutionary connection. Like nhr-23 in C. elegans, the circadian rhythm-governed expression of such genes in the soma is constitutive once it is turned on in the germline. As such, this study has broad potential interest to the readers of Development. It is well-executed and should be published after the issues listed below are addressed.

Comments for the author

Significant issue requiring an experiment
Page 8, lines 20-28: This is a tricky experiment to interpret because reducing/eliminating NHR-23 function in the testis will cause males to accumulate primary spermatocytes that probably are not ejaculated. In looking at the Materials and Methods, there is no discussion of how the mating experiments were performed and what assurances, if any, that the males actually mated. Ubiquitin-mediated degradation of NHR-23 is initiated due to MEX-5::Tir1 expression. MEX-5 expression is not specific to the germline, it is expressed in a number of other locations, including the nervous system, as is NHR-23, which is nicely documented in Figure 2A of this manuscript. So, one worry is that the males did not actually mate, either because they weren't interested in mating, or something about their copulatory structure and/or its associated nervous system was defective. This needs to be sorted out. First, when watching males, do they seem to be copulating? Second, try to determine if seminal fluid is transferred using an assay developed by Shakes and Ward (1989) Dev Biol 134: 189-200; Dr. Shakes is an author on this manuscript.

Technical questions
Page 8, line 18: How was it determined that knockdown was ~70%. Was this based on scanning the blot, some other approach or just an eyeballed guess? It would be worth mentioning that because of the way the experiment had to be done, residual expression is an expected result because NHR-23 in non-germline tissue would be expected to occur after auxin induction in the germline.

Page 15, lines 6-15: There is no discussion of whether or not the spe-44::mScarlet::3xMyc allele is indistinguishable from wild type. Explain whether or not this is the case and describe how this was assessed. This does not have to be very extensive and can be a supplemental information matter.

Page 22, lines 13-19: Please provide more details. When comparing control to experimental, were all Photoshop adjustments done identically and, if not, explain why.

Page 22, lines 29-30: What buffer was used for these experiments?

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Page 12, line 12: Replace “may” with “might”
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“These results are consistent with NHR-23-depletion associated defects”
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Figure Legends and Figures
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“Following separation from the RB, FBs disassemble and release unpolymerized MSP and the MOs dock with the plasma membrane. Males store sperm in this inactive spermatid state. During spermatid activation, MOs fuse with the plasma membrane and unpolymerized MSP localizes to the pseudopod where it forms fibers that are required for spermatozoon motility.”
Figure 2 legend, page 41, line 14: replace “used” with “analyzed”
Figure 3: In D, label the Western blot lanes a, b and c for both panels. Presumably, the anti-Flag and Total Protein are from the same gel, and these should be printed at the same magnification and include size standard numbers on the Total Protein Y axis.
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“Differential interference contrast (DIC) images of cells were overlaid by epifluorescence images of their Hoechst stained nuclei”.
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page 44, line 30: State the size of the scale bars in panel B page 44, line 30 Change: “+ 1 day 30 nhr-23::GFP::Biotag::AID*::3xFLAG; sun-1p::TIR1 hermaphrodites.”
to
“+ 1 day 30 nhr-23::GFP::Biotag::AID*::3xFLAG; sun-1p::TIR1 hermaphrodites in the absence (top) or presence (bottom) of 4 mM auxin”

First revision

Author response to reviewers' comments

We thank both reviewers for their helpful and constructive suggestions, and have a point-by-point response to their comments below. In the re-submitted manuscript, major changes and changes suggested by the reviewers are denoted by red text.

Reviewer 1 Comments for the Author:
Overall, this is a technically impressive study that provides new insights. I have two suggestions, however:

1. Spermatocytes are actually unusual among germ cells of the worm in that cell type-specific mRNA transcription seems to be the main driver of their gene expression, as is often the case in the soma. In contrast, germline stem cells and oocytes appear to primarily localize/time protein production via translational control and RNA-binding proteins acting via 3' UTRs. Mentioning this provides further incentive for the experiments described. Citing the work of Geraldine Seydoux’s lab, by Merritt et al. (2008), would be appropriate.
>>>This point is well-taken and we have added text to the introduction (pages 4and 5) to better set up the motivation for our experiments.

2. It would be really cool to compare the transcriptomes of the NHR-23 AID strain with and without auxin. This could provide positive data about genes that depend upon NHR-23 for activation or repression, and would complement the SPE-44 study of Kulkarni et al. nicely. It's not a hard experiment, but would add a great deal to the impact of the study.
>>>We agree with the reviewer that such an experiment is valuable and important. However, we feel such an experiment is beyond the scope of this manuscript. Given the scaled-back access to our labs during the pandemic, the severe disruption caused by power outages and wildfires in California, these analysis would take much longer than usual and significantly delay resubmission of our paper. Additionally, to be maximally informative, we would need to do RNA-seq on NHR-23-depleted males, SPE-44-depleted males, and NHR-23+SPE-44-depleted males. These experiments would require in-depth bioinformatic analysis, including comparison with the SPE-44 study of Kulkarni et al. Including such RNA-seq and bioinformatic analysis in this paper would require moving much of the current findings to Supplementary Material, as we were previously tightly against the word limit, and addressing the reviewer comments has resulted in addition of content. Finally, the RNA-seq analysis may or may not yield interesting results that would boost the current study. We humbly suggest that the RNA-seq experiments would be an ideal starting point of a manuscript following up our initial discovery of this role for NHR-23 in spermatogenesis.

3. As a nuclear hormone receptor, there is at least a chance that NHR-23 actually has a hormonal ligand. Meanwhile, the timing of germline development in relation to the soma is precisely coordinated in both sexes, yet the mechanism for that remains unknown. Are there any candidates that could be discussed, even at the level of broad biochemical category? To me this is a clear future direction.
>>>We do think that a hormonal ligand for NHR-23 is a good possibility and are actively testing this hypothesis. We have added text in the last paragraph of the Discussion to bring up the possibility of a ligand coordinating spermatogenesis and somatic development.

Reviewer 2 Comments for the Author:
Significant issue requiring an experiment
Page 8, lines 20-28: This is a tricky experiment to interpret because reducing/eliminating NHR-23 function in the testis will cause males to accumulate primary spermatocytes that probably are not ejaculated. In looking at the Materials and Methods, there is no discussion of how the mating experiments were performed and what assurances, if any, that the males actually mated. Ubiquitin-mediated degradation of NHR-23 is initiated due to MEX-5::Tir1 expression. MEX-5 expression is not specific to the germline, it is expressed in a number of other locations, including the nervous system, as is NHR-23, which is nicely documented in Figure 2A of this manuscript. So, one worry is that the males did not actually mate, either because they weren't interested in mating, or something about their copulatory structure and/or its associated nervous system was defective. This needs to be sorted out. First, when watching males, do they seem to be copulating? Second, try to determine if seminal fluid is transferred using an assay developed by Shakes and Ward (1989) Dev Biol 134: 189-200; Dr. Shakes is an author on this manuscript.
>>>To address these questions, we watched the males and observed that they do indeed mate. We also set up 24 hour crosses of NHR-23 depleted males with individual *spe-8(hc50)* hermaphrodites. These crosses can be tricky, but L4 + 24 hour males produced no outcross progeny and some crosses yielded a few *spe-8* self-progeny indicating that seminal fluid was indeed transferred. Twenty-four hour crosses with L4 males were more variable as some of these males sired outcross progeny. In our immunofluorescence experiments, we do sometimes observe spermatids in young adult males before the strong spermatocyte arrest phenotype predominates. The production of some sperm early is also common in many spe mutants.

Technical questions
Page 8, line 18: How was it determined that knockdown was ~70%. Was this based on scanning the blot, some other approach or just an eyeballed guess? It would be worth mentioning that because of the way the experiment had to be done, residual expression is an expected result because NHR-23 in non-germline tissue would be expected to occur after auxin induction in the germline.
>>>We have updated our Methods section to clarify the quantitation. Western blots were imaged using a BioRad ChemiDoc MP imaging system, taking care to ensure band pixels were not saturated. The imaging software has a band densitometry function, which allowed us to quantitate pixels to determine the difference in band intensity. While we do not see NHR-23::GFP expression in adult male somatic tissue, the reviewer is correct that some of the residual NHR-23 expression could reflect NHR-23 expression in somatic tissue, which would make the actual NHR-23 germline depletion even more robust in that case. We have added language to address this possibility.

Page 15, lines 6-15: There is no discussion of whether or not the spe-44::mScarlet::3xMyc allele is indistinguishable from wild type. Explain whether or not this is the case and describe how this was assessed. This does not have to be very extensive and can be a supplemental information matter.
>>>We thank the reviewer for catching this omission. We have added a supplemental figure (Figure S4) characterizing *the spe-44::mScarlet::3xMyc* allele. This figure also confirms that our *mex-5p::TIR1* transgene causes complete sterility with the *spe-44(fx110)* degron strain described by Kasimatis et al., 2018.

Page 22, lines 13-19: Please provide more details. When comparing control to experimental, were all Photoshop adjustments done identically and, if not, explain why.
>>> We have added this information in the methods. When the experiment was designed to compare levels of fluorescence between control and experimental samples, images from the same experimental preparation were captured with the same exposure and any post experimental

processing were done identically. However when the critical imaging information was to distinguish between the structures of chromatin, microtubule array, or 1CB4 staining structures, the images were optimized for the individual cells.

Page 22, lines 29-30: What buffer was used for these experiments?
>>> We have added this information.

Page 26, line 3: Was the auxin added to the hot agar prior to preparing media plates or was it infused into already- prepared agar plates. Please provide these details.
>>>Yes, the auxin was added when the agar was at 55ºC. We monitor media temperature using an infrared thermometer gun. We have added these details to the methods.

Minor wording, grammatical and interpretation issues
Generally, copyedit the entire manuscript and make sure there is a space between the number and mM or uM for example, it should be 4 mM not 4mM
>>> We have made these corrections throughout.

Page 6, line 24: Change “To address this,” to “To address this issue,”
>>> We have made this correction.

Page 8, line 26: Change “auxin did not” to “auxin did not sire any progeny”
>>> We have made this correction.

Page 9, line 1: Cite an appropriate reference for the origin of Figure 4A.
>>> We have added a reference to Ellis and Schedl, 2007.

Page 9, line 15: Presumably, the authors meant to say Figure S1 as there is no Figure S2.
>>> Yes, we should have referred to Figure S1. This error has been corrected.

Page 10, line 4: Change “These results indicate NHR-23…” to “These results indicate that NHR-23……”
>> We have made this correction.

Page 10, line 28 and 29: What vacuoles? This needs to be indicated with an arrowhead, preferably on a larger figure panel, as it is very difficult to see them.
>> We have removed mention of the vacuoles at this point and wait to discuss this phenotype until the FB-MO results from Fig 7 are presented in the manuscript.

Page 10, line 31: Change Fig. 6J (which is not nuclear pores) to 6G and H
>> We have made this correction.

Page 11, lines 5-17: As written, this text is difficult to follow. Figure 6C and D need more extensive labeling. Put dashed vertical lines to delineate the K region, for instance. Use arrows and arrowheads to point to specific- representative cells referred to in the text. Use equivalent labeling in C and D, for instance, there is no Div, in D, yet Div is talked about in the text.
>>>We added dashed lines in the DAPI images to better separate pachytene, karyosome, division and spermatid zones. We changed T to Div/T in 6D to reflect the "extended division zone" referred to in the paper text. We opted to not add any arrows or arrow heads because we felt that the point of these panels is to show the gonad overview and how the division zone is expanded with depletion of NHR-23. We separated the zoomed-in images and labeled them Figure 6E and F, which we hope will help the reader navigate this paragraph and highlight the expected morphology at each stage.

Page 12, line 12: Replace “may” with “might”
>>> This change has been made.

Page 12, line 29: This process is highly organized and the use of the word “lost” is misleading. What occurrs is that actin is being placed into the residual body. Label the residual body (rb) in the Late P and RB/S panels and refer to it in the text.
>>> We agree that the sentence was misleading. We have revised accordingly.

Page 13, line 1: The effect could be quite indirect and well downstream from the primary defect. Change: “These results are consistent with NHR-23-depletion causing defects” to “These results are consistent with NHR-23-depletion associated defects”
>>>We agree that the effect could be indirect. We deleted that sentence, as the revisions were bringing us against the word limit.

Page 13, line 8: Change “a vacuolated phenotype by DIC very similar” to “a vacuolated phenotype by DIC that is very similar”
>>> This change has been made.

Page 14, lines 9-32: When discussing the black and white insets, refer to them explicitly, not to Figure 7E generally, which is confusing.
>>> We now refer explicitly to the insets.

Page 15, line 17: Change “spermatogenesis coordinated by” to “spermatogenesis is coordinated by”
>>> This change has been made.

Page 20, line 2: cite an appropriate reference for MYOB media, which is not standard and unfamiliar to many investigators.
>>> We have added a citation to the MYOB recipe.

Page 26, line 5 and 6: Jargon. Replace, ” were singled onto equivalent media to lay eggs” with “were individually placed onto equivalent media and allowed to lay eggs”
>>> This change has been made.

Page 26, line 9: Change “matings, F1 progeny were grown to L4, then singled or mated with L4 males for 24” to “matings, F1 hermaphrodite progeny were grown to L4, then individually picked to plates and either left alone or mated with males (picked as L4’s) for 24”
>>> This change has been made.

Figure Legends and Figures
Figure 1: The FB is composed of fibers of MSP, the way it is depicted here as a uniform, red field is inaccurate. Also, the FB-MO’s are concentrated at the spindle poles during cell division, not randomly dispersed around the cytoplasm. The MSP in the pseudopod forms densely pack filaments, which should be depicted as such, not a uniform red colored area. Consult one of the reviews and re-draw.

>>> This correction has been made. In the revised diagram, the FB has lines to represent the oriented filaments.

Figure 1 legend, page 40, lines 24-27: Change “Following separation from the RB, FBs disassemble to release their MSP and the MOs dock with the plasma membrane. Males store sperm in this inactive spermatid state. During sperm activation, MOs fuse with the plasma membrane and MSP localizes to the pseudopod.” to
“Following separation from the RB, FBs disassemble and release unpolymerized MSP and the MOs dock with the plasma membrane. Males store sperm in this inactive spermatid state. During spermatid activation, MOs fuse with the plasma membrane and unpolymerized MSP localizes to the pseudopod where it forms fibers that are required for spermatozoon motility.”
>>> This change has been made.

Figure 2 legend, page 41, line 14: replace “used” with “analyzed”
>>> This change has been made.

Figure 3: In D, label the Western blot lanes a, b and c for both panels. Presumably, the anti-Flag and Total Protein are from the same gel, and these should be printed at the same magnification and include size standard numbers on the Total Protein Y axis.
>>> We adjusted the figure to make both the Total Protein and the anti-FLAG blot the same magnification and then cropped the relevant bands. The whole blot is shown in Figure S2 for data

transparency.

Figure 3 legend, page 42, line 6: What appears in the figure is an arrow, not an arrowhead
>>> The relevant bands have been cropped making the arrow unnecessary. The arrow has been removed and the text has been adjusted to reflect this.

Figure 6 legend, page 43, line 6 and 7, replace “unfixed” with “live”. Replace “Cells imaged with differential interference contrast (DIC), and DNA imaged with Hoechst stain”.
with
“Differential interference contrast (DIC) images of cells were overlaid by epifluorescence images of their Hoechst stained nuclei”.
>>> These corrections have been made.

Figure 6 legend, page 43, line 9: Change “Gonad images show proximal” to “Gonad images show the proximal”
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Figure 6 legend, lines 13 and 14: Change “Higher resolution images of individual spermatocytes below. (E-F). Isolated control (E) and NHR-23-depleted (F) proximal male gonads (pachytene and beyond)” to “Higher magnification images of individual spermatocytes. (E-F). Isolated control (E) and NHR-23-depleted (F) proximal male gonads (pachytene and later meiotic stages)”
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to
“Black and white images at the lower left of the E panels show DAPI (left) and 1CB4-stained MOs (right) in”
>>> The reviewer is correctly interpreting the figure legend, and we have changed the legend to their better wording.

Figure 8 legend, page 44 lines 11 and 12: Change “and a mScarlet::3xMyc knock-in to the endogenous spe-44 gene to create a C-terminal translational fusion.”to
”and spe-44::mScarlet.” (This information is already described in the Results and the Materials and Methods sections)
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Figure S1 legend, Page 44, line 27 and 28: Unfertilized oocytes are not progeny. Change “(A) Count of L1 larvae and fertilized eggs or unfertilized oocyte progeny from parents grown from L1 onwards with or without 4mM auxin.”
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>>> This correction has been made.

page 44, line 30: State the size of the scale bars in panel B
>>> Scale bar information has been added.

page 44, line 30 Change: “+ 1 day 30 nhr-23::GFP::Biotag::AID*::3xFLAG; sun-1p::TIR1 hermaphrodites.”

to “+ 1 day 30 nhr-23::GFP::Biotag::AID*::3xFLAG; sun-1p::TIR1 hermaphrodites in the absence (top) or presence (bottom) of 4 mM auxin”
>>> This correction has been made.

Second decision letter

MS ID#: DEVELOP/2020/193862

MS TITLE: The conserved molting/circadian rhythm regulator NHR-23/NR1F1 serves as an essential co-regulator of *C. elegans* spermatogenesis

AUTHORS: James Matthew Ragle, Abigail L. Aita, Kayleigh N. Morrison, Raquel Martinez-Mendez, Hannah N. Saeger, Guinevere A. Ashley, Londen C. Johnson, Katherine A. Schubert, Diane C. Shakes, and Jordan D. Ward
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. Please consider taking into account the minor suggestions of the second reviewer at the proof stage.

Reviewer 1

Advance summary and potential significance to field

(see first review)

Comments for the author

The authors generally took my review to heart. I was disappointed that the transcriptome comparison I suggested was not possible, but I agree that these are rather nutty times in the USA in general, and in California especially, and I think it would be a mistake to delay the MS for months.

Reviewer 2

Advance summary and potential significance to field

A key regulator of C. elegans spermatogenesis has been revealed by sophisticated tissue-specific protein depletion. This turned out to be a transcription factor associated with an early lethal phenotype that precluded the identification of its role during spermatogenesis. The authors place this transcription factor with the context of previous work, including the SPE-44 transcription factor that they show functions in a parallel, non redundant pathway. The work is well-executed, the results are interesting and it will be of wide interest to the audience of Development.

Comments for the author

The revision of the manuscript “The conserved molting/1 circadian rhythm regulator NHR-23/NR1F1 serves as an essential co-regulator of C. elegans spermatogenesis by Ragle, J. M. et al.” that is being considered for publication in Development has, in my opinion now been suitable revised except to the few minor points I mention below that are easily fixed.

Page 3, lines 56 and 57, replace “regulates the expression of multiple sperm genes” with “regulates the expression of multiple genes required for spermatogenesis”
Page 4, line 88 replace “Many C. elegans germline genes” with “Many C. elegans germline expressed genes”

Page 5, line 95, replace “as well as sperm function genes such” with “as well as genes whose expression is required for sperm function, such Page 5, lines 106-107, replace “Transcription of spermatogenesis genes” with “Transcription of genes required for spermatogenesis Page 6, line 132, replace “in spermatogenesis which we describe here.” with “during spermatogenesis, which we describe here.
Page 6, line 137, replace “NHR-23 promotes spermatogenesis in a distinct pathway from” with “NHR-23 promotes spermatogenesis in a pathway that is distinct from that of”
Page 7, line 157, right after the word “germline”, cite Kelly, W. G., Xu, S., Montgomery, M. K. and Fire, A. (1997). Distinct requirements for somatic and germline expression of a generally expressed Caernorhabditis elegans gene. Genetics 146, 227-238. and add to the list of cited references.
Page 8, line 206, replace “in” with “during”
Page 9, lines 217-219, The preferred way to do this experiment is to use a morphological marker in the spe-8 hermaphrodite, such as a dpy, and not have a him in the strain. Done in this manner, it is easily possible to distinguish self from outcross progeny, as self will be Dpy (and there will be no males) and outcross will be nonDpy and 50% male. So, the way the experiment was done here was odd. Nonetheless, the authors have shown that reducing/knocking out NHR-23 does not preclude seminal fluid transfer, so all of the plumbing and neural circuits needed for that to occur are OK, which was what I asked during the first review. However, in order to make a statement that they know anything about the paternity of the observed progeny (self vs outcross) more details need to be stated, such as maybe transgene expression was observed in the progeny, a pedigree analysis was performed or whatever reliable indicator allowed assessing paternity.
Page 12, line 342, (Fig. 6K) should be (Fig, 6M)
Page 17, line 471 replace “may” with “might”. May is used when asking for permission while might is used when discussing possibility.
Page 17, line 17 “The meiotic phenotypes following NHR-23-depletion are similar to, but distinct from,” (Add a comma on either side of the appositional phrase)
Page 18, line 521, replace “which” with “that”. What follows “which” is a dependent clause, hence the reason for replacing it with “that”.
Page 19, line 539, replace “may” with “could”
Page 19, line 546, Same issue with the “which”, it should be “that” here Page 22, line 656. While the images look OK, the authors should be aware that the Edgar egg buffer is hypertonic for C. elegans sperm. In the future, they should use the buffer optimized for sperm biochemistry first published by the Ward lab in the 1980’s and modified over the years. See for instance Machaca, DeFelice and L’Hernault (1996) Dev Biol 176: 1-16. Primary spermatocytes will develop all the way to spermatids that drop off the residual body in this medium.

Supplemental Figure 2: Presumably, what is affected is the protein at 102,000 kDa. Indicate this fact on the figure with an arrow or in some other matter.