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C. elegans molting requires rhythmic accumulation of the Grainyhead/LSF transcription factor GRH-1

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Review #1

1. Evidence, reproducibility and clarity:

Evidence, reproducibility and clarity (Required)

The manuscript by Meeuse and colleagues describes a series of detailed and elegant experiments addressing the molecular mechanisms underlying oscillatory gene expression patterns in the nematode Caenorhabditis elegans and how these are required for molting between larval stages. By performing ChIP-seq on synchronised populations at 12 different timepoints (each separated by 1h), the authors find that RNA pol II (RNAPII) occupancy at >2000 promoters shows an oscillating behaviour that is paralleled by similar changes in mRNA abundance. To identify transcription factors (TFs) involved in generating these cycles, the authors conducted a candidate RNAi screen on 92 TFs annotated as been expressed in an oscillating manner themselves. Monitoring developmental progression of individual animals by a luminescence-based assay, the authors identify 6 TFs that caused either death (or arrest) after aberrant molts (3 TFs) or prolonged duration on molts (3 TFs). One of these, the Grainyhead/LSF transcription factor GRH-1 is then investigated in further details. Temporal control of GRH-1 depletion is achieved by TIR1/auxin-mediated protein degradation. This revealed that GRH-1 is required during each larval molt. In agreement with the criteria for including grh-1 among the candidate RNAi clones, GRH-1 protein levels are shown to peak immediately before entry to each molt.

I am very enthusiastic about the manuscript at several levels: conceptualisation, experimental design, quality of data and clarity of text and discussion. I have only two comments in the category of "major comments":

1. I do not see how the experiments presented in Figure 7 can be used as argument to conclude that "Molting requires oscillatory GRH-1 activity" (title of last Results section and also reflected in the title of the manuscript). I think the experiment nicely shows that there is a timepoint beyond which removal of GRH-1 no longer interferes with the upcoming molt but that doesn't imply that GRH-1 necessarily needs to oscillate. Stronger evidence could be provided by inducible, non-oscillating GRH-1 expression.
2. After reading the manuscript, one is left with the obvious question: which of the many oscillating genes are direct targets of the oscillating TF GRH-1? Experiments to answer this are not strictly needed for the current manuscript, which stands perfectly on its own, but it would make a significant increase in the overall understanding of oscillating genes and GRH-1 in particular.

Minor comments:

The sampling used to generate the data represented in Figure 1 correspond to from 22 hours until 33 hours of post-embryonic development at 25{degree sign}C. It would be useful to indicate how these time points relate to larval development L1-L4.

The authors state that co-oscillation of RNAPII and mRNA is not observed for all genes. Although this might indeed be due to technical limitations are suggested by the authors, it would be relevant to provide more information (e.g. number or percentage of genes).

Are the images of GRH-1::GFP expression in larvae (Fig S8) and adults (Fig S9) acquired with identical settings? I assume so, but it is not obvious, particularly because the images are in separate figures.

Have the authors examined if GRH-1 activity is not only controlled by oscillating transcription, but also post-translationally (e.g. phosphorylation, nuclear import, etc.) as is the case for many TFs. The authors could potentially "overlay" the GRH-1::GFP data with transcriptional data (available at least for 22-33h) to see if the shapes coincide.

2. Significance:

Significance (Required)

Oscillating genes are found in many biological contexts and are fascinating examples of tightly controlled gene expression. The Großhans laboratory has previously identified close to 4,000 oscillating transcripts and is a leader in this field. The current manuscript incorporates a variety of sophisticated techniques that together enable the authors to identify six genes that are required for rhythmic molting in C. elegans. The protein most deeply studied in the manuscript, GRH-1, is homologous to Grainyhead, which is involved in ECM remodeling and other cyclic processes. The findings in this manuscript are therefore of potential relevance across a broad evolutionary scale.

3. How much time do you estimate the authors will need to complete the suggested revisions:

Estimated time to Complete Revisions (Required)

(Decision Recommendation)

Less than 1 month

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Reviewer Publons

Yes

Review #2

1. Evidence, reproducibility and clarity:

Evidence, reproducibility and clarity (Required)

Summary:

In this paper, Meeuse et al. analyze the determinants of rhythmical molting in C. elegans using a combination of

RNA-polymerase ChIP-seq, RNA-seq, RNAi and imaging coupled to targeted degradation. The main finding is the discovery of the importance for the molting process of GRH-1, a transcription factor homolog to Vertebrate Grainyhead, as well as the identification of 5 additional transcription factors for which molting is defective.

Major comments:

The experiments are generally well performed, and the results convincingly demonstrate the function of GRH-1 in molting.

The coupled RNA-seq and ChIP-seq experiment (Fig. 1A/B) was done only once.

One of the claims (p3) is that rhythmic transcription of oscillating genes is driven by rhythmic RNA pol II occupancy. If this is suggested visually by Figure 1A/B, I think this claim merits further statistical analysis. How is ChIP-seq correlated with RNA-seq globally and at the individual gene level? How good is the correlation? Can the authors provide a supplementary table with this correlation at the gene level? In the same paragraph, a couple examples of genes for which RNA polII ChIP does not correlate with RNA-seq would be helpful to the reader (they are currently cited as "instances where oscillating mRNA levels were not accompanied by rhythmic RNAPII promoter binding"). Please provide gene names.

The oscillatory transcription of several promoters is tested using GFP fusions coupled with q-RT PCR. It is not clear to me whether these experiments were repeated. Additionally, the authors state that each qPCR was repeated (only once), hence both data points should be shown in the graphs on Fig. 1C and S2.

The authors perform then RNAi knock-down of 92 transcription factors involved in molting using bioluminescence and identify 6 genes involved in molts, three of which have been characterized previously. They focus on one of the other three, grh-1, as its orthologs are involved in epidermal biology in other organisms. Targeted degradation of GRH-1 using the auxin degron confirms the function of GRH-1 in each molt, in an auxin-concentration dependent manner. GFP tagging of GRH-1 shows an accumulation prior to each molt, suggesting the transcription factor is necessary for the onset of molting. As the GRH-1 target site is known (PMID 12888489), the paper would be greatly strengthened if the authors could loop back to the RNA-seq/ChIP-seq dataset and highlight which cycling genes have indeed a GRH-1 binding site in their promoter sequence and whether this correlates with one specific phase of the molting cycle.

Similar to the ChIP/RNA-seq experiments, it is not clear to me whether the different bioluminescence experiments were performed once or twice.

As stated above, the results are very convincing and the conclusions quite clear. Additionally, all wet lab experiments are described in very many details. However, I feel that the description of the data analysis is too succinct to allow reproducing the experiments, even using the GEO data. I would expect the authors to provide a github public link with the scripts to perform the RNA-seq and ChIP-seq analyses, the Matlab scripts to analyze bioluminescence experiments (Fig. 2,5,7) and the CNN used to analyze single worm molting, even if the method is to be described elsewhere.

Minor comments:

The authors should provide descriptive statistics for their high-throughput sequencing (read number, mapping statistics etc...).

In figure 1C, it would be helpful to the reader to write peak phase and amplitude of the tested genes on the graph.

In the same figures, for gene F58H1.2, for which the correlation between the reporter and the endogenous gene is not perfect, the authors "suspect [...] that the reporter may lack relevant promoter or intronic enhancer

elements". An alternative explanation is that post-transcriptional regulation occurs for this mRNA, a hypothesis which should be added to the text.

2. Significance:

Significance (Required)

As stated above, this manuscript highlights convincingly that GRH-1 is involved in the molting cycle in the nematode C. elegans, a conserved function of the gene between evolutionary distant species for skin biology.

Field of expertise: C. elegans, high-throughput sequencing methods, imaging.

3. How much time do you estimate the authors will need to complete the suggested revisions:

Estimated time to Complete Revisions (Required)

(Decision Recommendation)

Between 3 and 6 months

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Yes

Review #3

1. Evidence, reproducibility and clarity:

Evidence, reproducibility and clarity (Required)

Summary:

The manuscript describes how a rhythmically active transcription factor is important for molting cycles.
The first part of the manuscript focuses on oscillating genes, and the authors nicely show a rhythmic transcription of these genes. Indeed, using RNAPolII Chip-Seq experiments, they show a rhythmic recruitment of the RNAPolII to the promoters of the oscillatory genes they have previously described. They then demonstrate that GFPs driven by the promoter of these oscillating genes, and inserted as a single copy, can very accurately recapitulate the rhythmic transcription of oscillating genes. It is interesting to see the weak impact of

introns and 3'UTR on the rhythmic expression.
In the second part of the manuscript, the authors perform an RNAi screen, looking for oscillating transcription factors (TF) important for molting. The goal of this approach is to identify core oscillators that could control molting cycles. Focusing their screen on oscillating TF allows them to exclude TF that would be required for embryonic or larval viability unrelated to molting. Among the 6 candidates they have identified, 5 have already been linked to molting. They focus their study on grh-1, which has never before been described during larval development. They characterize the molting phenotype of grh-1 defective worms using the AID degron system. They monitor the molting cycles using a luciferase assay in a liquid culture where transgenic worms for luciferase are grown in the presence of bacteria supplemented with luciferin. Using this approach (which is quantitative and allows high-throughput analysis), they show that grh-1 is required for each molt in a dose-dependent manner. The GRH-1 protein oscillates and peaks shortly before each molt entry, reinforcing the idea that GRH-1 is an important core TF for molting. The authors finally show that the oscillating activity of GRH-1 is crucial for the molting.

Major comments:

Overall, the data are clear and convincing, and the results are quantified with care. The first part of the manuscript represents a significant amount of work with the RNAPolII Chip-Seq, the different single-copy integrants and RT-PCRs. Then, the authors provide a quantitative assessment of the molting process by combining their grh-1-AID construct with the luciferase system. This strengthens the quality of the manuscript.

My substantial suggestion is that the authors could consider extending the scope of the study in two alternative ways:

- One question that immediately springs to mind is: what are the targets of grh-1? By applying their luciferase assay to further possible downstream targets of grh-1, they could attempt to phenocopy the grh-1 molting defect, and then look if the oscillating expression of these targets is eliminated in grh-1 defective animals. The binding site of grh-1 is apparently known (Venkatesan, 2003), so is it possible to reduce the potential target list among the oscillating genes using a bioinformatics approach? This requires a substantial amount of work (2 to 3 months) but it would help tell a more complete story.
- It is striking that myrf-1 and nhr-23 RNAi display the same molting defects as grh-1 RNAi as show in figure 2B. Have the authors considered testing the genetic interactions of grh-1 with these two other candidates? Do they belong to the same GRN? Does grh-1 depletion impact the expression of the nhr-23 and myrf-1, or vice versa? Do they have the same target genes (Chip-seq data for nhr-23 are available)? This, again, would significantly strengthen the paper and would make the second part more complete. This alternative piece of work would require less experiments than the first suggestion but would be also of great interest.
These two points are only suggestions as it represents a significant amount of work, and the paper could very well be published in its current form.

About the luciferase assay for grh-1, nhr-23 and myrf-1 RNAi, the authors observe "an apparent arrest in development or death following atypical molts". What do they mean by "atypical molt" at this stage of the paper? Indeed, for these candidates, the luminescence traces are highly perturbed after the second molt (for grh-1 and nrh-23 RNAi) or the third molt (for myrf-1), but these abnormal traces seem to reflect an arrest in development or larval lethality rather than an atypical molting. Can the authors clarify this point?

In the part on the phenotypic analysis of GRH-1 depleted animals, the authors conclude the paragraph with "GRH-1 is required for viability at least in part through its role in proper cuticle formation". This role in proper cuticle formation refers to the cuticle break in the head region as observed in time lapse. It would be useful to have a visual test of the cuticle permeability using an Hoechst staining.

The authors show GFP::GRH-1 pictures at different stages to describe a rhythmic protein accumulation (see also my minor comment on GFP picture quality). From the perspective of whether all tissues are oscillating, it would be interesting to see if all the cells they mention in the text are showing the same rhythmic fluorescence.

In relation to the previous comment, which tissue is responsible for the defects observed by the degradation of GRH-1? Is it possible to use a tissue-specific depletion of AID-tagged GRH-1 using Seam-cell specific, rectal cell specific, vulval precursors specific promoters, etc...?

In the last part of the results, the authors show that molting requires oscillatory GRH-1 activity by depleting GRH-1 at variable times in L2. It would be interesting to know what happens if a stable (non-oscillating) amount of GRH-1 protein is maintained over time in the worms (using a non-oscillating promoter).

Minor comments:

In figure 5 B, C, D, it seems that right before entering the M2, M3 and M4 respectively, there is a peak of luminescence (a thin bright line) and a strong luminescent signal is detected at the molt exit. Can the authors comment on that?

If I understand correctly, for the GRH-1 GFP CRISPR reporter (Fig S7, S8 and S9), the authors have imaged single worms in microchambers on a spinning disk microscope. I fail to see why they used such a sophisticated approach to describe the expression pattern of GRH-1. This imaging setup is ideal for timelapse. However, in the context of which cell express GRH-1, the resolution is not good enough to fully assess cell identity. Indeed, the GFP images are a bit blurry, and it is difficult to make out the difference between real GFP fluorescence and gut autofluorescence. It would be helpful to have better quality pictures with a more regular setup, i.e., a 2% agarose pad mounted on a regular microscope or confocal. For non-specialists of the C. elegans anatomy, small insets for each category of cells mentioned (seam cells, vulval precursors etc.) would be appreciated.

It would be easier to assess the GRH-1 expression decrease in adults if the pictures were shown in parallel with the larval pictures, with the same brightness/contrast correction (if any). Make insets to compare the same cells between different stages.

How can the authors quantify the duration of molts 3 and 4 in fig S4 when these molts are not seen in the luciferase assay in fig 2B? Can the authors clarify this point?

Writing/clarity:
For non-specialists, mention why the authors used a PEST sequence in their constructs and explain what the eft-3 promoter is (they mention it in the Luciferase assay, but it is not clear enough).

In Fig S4, make clearer that EV = MOCK.

In the methods, the authors refer to the we146 mutant strain, but they neither use it nor mention it in the body of the text. Producing such a mutant strain is great and it should be mentioned in the results, with an explanation as to why they are dying. Otherwise, it should be removed from methods.

In the Methods, the genotype of the strains is misleading. For example HW1372 : EG6699; xeSi... is not the regular way to write a C. elegans genotype. It should be written as:
HW1360 xeSi131[F58H1.2p::GFP::H2B::Pest::unc-54 3', unc-119+] II; unc-119(ed3) III as the strain used to generate the MosSci insertion is described in the paragraph on Transgenic reporter strain generation.

For the GFP CRISPR strain, the authors write either GFP::GRH-1 in Fig S7, S8 and S9, grh-1::gfp::3xflag in the methods or GRH-1-GFP fusion in the results. The authors should homogenize the way they write this reporter strain. Whether it is an Nterminal or a Cterminal fusion will determine how they should label it.

Enlarge the font size for Fig S8 and 9 for the scale bar.

2. Significance:

Significance (Required)

The author's prior analysis (Meeuse, et al 2020) showed that mRNA oscillations are coupled with developmental processes, including molting. The present paper extends that finding by showing that oscillating transcript levels are directly linked to a rhythmic recruitment of the RNAPolII on their promoter. Then Meeuse and her colleagues use the molting as a model system to access the importance of oscillating TF for rhythmic processes. Through an RNAi screen, they have identified 6 candidates involved in the molting process. One of the candidates, grh-1, is characterized further. They combine a quantitative-based analysis (luciferase assay) with a time and dose-controlled degradation of GRH-1 to clearly describe the impact of grh-1 depletion on molting. This time and dose control is very smart and key to their study.
Overall, the paper adds some interesting piece of information to the field of rhythmic control of molting cycles, as it shows that oscillating transcription factors provide a developmental clock in this process. But this notion is not completely new, as it has been shown in other developmental processes like the circadian clock. Moreover, how molting cycles are controlled by GRH-1 remains to be elucidated.

My field of expertise is GNRs studies, the genetic of C. elegans, embryonic and larval development in C.elegans, timelapse and confocal imaging. I do not have expertise in Chip-Seq analysis.

3. How much time do you estimate the authors will need to complete the suggested revisions:

Estimated time to Complete Revisions (Required)

(Decision Recommendation)

Between 3 and 6 months

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Reviewer Publons

No

1st Editorial Decision **24th Jun 2022**

Thank you again for transferring your Review Commons manuscript to The EMBO Journal, as well as your response to the referee comments. In light of the referees' positive comments and your revision plan, we would like to invite you to prepare and submit a revised manuscript.

As outlined in your revision plan, it will be important to address the reviewer's concerns experimentally or by revising the manuscript and to provide a detailed response to each of their comments. In addition, as we discussed, further characterization of GRH-1 target genes will be needed to address the main concern all reviewers noted (ref #1- point 2; ref#2- point 5; ref#3-point 1), for example through the proposed ChIPseq analyses of tagged GRH-1. When submitting the revised manuscript please consult the EMBO Journal guidelines for manuscript format (https://www.embopress.org/page/journal/14602075/authorguide#submissionofrevisions) and ensure all acquired datasets are accessible to the referees (see below for more details).

We thank the reviewers for their careful examination of, and constructive comments on, the manuscript and their strong endorsements of our work. We also appreciate their suggestions on future work directions, even where beyond the scope of the current study. In discussion with the editor, we have focused on one particular aspect, namely elucidation of candidate GRH-1 targets, using ChIP-seq analysis. This now provides further evidence for a phase-specific function of GRH-1 and identifies an interaction with some of the screen hits.

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

The manuscript by Meeuse and colleagues describes a series of detailed and elegant experiments addressing the molecular mechanisms underlying oscillatory gene expression patterns in the nematode Caenorhabditis elegans and how these are required for molting between larval stages. By performing ChIP-seq on synchronised populations at 12 different timepoints (each separated by 1h), the authors find that RNA pol II (RNAPII) occupancy at >2000 promoters shows an oscillating behaviour that is paralleled by similar changes in mRNA abundance. To identify transcription factors (TFs) involved in generating these cycles, the authors conducted a candidate RNAi screen on 92 TFs annotated as been expressed in an oscillating manner themselves. Monitoring developmental progression of individual animals by a luminescence-based assay, the authors identify 6 TFs that caused either death (or arrest) after aberrant molts (3 TFs) or prolonged duration on molts (3 TFs). One of these, the Grainyhead/LSF transcription factor GRH-1 is then investigated in further details. Temporal control of GRH-1 depletion is achieved by TIR1/auxin-mediated protein degradation. This revealed that GRH-1 is required during each larval molt. In agreement with the criteria for including grh-1 among the candidate RNAi clones, GRH-1 protein levels are shown to peak immediately before entry to each molt.

I am very enthusiastic about the manuscript at several levels: conceptualisation, experimental design, quality of data and clarity of text and discussion. I have only two comments in the category of "mayor comments":

We thank this reviewer for his/her enthusiastic endorsement of our work.

1) I do not see how the experiments presented in Figure 7 can be used as argument to conclude that "Molting requires oscillatory GRH-1 activity" (title of last Results section and also reflected in the title of the manuscript). I think the experiment nicely shows that there is a timepoint beyond which removal of GRH-1 no longer interferes with the upcoming molt but that doesn't imply that GRH-1 necessarily needs to oscillate. Stronger evidence could be provided by inducible, non-oscillating GRH-1 expression.

We agree with the reviewer's point of view and have explored possibilities of performing the suggested experiment. Unfortunately, we had to conclude that the experiment is currently not feasible. It requires a) a suitable induction system, b) a promoter with matching spatial activity and activity levels, and c) rescuing transgenes. We are currently wanting on all three. The only robust inducible system in *C. elegans* is the heat shock promoter, but the oscillation frequency is temperature dependent, rendering this system

unsuited. The inducible expression of such a hypothetic promoter would not only have to be stable but also occur in exactly the tissues where the *grh-1* promoter is active, and occur at levels that correspond to baseline, median, and/or peak levels of the endogenous promoter. Otherwise, phenotypes might result from expression in the wrong tissue (or lack of expression in some relevant tissues), or inappropriately high or low levels, rather than lack of rhythmic expression. Unfortunately, we have no data to tell us which promoter specifies which level of activity in which tissue. Finally, in initial explorations, we were unable to obtain full rescue of *grh-1(0)* mutant phenotypes with transgenes where we used the *grh-1* promoter to drive *grh-1* cDNA transcription. (cDNA is necessitated because of the gene size.) This could be for technical reasons (e.g., splicing is needed to achieve proper levels or timing of expression), or because both isoforms are needed (we tested them individually). Irrespectively, there are currently too many technical limitations and confounders for us to conduct these experiments. To acknowledge this limitation, we now conclude the section by writing "Although technical limitations have prevented us from testing whether constitutive expression of *grh-1* is incompatible with development, the dynamic GRH-1 accumulation and a recurring need for GRH-1 for molting thus supports a model of rhythmic GRH-1 activity during larval development." This said, the ChIP-seq analysis we performed in response to the next question (and detail below) provides further support for a rhythmic activity of GRH-1.

2) After reading the manuscript, one is left with the obvious question: which of the many oscillating genes are direct targets of the oscillating TF GRH-1? Experiments to answer this are not strictly needed for the current manuscript, which stands perfectly on its own, but it would make a significant increase in the overall understanding of oscillating genes and GRH-1 in particular.

This is indeed a very interesting question. Although the reviewer pointed out that this would be an extension of our work rather than data required to support it, we sought to obtain some insight into this matter. Specifically, we performed ChIP-seq analysis on endogenously GFP-tagged GRH-1. We found that peaks were enriched for a motif similar to that of the orthologous mammalian GRHL1/2 proteins and a motif proposed based on in vitro binding data. We find that targets appear enriched in a phase window before molt entry, thus agreeing both with the accumulation pattern of GRH-1 protein and the period of sensitivity to GRH-1 depletion. By contrast, genes peaking in antiphase were depleted for GRH-1 binding. We interpret this as additional support for rhythmic GRH-1 activity. Finally, we find that GRH-1 binds to its own promoter as well as those of additional screen hits, tentatively supporting its placement in a gene regulatory network generating oscillations.

Minor comments:

The sampling used to generate the data represented in Figure 1 correspond to from 22 hours until 33 hours of post-embryonic development at 25{degree sign}C. It would be useful to indicate how these time points relate to larval development L1-L4.

We have updated the figure to include a panel that provides an overview on the approximate timing of this experiment (new Figure 1A).

The authors state that co-oscillation of RNAPII and mRNA is not observed for all genes. Although this might indeed be due to technical limitations are suggested by the authors, it would be relevant to provide more information (e.g. number or percentage of genes).

We have performed further analysis of this matter which supports our notion that this is by and large a technical matter (although not excluding the possibility that there could be exceptions involving genuine biological phenomena). As shown in the new Expanded View Figure EV1B-D, we find that genes that lack a positive correlation between RNAPII ChIP-seq and mRNA-seq dynamics are on average expressed at lower levels and exhibit a lower amplitude than all oscillating genes. We have refrained from providing a percentage because this number would depend on the specific cut-offs used, and thus easily become misleading. Instead, we provide Table EV1 with our analysis so that interested readers can explore the data for themselves and decide whether individual genes deserve follow-up studies.

Are the images of GRH-1::GFP expression in larvae (Fig S8) and adults (Fig S9) acquired with identical settings? I assume so, but it is not obvious, particularly because the images are in separate figures.

We have replaced these figures with better ones according to a request from Reviewer #3. The previous Figure S8 (larvae) is now Figure EV4C. Moreover, we revised Fig. S9 (now Appendix Figure S5) to show larval and adult worm images (acquired with the same settings) side by side so that decreased adult levels are readily appreciated. Note that these are also apparent from the quantification in Figure 6.

Have the authors examined if GRH-1 activity is not only controlled by oscillating transcription, but also post-translationally (e.g. phosphorylation, nuclear import, etc.) as is the case for many TFs. The authors could potentially "overlay" the GRH-1::GFP data with transcriptional data (available at least for 22-33h) to see if the shapes coincide.

We consider it possible that such additional levels of regulation exist, but because RNA production and protein production are successive events and because proteins tend to have longer half-lives than RNAs, we expect that the protein is phase-shifted relative to the RNA. This is indeed what we observed when we did an analysis of the type that the reviewer suggests. However, without knowledge of specific production and decay rates, we cannot perform a quantitative analysis to determine whether the observed patterns imply post-translational regulation. Hence, we decided against including this analysis in the manuscript.

Reviewer #1 (Significance (Required)):

Oscillating genes are found in many biological contexts and are fascinating examples of tightly controlled gene expression. The Großhans laboratory has previously identified close to 4,000 oscillating transcripts and is a leader in this field. The current manuscript incorporates a variety of sophisticated techniques that together enable the authors to identify six genes that are required for rhythmic molting in C. elegans. The protein most deeply studied in the manuscript, GRH-1, is homologous to Grainyhead, which is involved in

ECM remodeling and other cyclic processes. The findings in this manuscript are therefore of potential relevance across a broad evolutionary scale.

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

Summary:
In this paper, Meeuse et al. analyze the determinants of rhythmical molting in C. elegans using a combination of RNA-polymerase ChIP-seq, RNA-seq, RNAi and imaging coupled to targeted degradation. The main finding is the discovery of the importance for the molting process of GRH-1, a transcription factor homolog to Vertebrate Grainyhead, as well as the identification of 5 additional transcription factors for which molting is defective.

Major comments:
The experiments are generally well performed, and the results convincingly demonstrate the function of GRH-1 in molting.

We thank the reviewer for her/his endorsement of our work.

The coupled RNA-seq and ChIP-seq experiment (Fig. 1A/B) was done only once.

This is correct, we performed a densely sampled time course instead of replicates at lower sampling density. This is consistent with best practice and traditional approaches to time series analysis in other systems (e.g., Koike et al., Science 2012, Sobel et al., PLoS Biol 2017, see discussion in Sefer et al., Cell Systems 2016). It is specifically backed up by theoretic analyses (Sefer et al., Cell Systems 2016): dense sampling without replicates is better than sparse sampling with replicates when aiming to infer dynamic patterns. (Intuitively, the benefit of this approach becomes clear when considering neighboring time points as partial replicates; in our case, if the signal were noise, we would not see oscillations but wiggles.) We now state this explicitly in the methods section.

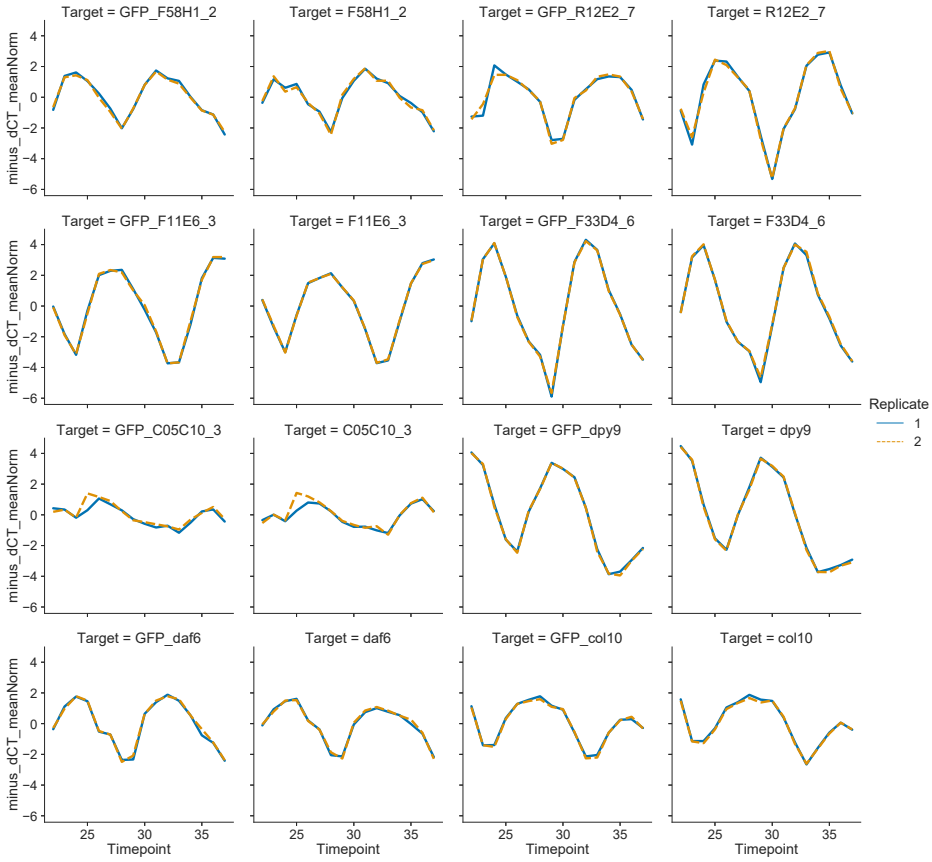
One of the claims (p3) is that rhythmic transcription of oscillating genes is driven by rhythmic RNA pol II occupancy. If this is suggested visually by Figure 1A/B, I think this claim merits further statistical analysis. How is ChIP-seq correlated with RNA-seq globally and at the individual gene level? How good is the correlation? Can the authors provide a supplementary table with this correlation at the gene level? In the same paragraph, a couple examples of genes for which RNA polII ChIP does not correlate with RNA-seq would be helpful to the reader (they are currently cited as "instances where oscillating mRNA levels were not accompanied by rhythmic RNAPII promoter binding"). Please provide gene names.

We now provide Table EV1 with the requested correlations and have performed further analyses according to the reviewer's suggestion, which we show in the new Expanded View Figure EV1B-D. These support our notion that our inability to detect rhythmic RNAPII occupancy on some oscillating genes is explained mostly by technical limitations (although not excluding the possibility that there could be exceptions involving genuine biological phenomena). Specifically, we find that genes that lack a positive correlation between RNAPII ChIP-seq and mRNA-seq are on average expressed at lower levels and exhibit a lower

amplitude than all oscillating genes. Given this situation, we decided against providing gene names (since we have no way of ascertaining those that could be 'genuine'), but we point out in the Discussion section the availability of (Table EV1) that interested readers can explore to select genes for further investigation.

The oscillatory transcription of several promoters is tested using GFP fusions coupled with q-RT PCR. It is not clear to me whether these experiments were repeated. Additionally, the authors state that each qPCR was repeated (only once), hence both data points should be shown in the graphs on Fig. 1C and S2.

For the same considerations that applied to our ChIP-seq time course, we elected to maximize the information content of these analyses through dense sampling of single time courses rather than replicates of sparsely sampled time courses. Indeed, here we sampled not only densely, but also over extended time periods: 16-h time courses (a separate one for each strain, sampled hourly!). They thus cover ~2 oscillation cycles, providing internal replication, and we provide such information for many genes. We consider this approach much preferable over the other logistically feasible alternative; repeat experiments for few genes, sparsely sampled, over short durations.
Below, we show the replicate data as requested; as expected from technical replicates, results are very similar.



Reviewer Figure: Technical replicates of RT-qPCR time course analyses. Transcripts of GFP transgenes and corresponding endogenous genes are plotted separately for better assessment of experimental reproducibility.

We found that the figure above would look cluttered and not readily transmit the key message (the similarity of endogenous transcript relative to transgene), but aggregating four traces in one panel made it difficult to see anything, with little gain of information. Hence, we decided to stick with the previous figures showing average data, and provide the raw data as Source Data 1 linked to the figure.

The authors perform then RNAi knock-down of 92 transcription factors involved in molting using bioluminescence and identify 6 genes involved in molts, three of which have been characterized previously. They focus on one of the other three, grh-1, as its orthologs are involved in epidermal biology in other organisms. Targeted degradation of GRH-1 using the auxin degron confirms the function of GRH-1 in each molt, in an auxin-concentration dependent manner. GFP tagging of GRH-1 shows an accumulation prior to each molt, suggesting the transcription factor is necessary for the onset of molting. As the GRH-1 target site is known (PMID 12888489), the paper would be greatly strengthened if the authors could loop back to the RNA-seq/ChIP-seq dataset and highlight which cycling genes have indeed a GRH-1 binding site in their promoter sequence and whether this correlates with one specific phase of the molting cycle.

We agree with the reviewer that this is an interesting question. Unfortunately, we found that the published motif has a low information content, since it is built on only ~15 sequences, hence yielding ~17,000 hits. Hence, and as a much stronger indicator of interaction, we performed a ChIP-seq analysis using endogenously tagged GRH-1 (new Figures 8, EV5, Appendix Figure S7). As predicted by the reviewer, this revealed preferred binding to genes with a specific peak phase, which gratifyingly overlaps with the peak accumulation of GRH-1 protein and the window of activity determined by the timed depletion in Figure 7. Notably, putative target genes also include additional hits from the screen.

Similar to the ChIP/RNA-seq experiments, it is not clear to me whether the different bioluminescence experiments were performed once or twice.

We apologize for our lack of clarity. For the auxin titration experiments, we initially performed a broad, exploratory concentration survey and found that µM to sub-millimolar auxin concentrations caused animal death specifically in *aid::grh-1* but not control animals (shown in Appendix Figures S3 and S4). Hence, we selected 250 µM as our standard concentration (in Figure 3), replicating the initially observed effect for animals hatching in the presence of auxin and extending it to other time points. Second, we focused on sub-µM concentrations to examine non-lethal effects in an independent experiment (in Figure 4). We have made textual additions to clarify the procedure. We also note that each experiment involves tens of animals per conditions that are examined individually and thus constitute biological replicates. Finally, we have done numerous experiments using RNAi to deplete GRH-1 and observed larval arrest/death – this effect is so robust that we have begun to include *grh-1* RNAi as a positive control in other luciferase experiments that we are doing.

As stated above, the results are very convincing and the conclusions quite clear. Additionally, all wet lab experiments are described in very many details. However, I feel that

the description of the data analysis is too succinct to allow reproducing the experiments, even using the GEO data. I would expect the authors to provide a github public link with the scripts to perform the RNA-seq and ChIP-seq analyses, the Matlab scripts to analyze bioluminescence experiments (Fig. 2,5,7) and the CNN used to analyze single worm molting, even if the method is to be described elsewhere.

We have now supplied this information as requested and provide a link in the Data and Materials availability section (https://github.com/fmi-basel/ggrosshans_grh-1_paper). We have also included an additional section on the CNN, although we point out that it was merely used to segment microscopy images to identify the location of the animal; it was not used to analyze molting.

Minor comments:
The authors should provide descriptive statistics for their high-throughput sequencing (read number, mapping statistics etc...).

We have assembled the requested statistics below for the reviewer's inspection. Since the values are all very similar, and given the exploding size of this manuscript and all its supplements, we have decided to mention only ranges in the methods section; as the data are available on GEO, readers interested in greater detail will be able to analyze them further.

PolII ChIP:

sampleID	mapped	unmapped	nb_reads	pMap
22h_polII	28782441	8609489	37391930	76.975
23h_polII	28153861	7959761	36113622	77.959
24h_polII	28472313	6478638	34950951	81.464
25h_polII	27072512	6461233	33533745	80.732
26h_polII	32678638	9237332	41915970	77.962
27h_polII	33240263	8332927	41573190	79.956
28h_polII	32673482	9621034	42294516	77.252
29h_polII	25183108	6393996	31577104	79.751
30h_polII	27632013	8108343	35740356	77.313
31h_polII	26258403	6304221	32562624	80.64
32h_polII	25058360	6666576	31724936	78.986
33h_polII	24029567	6653083	30682650	78.316

RNA-seq

sampleID	mapped	unmapped	nb_reads	pMap
22h	39339505	15634285	54973790	71.56
23h	39685931	10771926	50457857	78.652
24h	37724336	9632440	47356776	79.66
25h	36332352	8348102	44680454	81.316
26h	33122224	8247988	41370212	80.063
27h	22297229	18627229	40924458	54.484

28h	36686412	9253598	45940010	79.857
29h	34065659	11968228	46033887	74.001
30h	33130567	7836597	40967164	80.871
31h	34011574	8073678	42085252	80.816
32h	35353707	8876359	44230066	79.931
33h	34851143	9132066	43983209	79.237

In figure 1C, it would be helpful to the reader to write peak phase and amplitude of the tested genes on the graph.

Thank you for this suggestion, we have added this information.

In the same figures, for gene F58H1.2, for which the correlation between the reporter and the endogenous gene is not perfect, the authors "suspect [...] that the reporter may lack relevant promoter or intronic enhancer elements". An alternative explanation is that post-transcriptional regulation occurs for this mRNA, a hypothesis which should be added to the text.

We now mention this possibility.

Reviewer #2 (Significance (Required)):

As stated above, this manuscript highlights convincingly that GRH-1 is involved in the molting cycle in the nematode C. elegans, a conserved function of the gene between evolutionary distant species for skin biology.

Field of expertise: C. elegans, high-throughput sequencing methods, imaging.

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

Summary:
The manuscript describes how a rhythmically active transcription factor is important for molting cycles.
The first part of the manuscript focuses on oscillating genes, and the authors nicely show a rhythmic transcription of these genes. Indeed, using RNAPolII Chip-Seq experiments, they show a rhythmic recruitment of the RNAPolII to the promoters of the oscillatory genes they have previously described. They then demonstrate that GFPs driven by the promoter of these oscillating genes, and inserted as a single copy, can very accurately recapitulate the rhythmic transcription of oscillating genes. It is interesting to see the weak impact of introns and 3'UTR on the rhythmic expression.
In the second part of the manuscript, the authors perform an RNAi screen, looking for oscillating transcription factors (TF) important for molting. The goal of this approach is to identify core oscillators that could control molting cycles. Focusing their screen on

oscillating TF allows them to exclude TF that would be required for embryonic or larval viability unrelated to molting. Among the 6 candidates they have identified, 5 have already been linked to molting. They focus their study on grh-1, which has never before been described during larval development. They characterize the molting phenotype of grh-1 defective worms using the AID degron system. They monitor the molting cycles using a luciferase assay in a liquid culture where transgenic worms for luciferase are grown in the presence of bacteria supplemented with luciferin. Using this approach (which is quantitative and allows high-throughput analysis), they show that grh-1 is required for each molt in a dose-dependent manner. The GRH-1 protein oscillates and peaks shortly before each molt entry, reinforcing the idea that GRH-1 is an important core TF for molting. The authors finally show that the oscillating activity of GRH-1 is crucial for the molting.

Major comments:
Overall, the data are clear and convincing, and the results are quantified with care. The first part of the manuscript represents a significant amount of work with the RNAPolII Chip-Seq, the different single-copy integrants and RT-PCRs. Then, the authors provide a quantitative assessment of the molting process by combining their grh-1-AID construct with the luciferase system. This strengthens the quality of the manuscript.

My substantial suggestion is that the authors could consider extending the scope of the study in two alternative ways:

We thank the reviewer for his/her strong endorsement of our work. We are also in complete agreement with her/him on future research directions but like her/him consider these extensions. We have been able to follow one of these suggestions by providing novel experimental data on GRH-1 targets, while others were outside the scope of the current manuscript and a revision.

- One question that immediately springs to mind is: what are the targets of grh-1? By applying their luciferase assay to further possible downstream targets of grh-1, they could attempt to phenocopy the grh-1 molting defect, and then look if the oscillating expression of these targets is eliminated in grh-1 defective animals. The binding site of grh-1 is apparently known (Venkatesan, 2003), so is it possible to reduce the potential target list among the oscillating genes using a bioinformatics approach? This requires a substantial amount of work (2 to 3 months) but it would help tell a more complete story.

This is indeed a very interesting question, also raised by reviewer #1. Although the reviewer pointed out that this would be an extension of our work rather than data required to support it, we sought to obtain some insight into this matter. Unfortunately, we found that the published motif has a low information content, since it is built on only ~15 sequences, hence yielding ~17,000 hits. Therefore, and as a better predictor of functional interaction, we performed ChIP-seq analysis on endogenously GFP-tagged GRH-1 (new Figures 8, EV5, S7). We found that peaks were enriched for a motif similar to that of the orthologous mammalian GRHL1/2 proteins and the in vitro motif. We find that targets appear enriched in a phase window before molt entry, thus agreeing both with the accumulation pattern of GRH-1 protein and the period of sensitivity to GRH-1 depletion. By contrast, genes peaking in antiphase were depleted for GRH-1 binding. We interpret this as additional support for

rhythmic GRH-1 activity. Finally, we find that GRH-1 binds to its own promoter as well as those of additional screen hits, tentatively supporting its placement in a gene regulatory network generating oscillations.

- It is striking that myrf-1 and nhr-23 RNAi display the same molting defects as grh-1 RNAi as show in figure 2B. Have the authors considered testing the genetic interactions of grh-1 with these two other candidates? Do they belong to the same GRN? Does grh-1 depletion impact the expression of the nhr-23 and myrf-1, or vice versa? Do they have the same target genes (Chip-seq data for nhr-23 are available)? This, again, would significantly strengthen the paper and would make the second part more complete. This alternative piece of work would require less experiments than the first suggestion but would be also of great interest.

We would like to clarify that contrary to the reviewer's impression, the phenotypes observed for these genes are only superficially similar (failure to progress through all four molts). Closer inspection of animals by microscopy reveals differences: GRH-1-depleted animals die by bursting through their heads (Figure 3), NHR-23-depleted animals remain entrapped in a partially shed cuticle (Kostrouchova et al., Development 1998, Johnson et al., bioRxiv 2021), MYRF-1-depleted animals fail to ecdyse and the cuticle remains particularly attached to their mouths (our unpublished observations).

Hence, it is not evident that these factors would act through the same target genes, and we have not explored this further. Nonetheless, we agree with reviewer view that (some of) the screen hits could belong to the same GRN (since there is a priori no assumption that different perturbations of nonlinear interaction networks, required to generate oscillations, will result in similar phenotypes). Indeed, our GRH-1 ChIP-seq data provide some preliminary support for this notion, allowing us to discuss this now in the manuscript. Since the dynamic nature of the genes' expression and the lethality phenotypes make explicit experimental testing challenging, further validation will, however, await future experimental work.

These two points are only suggestions as it represents a significant amount of work, and the paper could very well be published in its current form.

We thank the reviewer for emphasizing that these are extensions to the current work, not requirements for publication.

About the luciferase assay for grh-1, nhr-23 and myrf-1 RNAi, the authors observe "an apparent arrest in development or death following atypical molts". What do they mean by "atypical molt" at this stage of the paper? Indeed, for these candidates, the luminescence traces are highly perturbed after the second molt (for grh-1 and nrh-23 RNAi) or the third molt (for myrf-1), but these abnormal traces seem to reflect an arrest in development or larval lethality rather than an atypical molting. Can the authors clarify this point?

We apologize for the lack of clarity. Briefly, we meant to say exactly what the reviewer observed, highly perturbed luminescence traces and specifically molt durations extended beyond the wild-type duration of 1.5-2hr. For GRH-1, we then characterize in Figure 3 what "atypical" means (namely bursting through the head cuticle). We have now revised the text to clarify this.

In the part on the phenotypic analysis of GRH-1 depleted animals, the authors conclude the paragraph with "GRH-1 is required for viability at least in part through its role in proper cuticle formation". This role in proper cuticle formation refers to the cuticle break in the head region as observed in time lapse. It would be useful to have a visual test of the cuticle permeability using an Hoechst staining.

This is an interesting suggestion although we note that a role in cuticle formation does not necessarily imply a defect in cuticular barrier function during intermolt. That said, we have now examined cuticle integrity using a sensor (*nlp-29::gfp)* previously used by others for this purpose (Johnson et al., bioRxiv 2021), which we find upregulated in GRH-1-depleted animals. Additionally, we have examined luciferase signal in *grh-1::aid* animals during molts (when signal is low in wild-type animals, because the substrate luciferin cannot be ingested and does not penetrate the cuticular barrier) under low auxin conditions and find it to be moderately elevated. These results are shown in the new Appendix Figure S2.

The authors show GFP::GRH-1 pictures at different stages to describe a rhythmic protein accumulation (see also my minor comment on GFP picture quality). From the perspective of whether all tissues are oscillating, it would be interesting to see if all the cells they mention in the text are showing the same rhythmic fluorescence.

We agree that this is an interesting question, but we have made no claims concerning the spatial extent of oscillations, which is currently unknown. Hence, we consider addressing this issue outside the scope of the current manuscript. Indeed, our current time-lapse imaging set-up does not provide the spatial resolution that we would need to address it. We are working on building better tools and analytic approaches, but these are currently not available.

In relation to the previous comment, which tissue is responsible for the defects observed by the degradation of GRH-1? Is it possible to use a tissue-specific depletion of AID-tagged GRH-1 using Seam-cell specific, rectal cell specific, vulval precursors specific promoters, etc...?

Yes, we expect that this will be feasible in future work, but for the reasons above, consider addressing this question outside the scope of the current work.

In the last part of the results, the authors show that molting requires oscillatory GRH-1 activity by depleting GRH-1 at variable times in L2. It would be interesting to know what happens if a stable (non-oscillating) amount of GRH-1 protein is maintained over time in the worms (using a non-oscillating promoter).

We share this reviewer's curiosity concerning these experiments and have therefore done some exploratory experiments, which, unfortunately, revealed that we cannot address this point with currently available technology: as the *grh-1* gene is large, we need to perform these manipulations using cDNA-based transgenes. When we tried this, we were unable to fully rescue *grh-1(0)* mutant animals with either one of the two *grh-1* isoforms. This could be for technical reasons (e.g., splicing is needed to boost expression, or to obtain the

relevant delays (e.g., doi.org/10.1073/pnas.1014418108)), or because indeed both isoforms are needed, but either way, this finding complicates such analyses. Separately, in *C. elegans*, we currently lack a good inducible expression system. The widely used heat-shock promoters are unsuited for our purpose because the period of development and oscillation is temperature-dependent, and we would not be able to disentangle real from confounding effect. Finally, we would need to have appropriate control of expression levels and spatial patterns (i.e., both would have to match endogenous expression), which we cannot currently obtain. Hence, although we absolutely agree on the benefits of this experiment, it is currently not feasible. We now discuss this limitation in the manuscript.

Minor comments:
In figure 5 B, C, D, it seems that right before entering the M2, M3 and M4 respectively, there is a peak of luminescence (a thin bright line) and a strong luminescent signal is detected at the molt exit. Can the authors comment on that?

Based on the experiments from Figure 3, the peak at molt exit likely reflects the rupturing of the animal (which increases cellular luciferin uptake because the cuticular barrier disintegrates). We do not know the meaning or cause of the smaller peak at molt entry, but it may similarly indicate some defect in cuticular barrier function.

If I understand correctly, for the GRH-1 GFP CRISPR reporter (Fig S7, S8 and S9), the authors have imaged single worms in microchambers on a spinning disk microscope. I fail to see why they used such a sophisticated approach to describe the expression pattern of GRH-1. This imaging setup is ideal for timelapse. However, in the context of which cell express GRH-1, the resolution is not good enough to fully assess cell identity. Indeed, the GFP images are a bit blurry, and it is difficult to make out the difference between real GFP fluorescence and gut autofluorescence. It would be helpful to have better quality pictures with a more regular setup, i.e., a 2% agarose pad mounted on a regular microscope or confocal. For non-specialists of the C. elegans anatomy, small insets for each category of cells mentioned (seam cells, vulval precursors etc.) would be appreciated.

We apologize for our lack of clarity, these were indeed epifluorescences images acquired on mounted animals, not time points from the time-lapse imaging experiments. We have now acquired better images using confocal microscopy and remade the new figures EV4 (embryos and larvae) and S5 (comparison larvae – adults).

It would be easier to assess the GRH-1 expression decrease in adults if the pictures were shown in parallel with the larval pictures, with the same brightness/contrast correction (if any). Make insets to compare the same cells between different stages.

We thank the reviewer for this suggestion, which we followed; this is now show in the new Appendix Figure S5.

How can the authors quantify the duration of molts 3 and 4 in fig S4 when these molts are not seen in the luciferase assay in fig 2B? Can the authors clarify this point?

The figure shows numbers for animals that go through all for molts (*nhr-25, blmp-1, bed-3)* and not those that fail to include certain molts. This was indicated in the figure caption "Quantification of molt and intermolt duration in screen hits that complete development" (emphasis new).

Writing/clarity:
For non-specialists, mention why the authors used a PEST sequence in their constructs and explain what the eft-3 promoter is (they mention it in the Luciferase assay, but it is not clear enough).

We now explain this in the methods section.

In Fig S4, make clearer that EV = MOCK.

We have now revised all figures to consistently use "mock" as a label in figures employing RNAi, explaining in the legend that this refers to the "empty vector L4440"

In the methods, the authors refer to the we146 mutant strain, but they neither use it nor mention it in the body of the text. Producing such a mutant strain is great and it should be mentioned in the results, with an explanation as to why they are dying. Otherwise, it should be removed from methods.

We have removed the strain since we have not examined it any further.

In the Methods, the genotype of the strains is misleading. For example HW1372 : EG6699; xeSi... is not the regular way to write a C. elegans genotype. It should be written as: HW1360 xeSi131[F58H1.2p::GFP::H2B::Pest::unc-54 3', unc-119+] II; unc-119(ed3) III as the strain used to generate the MosSci insertion is described in the paragraph on Transgenic reporter strain generation.

We have fixed this.

For the GFP CRISPR strain, the authors write either GFP::GRH-1 in Fig S7, S8 and S9, grh-1::gfp::3xflag in the methods or GRH-1-GFP fusion in the results. The authors should homogenize the way they write this reporter strain. Whether it is an Nterminal or a Cterminal fusion will determine how they should label it.

The reviewer is of course correct. We thank her/him for spotting our mistake, which we have fixed.

Enlarge the font size for Fig S8 and 9 for the scale bar.

We have fixed this by removing the label altogether and providing the information instead in the figure legend.

Reviewer #3 (Significance (Required)):

The author's prior analysis (Meeuse, et al 2020) showed that mRNA oscillations are coupled with developmental processes, including molting. The present paper extends that finding by showing that oscillating transcript levels are directly linked to a rhythmic recruitment of the RNAPolII on their promoter. Then Meeuse and her colleagues use the molting as a model system to access the importance of oscillating TF for rhythmic processes. Through an RNAi screen, they have identified 6 candidates involved in the molting process. One of the candidates, grh-1, is characterized further. They combine a quantitative-based analysis (luciferase assay) with a time and dose-controlled degradation of GRH-1 to clearly describe the impact of grh-1 depletion on molting. This time and dose control is very smart and key to their study.
Overall, the paper adds some interesting piece of information to the field of rhythmic control of molting cycles, as it shows that oscillating transcription factors provide a developmental clock in this process. But this notion is not completely new, as it has been shown in other developmental processes like the circadian clock. Moreover, how molting cycles are controlled by GRH-1 remains to be elucidated.

Here, we would beg to differ with the reviewer. The circadian clock is not a developmental clock, and, a priori, we cannot assume that principles of that oscillator (which syncs to external geophysical cycles) also pertain to other genetic oscillators. They are clearly very different entities: one, the circadian clock, is a resonator with a fixed period, the other an integrator with a tunable period but a fixed amplitude. Indeed, although the reviewer is correct that transcription factors function at the heart of the circadian clock, as we state in our introduction "Counter-intuitively, rhythmic mRNA accumulation in the mammalian circadian clock appears to rely chiefly on co- and post-transcriptional mechanisms, including rhythmic splicing (Koike et al., 2012; Menet et al., 2012; Preußner et al., 2017)." This point notwithstanding, the temporal control of *C. elegans* molting is quite poorly understood and our findings provide a major advance over the current state of knowledge, particularly with the newly added GRH-1 ChIP-seq data.

My field of expertise is GNRs studies, the genetic of C. elegans, embryonic and larval development in C.elegans, timelapse and confocal imaging. I do not have expertise in Chip-Seq analysis.

Thank you for submitting your revised manuscript to The EMBO Journal. I again apologize for the delay, as mentioned, this was due to delayed referee responses and reports. We have now however received their comments and I am happy to say that we can proceed towards publication of your study in The EMBO Journal. Therefore I would ask you to please resolve a number of editorial issues that are listed in detail below. Please use the document that the data editors have added their comments to for any changes (see below). If you have any further questions regarding the specific points listed or the final manuscript version, please feel free to contact me. Once these final issues are resolved we will be happy to formally accept the manuscript for publication.

Referee #1:

I already provided my (enthusiastic) opinion about the manuscript's design, quality and finding in my original evaluation report to Review Commons so here I restrict myself to point out the improvements of the manuscript.
I wish to congratulate the authors on their revised manuscript and acknowledge their efforts to identify directly GRH-1 target genes by ChIP-seq (included as new Figure 8 and supplementary data). I am satisfied with the responses of the authors to the comments raised by the three reviewers and I highly recommend the study to be published.

Referee #2:

The authors have addressed all my requests from the first round of reviews, strengthening the conclusions. The paper should therefore be accepted in its revised form.

Referee #3:

The authors have done a thorough job of responding to reviewers' requests to clarify the manuscript.
As suggested by Ref #1 and 3, the authors have now tackled the issue of GRH-1 targets head-on using ChIP-seq analysis on endogenously GFP-tagged GRH-1. They give a very good insight into this question in a new dedicated paragraph at the end of the results section. This opens very interesting perspectives for the future direction of the project.
Overall, I found it very easy to see the improvements made and all the added data addresses the minor/major concerns from the original submission.
Therefore, I recommend publication of the revised manuscript.

All editorial and formatting issues were resolved by the authors.

Thank you again for submitting the final revised version of your manuscript and addressing the remaining points. I am happy to inform you that we have formally accepted your study for publication in The EMBO Journal.

EMBO Press Author Checklist

Corresponding Author Name: Helge Großhans	
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This checklist is adapted from Materials Design Analysis Reporting (MDAR) Checklist for Authors. MDAR establishes a minimum set of requirements in transparent reporting in the life sciences (see Statement of Task: 10.31222/osf.io/9sm4x). Please follow the journal's guidelines in preparing your
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Abridged guidelines for figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- ideally, figure panels should include only measurements that are directly comparable to each other and obtained with the same assay.
- plots include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical
- if n<5, the individual data points from each experiment should be plotted. Any statistical test employed should be justified.
- Source Data should be included to report the data underlying figures according to the guidelines set out in the authorship guidelines on Data

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements.
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.
- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

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Materials

Newly Created Materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
New materials and reagents need to be available; do any restrictions apply?	Yes	**Data and materials availability section**

Antibodies	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
For **antibodies** provide the following information: - Commercial antibodies: RRID (if possible) or supplier name, catalogue number and or/clone number - Non-commercial: RRID or citation	Yes	Materials and Methods

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Short novel DNA or RNA including primers, probes: provide the sequences.	Yes	Materials and Methods

Cell materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Cell lines: Provide species information, strain. Provide accession number in repository **OR** supplier name, catalog number, clone number, and/**OR** RRID.	Not Applicable	
Primary cultures: Provide species, strain, sex of origin, genetic modification status.	Not Applicable	
Report if the cell lines were recently **authenticated** (e.g., by STR profiling) and tested for mycoplasma contamination.	Not Applicable	

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Laboratory animals or Model organisms: Provide species, strain, sex, age, genetic modification status. Provide accession number in repository **OR** supplier name, catalog number, clone number, **OR** RRID.	Yes	Materials and Methods
Animal observed in or captured from the field: Provide species, sex, and age where possible**.**	Not Applicable	
Please detail **housing and husbandry conditions**.	Not Applicable	

Plants and microbes	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Plants: provide species and strain, ecotype and cultivar where relevant, unique accession number if available, and source (including location for collected wild specimens).	Not Applicable	
Microbes: provide species and strain, unique accession number if available, and source.	Not Applicable	

Human research participants	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
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Core facilities	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If your work benefited from core facilities, was their service mentioned in the acknowledgments section?	Yes	Acknowledgements

Design

Study protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If study protocol has been **pre-registered, provide DOI in the manuscript**. For clinical trials, provide the trial registration number **OR** cite DOI.	Not Applicable	
Report the **clinical trial registration number** (at ClinicalTrials.gov or equivalent), where applicable.	Not Applicable	

Laboratory protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Provide DOI OR other citation details if **external detailed step-by-step protocols** are available.	Not Applicable	

Experimental study design and statistics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Include a statement about **sample size** estimate even if no statistical methods were used.	Yes	Material and Methods
Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. **randomization procedure**)? If yes, have they been described?	Not Applicable	
Include a statement about **blinding** even if no blinding was done.	Yes	Material and Methods
Describe **inclusion/exclusion criteria** if samples or animals were excluded from the analysis. Were the criteria pre-established? If sample or data points were omitted from analysis, report if this was due to attrition or intentional exclusion and provide justification.	Yes	For auxin titration, Fig. 4B, C, animals were excluded from analysis as specified in figure legend (i.e., when they did not develop beyond M3, according to data in Fig. 4A)
For every figure, are **statistical tests** justified as appropriate? Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. Is there an estimate of variation within each group of data? Is the variance similar between the groups that are being statistically compared?	Yes	We used a non-parametric Wilcoxon rank sum test for luciferase assay analyses, which does not assume normal distribution of the data

Sample definition and in-laboratory replication	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
In the figure legends: state number of times the experiment was **replicated** in laboratory.	Yes	Figure 1, Figure 8 legends
In the figure legends: define whether data describe **technical or biological replicates**.	Yes	Figure 1, Figure 8 legends

Ethics

Ethics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Studies involving **human participants**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval.	Not Applicable	
Studies involving **human participants**: Include a statement confirming that **informed consent** was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	Not Applicable	
Studies involving **human participants:** For publication of **patient photos**, include a statement confirming that consent to publish was obtained.	Not Applicable	
Studies involving experimental **animals**: State details of **authority granting ethics approva**l (IRB or equivalent committee(s), provide reference number for approval. Include a statement of compliance with ethical regulations.	Not Applicable	
Studies involving **specimen and field samples:** State if relevant **permits** obtained, provide details of authority approving study; if none were required, explain why.	Not Applicable	

Dual Use Research of Concern (DURC)	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Could your study fall under dual use research restrictions? Please check biosecurity documents and list of **select agents and toxins** (CDC): https://www.selectagents.gov/sat/list.htm	Not Applicable	
If you used a select agent, is the security level of the lab appropriate and reported in the manuscript?	Not Applicable	
If a study is subject to dual use research of concern regulations, is the name of the **authority granting approval and reference number** for the regulatory approval provided in the manuscript?	Not Applicable	

Reporting

The MDAR framework recommends adoption of discipline-specific guidelines, established and endorsed through community initiatives. Journals have their own policy about requiring specific guidelines and recommendations to complement MDAR.

Adherence to community standards	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
State if relevant guidelines or checklists (e.g., **ICMJE, MIBBI, ARRIVE, PRISMA**) have been followed or provided.	Not Applicable	
For **tumor marker prognostic studies,** we recommend that you follow the **REMARK** reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	Not Applicable	
For **phase II and III randomized controlled trials**, please refer to the **CONSORT** flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	Not Applicable	

Data Availability

Data availability	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Have **primary datasets** been deposited according to the journal's guidelines (see 'Data Deposition' section) and the respective accession numbers provided in the Data Availability Section?	Yes	Data Availability
Were **human clinical and genomic datasets** deposited in a public access-controlled repository in accordance to ethical obligations to the patients and to the applicable consent agreement?	Not Applicable	
Are **computational models** that are central and integral to a study available without restrictions in a machine-readable form? Were the relevant accession numbers or links provided?	Yes	Data Availability
If publicly available data were reused, provide the respective **data citations in the reference list**.	Not Applicable	