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Global histone H2B degradation regulates insulin/IGF signaling-mediated nutrient stress

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

Dear Guangshuo,

Thank you for submitting your manuscript for consideration by the EMBO Journal. I sincerely apologise for the protracted review process due to the holiday period and delays in referee report submission. We have now received comments from three reviewers, which are included below for your information.

As you will see from the reports, all reviewers find the presented regulation of histone proteasomal degradation in adaptation to nutrient stress of interest. However, they also raise a number of important concerns that would need to be addressed in a revision, in particular asking for further strengthening of the evidence for direct histone ubiquitination and degradation upon starvation, more support for the link between starvation-induced histone turnover and insulin signalling, and more detailed analysis of the specificity of this phenomenon for particular C. elegans cell types, as well as its conservation in mammalian cells.

Based on these broadly positive assessments and the interest expressed by all reviewers, I would like to invite you to address the issues raised by the referees in a revised manuscript. I think it would be useful to discuss the revision in more detail via email or phone/videoconferencing - please let me know which option you prefer.

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, please contact me as soon as possible upon publication of any related work to discuss the appropriate course of action. Should you foresee a problem in meeting this three-month deadline, please contact us to arrange an extension.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: https://www.embopress.org/page/journal/14602075/authorguide#transparentprocess. Please also see the attached instructions for further guidelines on preparation of the revised manuscript.

Please feel free to contact me if you have any further questions regarding the revision. Thank you for the opportunity to consider your work for publication. I look forward to your revision.

With best regards,

Ieva

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We realize that it is difficult to revise to a specific deadline. In the interest of protecting the conceptual advance provided by the work, we recommend a revision within 3 months (4th May 2023). Please discuss the revision progress ahead of this time with the editor if you require more time to complete the revisions.

Referee #1:

In their manuscript 'Global histone H2B degradation regulates insulin/IGF-1 signaling-mediated nutrient stress' the authors describe the phenomenon of histone H2B protein turnover as a response to nutrient deprivation in the model organism Caenorhabditis elegans (C. elegans). The authors propose that histone H2B decrease coordinates appropriate insulin signaling, critical for physiological response to starvation. Further, the authors suggest that a general turnover of histones in an ubiquitin-proteasome-system (UPS)-dependent manner is evolutionarily conserved in mammalian cells. They propose that histone turnover represents an epigenetic mechanism that has not been appreciated.

Initially, the authors describe their striking observation that starvation results in the depletion of histone 2B (H2B) protein levels in C. elegans. Systematic analysis of H2B variants reveals that HIS-41, a unique histone variant (not associated with cell proliferation), emerges as an exception to this rule. Accordingly, HIS-41 protein levels remain stable upon starvation, or even increase. The authors hypothesize that decreased protein levels are resulting from enhanced protein turnover. In support of this, they isolated critical factors of selective protein degradation in a genetic screen for suppressors of histone protein decrease upon starvation. Further, they suggest a specific lysine residue in H2B proteins (K31) to be critical for histone protein depletion. Elucidating the physiological relevance of histone depletion upon starvation, the authors present data correlating histone depletion with a transcriptional response that is mediated through the insulin/IGF1 signaling pathway involving the receptor DAF-2 and the FOXO transcription factor DAF-16. According to their model, failure in histone composition causes an aberrant DAF-16-dependent transcriptional response in turn dampening appropriate response to starvation conditions. Animals with aberrant insulin signaling are sensitized towards impaired UPS-function as well as expression of histone K31A mutants. The manuscript closes with tissue culture data implying that histone turnover is a general phenomenon also observed in vertebrates.

In general, the authors' technical efforts throughout their analyses are well executed. However, the physiological relevance of starvation-mediated histone flux and the insulin signaling pathway remains speculative. While the authors' conclusion about a starvation-dependent reduction in histone protein levels is well supported by the data, the presented data linking H2B turnover to appropriate insulin/IGF-1 signaling remains sketchy. In particular, experimental evidence of histone ubiquitylation and protein degradation under respective conditions is missing. Starvation induced histone turnover on one hand and the insulin/IGF-1 signaling on the other might represent two independent pathways, separately determining response to starvation. It remains unclear how critical H2B composition in particular is during starvation response. Revision of the manuscript to address the concerns described below will make it more relevant to the broad readership of EMBO Journal.
Major concerns:

1) The manuscript does not reveal how nucleosome composition is actually affected in response to starvation or mutant conditions (steady-state levels are analyzed, not nucleosome composition). Moreover, it remains unclear how altered nucleosome composition might affect DAF-16 transcriptional activity. Further, the speculated effect of histone re-composition on the epigenome lacks experimental validation.
Based on the presented data, it is expected that protein levels of presumably all H2B variants will increase in ubc-20 and hecd-1 mutants: How do the authors envision specific changes in nucleosome composition and the epigenome?
2) While HIS-41 protein level are uniquely regulated in response to starvation compared to other H2B proteins, the authors generalize their conclusion to global H2B regulation. Particularly in the context of HIS-41 protein levels it remains elusive

whether nutrient deprivation (and consequently insulin/IGF-1 signaling) is a critical physiologic parameter.
3) The manuscript lacks experimental evidence validating histone ubiquitylation and subsequent protein degradation upon starvation. A possible contribution of mRNA level regulation to the observed decrease in H2B proteins upon starvation is not addressed.

Minor concerns:
4) In Figure 1 the authors show that HIS-41 is the only analyzed H2B protein that does not decrease in abundance upon starvation, it rather increased in protein level. In Figure 2E and 3E the authors show that HIS-41 steady-state levels remarkably increase in ubc-20 and hecd-1 mutants as well as in his-41K31A mutants. It remains unclear why HIS-41 is more stable under starvation conditions while it is supposed to be regulated in a UPS-dependent manner, alike other H2B proteins. The protein levels of HIS-41 upon starvation is unique amongst H2B proteins, but its regulation is anticipated to rely on the same mechanism as HIS-4 (Figures 2E, 3E): how do the authors link this molecular discrepancy with the follow-up physiological analyses of Dauer larvae formation and survival of nutrient deprivation? Why is the increase in both ubc-20/hecd-1 mutants and upon K31A mutation so pronounced under unchallenged conditions (Figures 2E, 3E), if the physiological condition was response towards starvation? These observations suggest that HIS-41 regulation is not critical under starvation conditions (and probably insulin/IGF1 signaling). Further, the effect of neither ubc-20/hecd-1 nor K31A mutation on HIS-41 upon nutrient deprivation has been experimentally addressed. In conclusion, it remains unclear whether HIS-41 level regulation is central to DAF-16-mediated starvation response.
5) Following the argumentation of 4) According to the authors' conclusion, the H2B variant his-41K31A is stabilized, supposed to affect nucleosome composition and consequently DAF-16 activity/recruitment. Figure 6A shows that the stable his-41K31A variant has no effect on DAF-16 recruitment on its own; his-41K31A mimics the WT condition. The same is actually true in ubc-20 and hecd-1 mutants. Both mimic the WT condition, despite the fact that these mutations should disturb H2B levels in fed conditions (Figure 2E, 3E). How do the authors explain the contradictory observation of remarkably increased HIS-41 protein levels in ubc-20/hecd-1 and his-41K31A on the one hand, and WT-like DAF-16 activity on the other if H2B re-composition had a crucial role in DAF-16-mediated response to starvation? Can the others trigger DAF-16 activation in his-41K31A background by other means than daf-2 mutation?
6) A possible contribution of H2B mRNA levels is neglected in the manuscript. One critical experiment should analyze the mRNA levels of histone variants in the isolated UPS-suppressor mutations as well as upon starvation, importantly not in the context of wrt-2 promoter driven transgenes as these are expected to be transcriptionally responsive to nutrient deprivation, as mentioned by the authors (shown in Figure 2D).
7) Throughout the manuscript the authors interpret the reduction of H2B protein levels as a consequence of enhanced protein turnover by the ubiquitin-proteasome-system (UPS). The isolation of mutations in representative UPS-factors might indicate that histone protein turnover plays a role. However, the manuscript does not present any careful analysis validating this conclusion. Direct experimental validation should include, documentation of H2B ubiquitylation and degradation in vivo, including meaningful controls. In order to exclude unspecific enrichment of proteins in the IP reactions, a negative control (e.g. GFP) is essential. Data shown in Figure 3F might be suited to address a direct ubiquitylation of H2B proteins. In these experiments the authors should show the level of poly-ubiquitin also in the lysates as the KOs of UBE2K and HECTD1 are expected to affect general poly-ubiquitylation as well. It appears unclear, why histone variant levels in the cell lysates are not increased in UBE2K-KO and HECDT1-KO cells (and K31A mutant) compared to WT controls. Why are H2Bs subject to poly-ubiquitylation in unchallenged conditions?
8) The authors isolate central factors of the ubiquitin-proteasome system (UPS) as genetic suppressors of H2B decrease. Though the molecular nature of the isolated mutations remains to be elucidated, it is striking that these mutations maintain H2B levels under starvation conditions (at least in the wrt-2 driven transgenes), suggesting loss-of-function or hypomorphic alleles. As all of the isolated mutations either affect a basic step in the ubiquitylation cascade or have documented broad function in protein quality control, it would be interesting how the authors envision specificity towards H2B and more importantly, how they can exclude global effects on protein turnover. In order to elucidate the direct contribution of H2B regulation respective RNAi knockdown in the Dauer formation/larval arrest assays should be included (Figure 4F, 5C). If H2B regulation is the critical determinant in this context, RNAi-mediated depletion of H2B in daf-2; ubc-20 or daf-2; hecd-1 would be expected modulate the phenotype of the double mutants into the respective phenotype of the daf-2 single mutation (increased Dauer formation, decreased larval lethality).
9) The authors interpret the histone exchange assay in mammalian tissue cell culture as a validation of an evolutionarily conserved histone protein turnover by the UPS.
As the incorporation of 'new vs. old' histones will be determined by the proliferative activity of the MG132-treated or UBE2K/HECTDT1-depleted cells the authors need to document the proliferation profile of the respective cell lines. The histone exchange assay does not serve as an appropriate method to determine a direct protein degradation of histone proteins. Furthermore, this data set is interpreted independent of the aforementioned connection to nutrient deprivation in C. elegans. In light of this experiment it should also be considered that UBE2K-KO, HECTD1-KO as well as H2BK31A mutation did not result in increased H2B levels (Figure 3F). Thus, the regulation of H2B levels in C. elegans appears to be distinct in mammalian cells.

Referee #2:

Zhu et al investigate histone H2B turnover during starvation in the nematode C. elegans. They use state-of-the-art reporter

genes to image each endogenous H2B protein, discovering that all but one of them are degraded in starved L1 larvae. The exception, his-41, is a replication-independent histone, apparently in contrast to the others, and they argue that it is subjected to ubiquitination and degradation just like all of the others but that its transcript maintains abundance such that it is continuously translated thus maintaining protein levels so that HIS-41 largely replaces the other H2Bs. They use forward genetics to identify E1, E2, and E3 ubiquitin ligases required for H2B degradation. They use mass spec to identify the amino acid required for ubiquitination as well, and they show that these ligases have analogous function in human cells. They also demonstrate physiological significance in worms by showing that daf-2/Insulin/IGF receptor mutants grown at the restrictive temperature (dauer-forming conditions) die without these ubiquitin ligases. They perform an additional forward genetic screen to suppress lethality under these conditions and identify the very well characterized effector of daf-2 signaling, daf-16/FOXO. They show that the "starvation response" is misregulated in the absence of the ubiquitin ligases, arguably accounting for lethality, and they show that mutation of daf-16 reverses this misregulation. They use ChIP-seq to further show that DAF-16 and H2B do in fact colocalize in chromatin and that lack of the ubiquitin ligases or lack of ubiquitination increases retention of H2B as well as DAF-16, providing mechanistic explanation for misregulation. The paper includes a lot of high-quality data (~20 CRISPR edits analyzed by imaging and western blot, 2 forward genetics screen each yielding valuable and insightful mutants, RNA-seq and ChIP-seq in a variety of genetic backgrounds, experiments in human cells to show conservation, etc), and it is generally well-written, clearly presented, and without overstated conclusions. This work is interesting, significant, and timely given that the function and regulation of individual histones is generally ignored though consequential, and because nutritional control of chromatin structure is understudied.

Major concerns

The authors provide very nice mRNA-seq and ChIP-seq analyses in Fig 5 and 6. However, these two analyses are not as well integrated as they could be, which has the potential to strengthen their conclusions. They provide genome browser shots for a pair of representative genes in Fig 6, but it is unclear how representative the correlations between the two data types revealed here are. In fact, they point out that not all genes with increased DAF-16 binding display altered expression, and they present a pair of representative genes for this in Fig S4. How many genes fit each pattern? Is it more common that expression is affected when binding is? The authors should present the overlaps (and their statistical significance) for the differentially expressed genes identified by RNA-seq and the differentially bound genes identified by ChIP-seq. This could be done with arbitrary cut-offs to define gene lists and venn diagrams, or the authors could consider scatter plots of fold-changes (potentially color coded by significance). A hybrid approach is also possible, where a discrete list is generated by one data type (eg, RNA-seq) and that gene set is plotted along with the set of all detected genes as a cumulative distribution of fold-changes in the other data type (eg, ChIP-seq). There may be other ways to satisfy this as well.

The demonstration that HIS-58/H2B is also degraded in epidermal seam cells upon asymmetric division in fed, developing larvae (in addition to starved larvae) is interesting. Notably, this is seen in the anterior daughter which fuses with the syncytium and ceases proliferation, as opposed to the posterior daughter which remains as a blast cell that will divide asymmetrically again in the next larval stage. As for their cell culture work, I'm thinking about cellular quiescence here. This piece would be strengthened if the authors could demonstrate that HIS-58 is replaced by HIS-41/H2B in the anterior daughter, analogous to what they've shown for starved larvae.

The authors refer to some histones as replication-dependent and others as replication-independent, and they cite Roberts, Sanicola et al 1987 (p-5; with mention of this again on p-7 with respect to his-4 and his-41 and possibly elsewhere). This is a very important distinction given their findings and overall model of histone turnover and replacement in fed vs. starved conditions. However, it isn't clear to me how this classification is drawn from the cited paper or what other, perhaps more recent data pertaining to transcription/mRNA abundance it is based on.

Minor concerns

It is my understanding that DAF-2 is homologous to mammalian insulin receptor and IGF receptor. We therefore refer to insulin/IGF signaling in worms. This is the case in some places, but not all, as if insulin and IGF signaling are synonomous. For example, at the bottom of p-3, "reduced insulin signaling enables DAF-16 to translocate to the nucleus". And on p-8, "DAF-2 encodes the human ortholog of insulin-like growth factor 1 receptor (IGF1R), and the daf-2(e1370) allele is a temperature-sensitive mutant with reduced insulin receptor activity."

On p-10, this finding is reported: "Previous RNAi experiments revealed that inhibiting 172 genes among those downregulated ones caused embryonic or larval lethality (Supplemental Table S6), which suggests that the aberrant transcription program may account for the double mutant phenotypes." Does this overlap reflect a statistically significant enrichment of genes with this phenotype in this gene set? The authors should perform a hypergeometric test to compute a p-value for this (as is done for GO term analysis).

The authors offer this conclusion on p-10: "These findings indicate that aberrant H2B retention induced ectopic DAF-16-dependent transcription that causes animal lethality." Because they have not directly demonstrated that ectopic DAF-16 activity

at these loci results in lethality the conclusion is stated too strongly with "indicate" and something like "suggests" is more appropriate.

Fig 4C and 5E,F: The authors should provide hypergeometric p-values demonstrating that these overlaps are statistically significant.

Suggestions to improve clarity

Abstract: "ligating enzymes *that* blocked"

Throughout: "chromatin" rather than "chromatins"

Top of p-5: "the rest *of the* strains showed the expected"

Twice near the bottom of p-6: "point mutations or early stop codons" - stop codons can result from point mutations.

p-13: "these findings reveal the widespread *nature* of the"

Referee #3:

The manuscript submitted by Zhu et al. is a thoroughly detailed analysis of a newly described process that involves the regulated turnover of histone proteins in response to physiological (starvation/quiescence) and/or developmental conditions. The turnover is dependent on specific ubiquitylation of lysine residues on many (replication dependent) H2B and H4 variants resulting in their subsequent removal and degradation. They have effectively used multiple approaches to provide a detailed understanding of the histone targets, the genes involved and how this might occur in specific cell types, including human cells.
The writing was generally very good to excellent and both the logic and the interpretation of most of the experiments was solid. Although I was mostly impressed with the quality of this manuscript I did have a few issues that may or may not be important for the authors to consider during their revision. Namely, it is not clear why they were examining the hypodermal lineage nor is it clear whether, in addition to starvation, this process is regulated in any other physiological or perhaps more relevant to the final experiment, during any other developmental contexts. The V cell lineage is already a bit particular due to the uncommon fates of the the daughter cells following the first division; one joins a syncytium (Vn.a) and the other (Vn.p) adopts stem cell-like properties. In Vn.a the histones are turned over while in Vn.p they are not. Does this happen in other asymmetrically dividing lineages where the cell fates of the daughters do not have the same dramatic outcomes. The QR divisions would suggest that this does not always happen, but I am not sure if QR.a and QR.p maintain similar fates. Perhaps examining the first Pn.p cell division or even the P cells themselves might complete this final experiment better and answer this question more thoroughly. The P cells are still hypodermal, but the outcomes are not as dramatically different as the V daughters. Alternatively, perhaps examining an M cell division or another asymmetric division might enhance this part of the analysis.
Keeping with this same concern, the SILAC experiment described on Page 12 and in Figure X is intriguing and the choice of using a confluent culture is quite appropriate, since replication should be halted and a quiescence program is initiated. However, with only one condition it is hard to know if the turnover that is observed is indeed regulated in a quiescence- dependent manner or whether it is simply homeostatic histone replacement. The authors very appropriately point out this caveat, however, It would be more informative if the experiment was also repeated with subconfluent cultures or a continously growing population of cells as a kind of control, to better understand if this process is part of general cellular homeostasis or whether is is regulated in a quiescence-dependent manner.
To complete this study it would also be informative to test if the orthologues of the genes that were identified in the initial genetic screen also contribute to the turnover of these histones in mammalian cells.
On page 14 in the Discussion, the authors speculate that the levels of HIS-41 may compariatively high and this could explain the observed changes. This should be easily testable simply by measuring the levels of HIS-41 at different life stages of the growing animal and comparing to that of the other histones.
During my reading I am came across a number of small grammatical/typo errors that should be addressed to further imporve the text of the manuscript. I have listed them in the lines below.

in the Abstract- ...uncovered "mutants"defective in....
"chromatin", not chromatins (change this throughout the manuscript)
Page 6- Progeny (not progenies); that retained (not retaining); conditions of starvation or during starvation (not starvation

condition-this might be changed throughout the manuscript as well)
Page 7-has been far less explored (not was); remove "the" from transfected human HEK293T cells
Page 8-DAF-2 is not a protein in this case is should be written as a gene daf-2; executed the dauer stage (not survived by entering..); all died on the third day following the shift to 25˚C (remove "exposed")
Page 9-the section concerning H4 K31 reduncancy is confusing. Could this section be re-worked to clearly specify why H4 woud be redundant in the first place.; 71 mutations that bear mutations in...
Page 10-the paragraph indicating that 172 of the identified genes that were in the downregulated subset were previously shown to be embryonic or larval lethal when compromised by RNAi could be written as such...
Page 13-Decorating histones at multiple locations around the histone core
Page 14-their abnormally high basic amino acid content

RESPONSE TO THE REVIEWERS

Referee #1:

In their manuscript 'Global histone H2B degradation regulates insulin/IGF-1 signaling-mediated nutrient stress' the authors describe the phenomenon of histone H2B protein turnover as a response to nutrient deprivation in the model organism Caenorhabditis elegans (C. elegans). The authors propose that histone H2B decrease coordinates appropriate insulin signaling, critical for physiological response to starvation. Further, the authors suggest that a general turnover of histones in an ubiquitin-proteasome-system (UPS)-dependent manner is evolutionarily conserved in mammalian cells. They propose that histone turnover represents an epigenetic mechanism that has not been appreciated.

Initially, the authors describe their striking observation that starvation results in the depletion of histone 2B (H2B) protein levels in C. elegans. Systematic analysis of H2B variants reveals that HIS-41, a unique histone variant (not associated with cell proliferation), emerges as an exception to this rule. Accordingly, HIS-41 protein levels remain stable upon starvation, or even increase. The authors hypothesize that decreased protein levels are resulting from enhanced protein turnover. In support of this, they isolated critical factors of selective protein degradation in a genetic screen for suppressors of histone protein decrease upon starvation. Further, they suggest a specific lysine residue in H2B proteins (K31) to be critical for histone protein depletion. Elucidating the physiological relevance of histone depletion upon starvation, the authors present data correlating histone depletion with a transcriptional response that is mediated through the insulin/IGF1 signaling pathway involving the receptor DAF-2 and the FOXO transcription factor DAF-16. According to their model, failure in histone composition causes an aberrant DAF-16-dependent transcriptional response in turn dampening appropriate response to starvation conditions. Animals with aberrant insulin signaling are sensitized towards impaired UPS-function as well as expression of histone K31A mutants. The manuscript closes with tissue culture data implying that histone turnover is a general phenomenon also observed in vertebrates.

In general, the authors' technical efforts throughout their analyses are well executed. However, the physiological relevance of starvation-mediated histone flux and the insulin signaling pathway remains speculative. While the authors' conclusion about a starvation-dependent reduction in histone protein levels is well supported by the data, the presented data linking H2B turnover to appropriate insulin/IGF-1 signaling remains sketchy. In particular, experimental evidence of histone ubiquitylation and protein degradation under respective conditions is missing. Starvation induced histone turnover on one hand and the insulin/IGF-1 signaling on the other might represent two independent pathways, separately determining response to starvation. It remains unclear how critical H2B composition in particular is during starvation response.

Revision of the manuscript to address the concerns described below will make it more relevant to the broad readership of EMBO Journal.

We are grateful to the reviewers for providing constructive comments and supporting the potential publication of our paper in the EMBO journal.

Major concerns:

1) The manuscript does not reveal how nucleosome composition is actually affected in response to starvation or mutant conditions (steady-state levels are analyzed, not nucleosome composition). Moreover, it remains unclear how altered nucleosome composition might affect DAF-16 transcriptional activity. Further, the speculated effect of histone re-composition on the epigenome lacks experimental validation. Based on the presented data, it is expected that protein levels of presumably all H2B variants will increase in ubc-20 and hecd-1 mutants: How do the authors envision specific changes in nucleosome composition and the epigenome?

We fully agree with the Referee's assessment. As is commonly acknowledged, isolating cells of the same type from *C. elegans* is a considerable challenge, making it a daunting task to accurately depict the legible nucleosome composition and chromatin landscape in a specific region. Our imaging and biochemistry data demonstrate the replacement and degradation of all H2B variants during development and starvation stress (Fig. 1, 8 and S8). Notably, the replication-dependent *his-4* was largely replaced by the replication-independent *his-41* in adult and starved animals. Therefore, we speculate that the composition of chromatin has undergone tremendous changes and acknowledge that understanding specific changes of nucleosome composition and the epigenome requires the development of advanced methodologies. We have discussed these issues in the revised Discussion on page 16, line 2:

"Isolating cells of the same type from *C. elegans* is a considerable challenge, making it a daunting task to accurately depict the legible nucleosome composition and chromatin landscape in a specific region. Nevertheless, our imaging and biochemistry data demonstrate the replacement and degradation of all H2B variants during development and starvation stress (Fig. 1, 8 and S8). Notably, the replication-dependent *his-4* was largely replaced by the replication-independent *his-41* in adult and starved animals, which suggests that the composition of chromatin has undergone tremendous changes. Future studies will develop tools to understand specific changes of nucleosome composition and the epigenome in *C. elegans*."

2) While HIS-41 protein level are uniquely regulated in response to starvation compared to other H2B proteins, the authors generalize their conclusion to global H2B regulation. Particularly in the context of HIS-41 protein levels it remains elusive whether nutrient deprivation (and consequently insulin/IGF-1 signaling) is a critical physiologic parameter.

We thank the Referee for pointing out this issue and agree with the Referee for the suggestion. We discussed this issue in the revised Discussion on page 16, line 20:

"…Although all the examined replication-dependent H2B proteins degrade, HIS-41 protein level is uniquely regulated in response to starvation, making it inappropriate to generalize the global H2B degradation. In particular, in the context of HIS-41 protein levels, it remains elusive whether nutrient deprivation and consequently insulin/IGF signaling is a critical physiologic parameter. Indeed, we cannot exclude the possibility that the others may trigger DAF-16 activation in the *his-41K31A* mutant background by other means than *daf-2* mutation."

3) The manuscript lacks experimental evidence validating histone ubiquitylation and subsequent protein degradation upon starvation. A possible contribution of mRNA level regulation to the observed decrease in H2B proteins upon starvation is not addressed.

We apologize for lacking the direct data. As suggested by the Referee, we performed RNA-seq and data analysis to determine whether the transcriptional regulation of histone genes contributes to the observed decrease in H2B proteins upon starvation. In *wild-type*, the *his-4* was expressed with ample food and sharply decreased upon starvation stress, but the expression of *his-41* mRNA level remained unchanged during starvation. Importantly, the depletion of UBC-20 and HECD-1 did not alter the transcriptional pattern of *his-4* and *his-41* mRNA upon starvation stress. These results suggest that UBC-20 and HECD-1 regulate H2B degradation at the protein level. We have included the new results in the revised text on page 7, line 18:

"…Furthermore, we performed RNA-seq and data analysis to examine a possible contribution of mRNA level regulation to the observed decrease in H2B proteins upon starvation. In *wild-type*, the *his-4* was expressed with ample food and sharply decreased upon starvation stress, but the expression of *his-41* mRNA level remained unchanged during starvation (Fig. S2G). Importantly, the depletion of UBC-20 and HECD-1 did not alter the transcriptional pattern of *his-4* and *his-41* mRNA upon starvation stress (Fig. S2G)…"

Minor concerns:

4) In Figure 1 the authors show that HIS-41 is the only analyzed H2B protein that does not decrease in abundance upon starvation, it rather increased in protein level. In Figure 2E and 3E the authors show that HIS-41 steady-state levels remarkably increase in ubc-20 and hecd-1 mutants as well as in his-41K31A mutants. It remains unclear why HIS-41 is more stable under starvation conditions while it is supposed to be regulated in a UPS-dependent manner, alike other H2B proteins. The protein levels of HIS-41 upon starvation is unique amongst H2B proteins, but its regulation is anticipated to rely on the same mechanism as HIS-4 (Figures 2E, 3E): how do the authors link this molecular discrepancy with the follow-up physiological analyses of

Dauer larvae formation and survival of nutrient deprivation? Why is the increase in both ubc-20/hecd-1 mutants and upon K31A mutation so pronounced under unchallenged conditions (Figures 2E, 3E), if the physiological condition was response towards starvation? These observations suggest that HIS-41 regulation is not critical under starvation conditions (and probably insulin/IGF1 signaling). Further, the effect of neither ubc-20/hecd-1 nor K31A mutation on HIS-41 upon nutrient deprivation has been experimentally addressed. In conclusion, it remains unclear whether HIS-41 level regulation is central to DAF-16-mediated starvation response.

We apologize for any confusion caused. We conducted a H2B dosage analysis during starvation by genetically crossing GFP::HIS-4 KI into *ubc-20(cas916)* and *hecd-1(cas908)* mutants. We synchronized the worms and subjected them to six days of starvation before imaging them using an identical setup. Our new results show depletion of UBC-20 and HECD-1 significantly inhibited GFP::HIS-4 degradation during starvation (Fig. S2E-G). In addition, we found mutations in *his-41K31A* also inhibited their own degradation during starvation (Fig. S3C-E). Combined with our previous data (Fig. 2E and 3E), these results indicate that UBC-20, HECD-1, and H2BK31 collectively regulate H2B degradation during development or starvation.

We have explained new results in the revised text on page 7, line 13:

"…In addition, we conducted a H2B dosage analysis during starvation by genetically crossing GFP::HIS-4 KI into *ubc-20(cas916)* and *hecd-1(cas908)* mutants, and synchronized the worms and subjected them to six days of starvation before imaging them using an identical setup. We show that depletion of UBC-20 and HECD-1 significantly inhibited GFP::HIS-4 degradation during starvation (Fig. S2E-F)..."

We have explained new results in the revised text on page 8, line 9:

"…In addition, mutations in *his-41K31A* inhibited their own degradation during starvation (Fig. S3C-E)..."

The stability of HIS-41/H2B appears to be higher compared to other H2B variants due to the cooperative effect of transcriptional regulation and protein degradation during development and starvation stress. Unlike the other H2B variants, *his-41* mRNA exhibits continuous expression. As suggested by the Referee in the Point #8, we have performed additional RNAi experiments of *his-41* to show HIS-41 level regulation is involved in DAF-16-mediated pathway. We have included the new data in the revised text on page 9, line 17:

"…To elucidate the direct contribution of H2B regulation in the dauer formation or larval arrest, we conducted RNAi experiments in the *daf-2*; *daf-2, ubc-20;* and *daf-2, hecd-1* mutant animals. RNAi against *his-41*/H2B did not reveal novel phenotypes in the *daf-2* background. Importantly, knockdown of *his-41*/H2B partially suppressed

larval lethality and rescued dauer formation in *daf-2, ubc-20* and *daf-2, hecd-1* (Fig. S4A). These results suggest that maintaining appropriate levels of histone H2B is critical for animal development and survival under the conditions of reduced insulin/IGF receptor activity."

5) Following the argumentation of 4) According to the authors' conclusion, the H2B variant his-41K31A is stabilized, supposed to affect nucleosome composition and consequently DAF-16 activity/recruitment. Figure 6A shows that the stable his-41K31A variant has no effect on DAF-16 recruitment on its own; his-41K31A mimics the WT condition. The same is actually true in ubc-20 and hecd-1 mutants. Both mimic the WT condition, despite the fact that these mutations should disturb H2B levels in fed conditions (Figure 2E, 3E). How do the authors explain the contradictory observation of remarkably increased HIS-41 protein levels in ubc-20/hecd-1 and his-41K31A on the one hand, and WT-like DAF-16 activity on the other if H2B re-composition had a crucial role in DAF-16-mediated response to starvation? Can the others trigger DAF-16 activation in his-41K31A background by other means than daf-2 mutation?

We thank the Referee for pointing out this issue. In the presence of ample food, high insulin/IGF signaling inactivates and sequestrates DAF-16 in the cytoplasm. As reported by the Cynthia Kenyon lab, forcibly translocating DAF-16 into the nucleus causes larval arrest and death (Lin et al., 2001). However, under dire conditions such as starvation, cues that reduce insulin/IGF signaling enables DAF-16 to translocate into the nucleus, and promotes dauer formation, which likely requires H2B re-composition by H2B degradation and replacement. We agree with the Referee that the others may trigger DAF-16 activation in his-41K31A background by other means than *daf-2* mutation.

We have carefully discussed these issues in the revised text on page 16, line 25:

"…Indeed, we cannot exclude the possibility that the others may trigger DAF-16 activation in the *his-41*^{K31A} mutant background by other means than *daf-2* mutation."

6) A possible contribution of H2B mRNA levels is neglected in the manuscript. One critical experiment should analyze the mRNA levels of histone variants in the isolated UPS-suppressor mutations as well as upon starvation, importantly not in the context of wrt-2 promoter driven transgenes as these are expected to be transcriptionally responsive to nutrient deprivation, as mentioned by the authors (shown in Figure 2D).

We thank the Referee for pointing out this critical experiment! In response to this suggestion, we have evaluated the mRNA level of *his-4* and *his-41* in wild-type, *ubc-20(cas916),* and *hecd-1(cas908)* under the conditions of well-fed or starvation. Our results show the replication-dependent *his-4* is expressed with ample food, but sharply decreased upon starvation stress. The replication-independent *his-41* mRNA

level remains unchanged during starvation. Consistent with the *wrt-2* promoter driven transgenes, mutations in *ubc-20* and *hecd-1* do not affect the transcriptional pattern of *his-4* and *his-41* mRNA upon starvation stress (Fig. S2G). These results provide additional support to the notion that the ubiquitin-proteasome system regulates H2B degradation.

We have presented these data in Fig. S2 and described the results in the revised text on page 7, line 18:

"…Furthermore, we performed RNA-seq and data analysis to examine a possible contribution of mRNA level regulation to the observed decrease in H2B proteins upon starvation. In *wild-type*, the *his-4* was expressed with ample food and sharply decreased upon starvation stress, but the expression of *his-41* mRNA level remained unchanged during starvation (Fig. S2G). Importantly, the depletion of UBC-20 and HECD-1 did not alter the transcriptional pattern of *his-4* and *his-41* mRNA upon starvation stress (Fig. S2G)…"

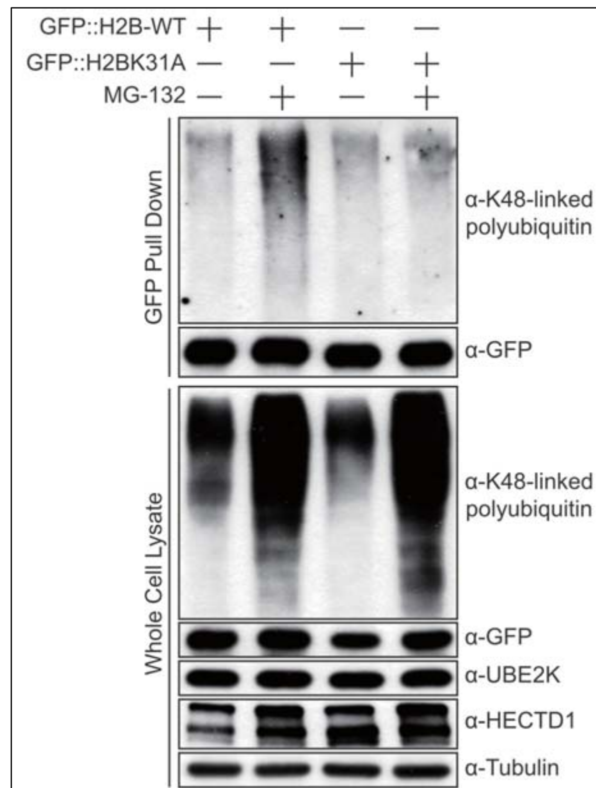
7) Throughout the manuscript the authors interpret the reduction of H2B protein levels as a consequence of enhanced protein turnover by the ubiquitin-proteasome-system (UPS). The isolation of mutations in representative UPS-factors might indicate that histone protein turnover plays a role. However, the manuscript does not present any careful analysis validating this conclusion. Direct experimental validation should include, documentation of H2B ubiquitylation and degradation in vivo, including meaningful controls. In order to exclude unspecific enrichment of proteins in the IP reactions, a negative control (e.g. GFP) is essential. Data shown in Figure 3F might be suited to address a direct ubiquitylation of H2B proteins. In these experiments the authors should show the level of poly-ubiquitin also in the lysates as the KOs of UBE2K and HECTD1 are expected to affect general poly-ubiquitylation as well. It appears unclear, why histone variant levels in the cell lysates are not increased in UBE2K-KO and HECDT1-KO cells (and K31A mutant) compared to WT controls. Why are H2Bs subject to poly-ubiquitylation in unchallenged conditions?

We fully agree with the Referee regarding these critiques. Indeed, direct experimental validation of H2B ubiquitylation and degradation is quite challenging in *C. elegans* due to the ineffectiveness of treating with the proteasome inhibitor MG-132, we therefore used the mammalian cells to examine H2B ubiquitylation and degradation.

We apologize for any confusion caused by the data in Figure 3F. In this experiment, we transfected plasmids encoding GFP-H2B-WT or the control GFP-H2BK31A into *wild-type*, *UBE2K-KO* and *HECDT1-KO* cells. The molecular weight of GFP-H2B/H2BK31A is approximately 40 KD, so the upper band represents the target protein indicated by the GFP antibody (whole cell lysate, the panel of α-GFP), and we have added an asterisk to labeled the unspecific protein band. Moreover, we have

examined the level of endogenous H2B in *wild-type*, *UBE2K-KO,* and *HECDT1-KO* cells. Our new results demonstrate that the depletion of UBE2K or HECDT1 could increase the level of endogenous H2B compared to the WT controls (Fig. S7B).

We have performed experiments to investigate H2B and H2BK31A ubiquitylation in mammalian cells with or without proteasome inhibitor MG-132. We examined the levels of poly-ubiquitin in cell lysates and IP (immunoprecipitation) reactions. Cells treated with the proteasome inhibitor MG-132 exhibited evident K48-linked polyubiquitin signaling in cell lysates. Moreover, consistent with the observation of H2BK31A inhibiting H2B degradation in worms, H2BK31A significantly reduced K48-linked polyubiquitylation compared to the WT controls (as followed).



We have revised Figure 3F and presented the new results in the revised text on page 13, line 7:

"By examining the level of endogenous H2B in wild-type, *UBE2K-KO*, and *HECDT1-KO* cells, we show that the depletion of UBE2K or HECDT1 increased the level of endogenous H2B compared to the WT controls (Fig. S7B)..."

8) The authors isolate central factors of the ubiquitin-proteasome system (UPS) as genetic suppressors of H2B decrease. Though the molecular nature of the isolated mutations remains to be elucidated, it is striking that these mutations maintain H2B levels under starvation conditions (at least in the wrt-2 driven transgenes), suggesting

loss-of-function or hypomorphic alleles. As all of the isolated mutations either affect a basic step in the ubiquitylation cascade or have documented broad function in protein quality control, it would be interesting how the authors envision specificity towards H2B and more importantly, how they can exclude global effects on protein turnover. In order to elucidate the direct contribution of H2B regulation respective RNAi knockdown in the Dauer formation/larval arrest assays should be included (Figure 4F, 5C). If H2B regulation is the critical determinant in this context, RNAi-mediated depletion of H2B in daf-2; ubc-20 or daf-2; hecd-1 would be expected modulate the phenotype of the double mutants into the respective phenotype of the daf-2 single mutation (increased Dauer formation, decreased larval lethality).

We appreciate the great suggestion from the Referee. In response to this suggestion, we conducted RNAi experiments in these mutants (*daf-2*; *daf-2, ubc-20;* and *daf-2, hecd-1*). RNAi against *his-41*/H2B did not reveal novel phenotypes in the *daf-2* background. Importantly, knockdown of *his-41*/H2B partially suppressed larval lethality and rescued dauer formation in *daf-2, ubc-20* and *daf-2, hecd-1* (Fig. S4A). These results suggest that maintaining appropriate levels of histone H2B is critical for animal development and survival under conditions of reduced insulin/IGF receptor activity. We have included the new data in the revised text on page 9, line 17:

"…To elucidate the direct contribution of H2B regulation in the dauer formation or larval arrest, we conducted RNAi experiments in the *daf-2*; *daf-2, ubc-20;* and *daf-2, hecd-1* mutant animals. RNAi against *his-41*/H2B did not reveal novel phenotypes in the *daf-2* background. Importantly, knockdown of *his-41*/H2B partially suppressed larval lethality and rescued dauer formation in *daf-2, ubc-20* and *daf-2, hecd-1* (Fig. S4A). These results suggest that maintaining appropriate levels of histone H2B is critical for animal development and survival under the conditions of reduced insulin/IGF receptor activity."

9) The authors interpret the histone exchange assay in mammalian tissue cell culture as a validation of an evolutionarily conserved histone protein turnover by the UPS. As the incorporation of 'new vs. old' histones will be determined by the proliferative activity of the MG132-treated or UBE2K/HECTDT1-depleted cells the authors need to document the proliferation profile of the respective cell lines. The histone exchange assay does not serve as an appropriate method to determine a direct protein degradation of histone proteins. Furthermore, this data set is interpreted independent of the aforementioned connection to nutrient deprivation in C. elegans. In light of this experiment, it should also be considered that UBE2K-KO, HECTD1-KO as well as H2BK31A mutation did not result in increased H2B levels (Figure 3F). Thus, the regulation of H2B levels in C. elegans appears to be distinct in mammalian cells.

We are grateful to the Referee for pointing out this possibility. We have performed the additional Western blot assay to assess the amount of endogenous H2B in *wild-type*, *UBE2K-KO,* and *HECTD1-KO* cells. Consistent with our observation in *C.*

elegans, depletion of human UBE2K and HECTD1 increased the level of endogenous H2B (Fig. S7B). However, we agree with the Referee that our histone exchange assay does not serve as an appropriate method to determine a direct protein degradation of histone proteins and that this data set is interpreted independent of the aforementioned connection to nutrient deprivation in *C. elegans*.

We have included the new data in the revised text on page 13, line 7:

"By examining the level of endogenous H2B in wild-type, *UBE2K-KO*, and *HECDT1-KO* cells, we show that the depletion of UBE2K or HECDT1 increased the level of endogenous H2B compared to the WT controls (Fig. S7B)..."

We have thus carefully discussed these possibilities in the revised Discussion on page 15, line 3:

"…Despite we cannot exclude the possibility that the regulation of H2B levels in *C. elegans* might be distinct in mammalian cells, these findings reveal the widespread nature of the UPS-based global histone degradation."

Referee #2:

Zhu et al investigate histone H2B turnover during starvation in the nematode C. elegans. They use state-of-the-art reporter genes to image each endogenous H2B protein, discovering that all but one of them are degraded in starved L1 larvae. The exception, his-41, is a replication-independent histone, apparently in contrast to the others, and they argue that it is subjected to ubiquitination and degradation just like all of the others but that its transcript maintains abundance such that it is continuously translated thus maintaining protein levels so that HIS-41 largely replaces the other H2Bs. They use forward genetics to identify E1, E2, and E3 ubiquitin ligases required for H2B degradation. They use mass spec to identify the amino acid required for ubiquitination as well, and they show that these ligases have analogous function in human cells. They also demonstrate physiological significance in worms by showing that daf-2/Insulin/IGF receptor mutants grown at the restrictive temperature (dauer-forming conditions) die without these ubiquitin ligases. They perform an additional forward genetic screen to suppress lethality under these conditions and identify the very well characterized effector of daf-2 signaling, daf-16/FOXO. They show that the "starvation response" is mis-regulated in the absence of the ubiquitin ligases, arguably accounting for lethality, and they show that mutation of daf-16 reverses this mis-regulation. They use ChIP-seq to further show that DAF-16 and H2B do in fact colocalize in chromatin and that lack of the ubiquitin ligases or lack of ubiquitination increases retention of H2B as well as DAF-16, providing mechanistic explanation for mis-regulation. The paper includes a lot of high-quality data (~20

CRISPR edits analyzed by imaging and western blot, 2 forward genetics screen each yielding valuable and insightful mutants, RNA-seq and ChIP-seq in a variety of genetic backgrounds, experiments in human cells to show conservation, etc), and it is generally well-written, clearly presented, and without overstated conclusions. This work is interesting, significant, and timely given that the function and regulation of individual histones is generally ignored though consequential, and because nutritional control of chromatin structure is understudied.

We thank the reviewer for the positive assessment of our study and our manuscript.

Major concerns

The authors provide very nice mRNA-seq and ChIP-seq analyses in Fig 5 and 6. However, these two analyses are not as well integrated as they could be, which has the potential to strengthen their conclusions. They provide genome browser shots for a pair of representative genes in Fig 6, but it is unclear how representative the correlations between the two data types revealed here are. In fact, they point out that not all genes with increased DAF-16 binding display altered expression, and they present a pair of representative genes for this in Fig S4. How many genes fit each pattern? Is it more common that expression is affected when binding is? The authors should present the overlaps (and their statistical significance) for the differentially expressed genes identified by RNA-seq and the differentially bound genes identified by ChIP-seq. This could be done with arbitrary cut-offs to define gene lists and venn diagrams, or the authors could consider scatter plots of fold-changes (potentially color coded by significance). A hybrid approach is also possible, where a discrete list is generated by one data type (eg, RNA-seq) and that gene set is plotted along with the set of all detected genes as a cumulative distribution of fold-changes in the other data type (eg, ChIP-seq). There may be other ways to satisfy this as well.

We apologize for missing the correlation analysis of RNA-seq and ChIP-seq. We thank the Referee for pointing out the approach to resolve this issue. As suggested by the reviewer, we have re-examined DAF-16::GFP binding sites and DAF-16-mediated gene expression in *daf-2, hecd-1* double mutant. Our new results show that 255 genes are bound by DAF-16::GFP but exhibit normal gene expression, such as *sod-3* or *F38A6.4*. Intriguingly, we identified 652 genes that are bound by DAF-16::GFP and display ectopic gene expression in *daf-2, hecd-1* double mutant, providing additional evidence for the notion that the inhibition of histone degradation affects the transcriptional activity of DAF-16 (Fig. S5D).

We have presented our new analyses in the revised Figure S5 and described in the revised text on page 12, line 13:

"…By examining DAF-16::GFP binding sites and DAF-16-mediated gene expression in *daf-2, hecd-1* double mutant, we showed that 255 genes are bound by

DAF-16::GFP but exhibit normal gene expression. Intriguingly, we identified 652 genes that are bound by DAF-16::GFP and display ectopic gene expression, revealing that the inhibition of histone degradation may affect the transcriptional activity of DAF-16 (Fig. S5D)…"

The demonstration that HIS-58/H2B is also degraded in epidermal seam cells upon asymmetric division in fed, developing larvae (in addition to starved larvae) is interesting. Notably, this is seen in the anterior daughter which fuses with the syncytium and ceases proliferation, as opposed to the posterior daughter which remains as a blast cell that will divide asymmetrically again in the next larval stage. As for their cell culture work, I'm thinking about cellular quiescence here. This piece would be strengthened if the authors could demonstrate that HIS-58 is replaced by HIS-41/H2B in the anterior daughter, analogous to what they've shown for starved larvae.

We thank the reviewer for pointing out this question. To address this issue, we have performed genetic crosses introducing GFP::HIS-58 KI or HIS-41::GFP KI into transgenic strains expressing mCherry::PH and mCherry::H1, which label the membrane and nucleus of epidermal seam cells, respectively. Consistent with our previous observations, GFP::HIS-58 was highly expressed in epidermal seam cells, and exhibited rapid degradation in the anterior daughter when fused with hyp 7 (Fig. S8A, indicated with yellow arrowheads). Indeed, HIS-41::GFP showed relatively low expression in epidermal seam cells but was enriched in the nucleus of hyp 7 (Fig.S8B, indicated with yellow and blue arrowheads). These results suggest that HIS-58 may be replaced by HIS-41 in the anterior daughter following fusion with hyp 7.

We have presented new data in Figure S8 and described the results in the revised text on page 13, line 25:

"…To examine whether endogenous HIS-58 is replaced by HIS-41 in the anterior daughter, we introduced GFP::HIS-58 KI or HIS-41::GFP KI into transgenic strains expressing mCherry::PH and mCherry::H1, which label the membrane and nucleus of epidermal seam cells, respectively. Consistent with our transgenic observations, endogenous GFP::HIS-58 was highly expressed in epidermal seam cells, and exhibited rapid degradation in the anterior daughter when fused with hyp 7 (Fig. S8A, indicated with yellow arrowhead). By contrast, HIS-41::GFP showed relatively low expression in epidermal seam cells but became enriched in the nucleus of hyp 7 (Fig. S8B, indicated with yellow and blue arrowhead). These results suggest that HIS-58 may be replaced by HIS-41 in the anterior daughter following fusion with hyp 7.…."

The authors refer to some histones as replication-dependent and others as replication-independent, and they cite Roberts, Sanicola et al 1987 (p-5; with mention of this again on p-7 with respect to his-4 and his-41 and possibly elsewhere). This is a very important distinction given their findings and overall model of histone turnover

and replacement in fed vs. starved conditions. However, it isn't clear to me how this classification is drawn from the cited paper or what other, perhaps more recent data pertaining to transcription/mRNA abundance it is based on.

We apologize for not citing appropriate publications for clear explanation of the differences between the replication-dependent histone and replication-independent histone in *C. elegans*. The replication-dependent histone genes lack intron and encode a special type of mRNA that lack a poly(A) tail, instead ending with a stem-loop sequencing (Marzluff et al., 2002). The stem-loop structure is essential for all steps in the mRNA metabolism, including the rapid degradation of histone mRNA at the end of the S-phase (Marzluff and Koreski, 2017). However, the replication-independent histone genes encode polyadenylated mRNAs, which are not regulated by cell-cycle. Studies conducted by the Berndt Müller lab have classified histone genes and demonstrated the essential role of stem-loop binding protein in the expression of replication-dependent histone genes in *C. elegans* (Pettitt et al., 2002). Their subsequent systematic analysis of histone mRNA in *C. elegans* further confirmed that *C. elegans* replication-dependent histone mRNAs indeed lack a poly(A) tail and terminate three to six nucleotides after the hairpin structure (Keall et al., 2007). We have cited these publications in the revised text.

Minor concerns

It is my understanding that DAF-2 is homologous to mammalian insulin receptor and IGF receptor. We therefore refer to insulin/IGF signaling in worms. This is the case in some places, but not all, as if insulin and IGF signaling are synonymous. For example, at the bottom of p-3, "reduced insulin signaling enables DAF-16 to translocate to the nucleus". And on p-8, "DAF-2 encodes the human ortholog of insulin-like growth factor 1 receptor (IGF1R), and the daf-2(e1370) allele is a temperature-sensitive mutant with reduced insulin receptor activity."

We thank the reviewer for pointing out the difference. We have corrected the writing on this throughout the manuscript, i.e. "reduced insulin/IGF signaling enables DAF-16 to translocate to the nucleus", "DAF-2 encodes the human ortholog of insulin-like growth", “the *daf-2(e1370)* allele is a temperature-sensitive mutant with reduced insulin/IGF receptor activity", and other parts of the manuscript.

On p-10, this finding is reported: "Previous RNAi experiments revealed that inhibiting 172 genes among those downregulated ones caused embryonic or larval lethality (Supplemental Table S6), which suggests that the aberrant transcription program may account for the double mutant phenotypes." Does this overlap reflect a statistically significant enrichment of genes with this phenotype in this gene set? The authors should perform a hypergeometric test to compute a p-value for this (as is done for GO term analysis).

We thank the reviewer for pointing out these questions. As suggested by the reviewer, we have performed GO term analysis, the new results show these genes are associated with endoplasmic reticulum, mitochondrion and protein synthesis, which may be also involved in embryo development. Our hypergeometric test to compute a p-value for this revealed that this overlap reflects a statistically significant enrichment of genes with this phenotype in this gene set. The new data and statistical analysis were presented in Supplemental Table S7. We described the new Table S7 in the revised text on page 11, line 14:

"…Using GO term analysis, we found that these essential genes are associated with endoplasmic reticulum, mitochondrion and protein synthesis, and this overlap reflects a statistically significant enrichment of genes with the phenotype (Supplemental Table S7)…"

The authors offer this conclusion on p-10: "These findings indicate that aberrant H2B retention induced ectopic DAF-16-dependent transcription that causes animal lethality." Because they have not directly demonstrated that ectopic DAF-16 activity at these loci results in lethality the conclusion is stated too strongly with "indicate" and something like "suggests" is more appropriate.

We have replaced "indicate" with "suggest", which is more appropriate. We thank the Referee.

Fig 4C and 5E,F: The authors should provide hypergeometric p-values demonstrating that these overlaps are statistically significant.

We have performed hypergeometric test and added the p-values to show that these overlaps are statistically significant in the revised Fig. 4C and Fig. 5E&F.

Suggestions to improve clarity

Thank the reviewer for pointing out these errors.

Abstract: "ligating enzymes *that* blocked"

We have added the "that".

Throughout: "chromatin" rather than "chromatins"

We have replaced "chromatins" with "chromatin" throughout the manuscript.

Top of p-5: "the rest *of the* strains showed the expected"

We have corrected the sentence as "…the rest of the strains showed the expected…"

Twice near the bottom of p-6: "point mutations or early stop codons" - stop codons can result from point mutations.

We have replaced the "point" with "missense".

p-13: "these findings reveal the widespread *nature* of the"

We have corrected it as "these findings reveal the widespread nature of the UPS-based global histone degradation"

Referee #3:

The manuscript submitted by Zhu et al. is a thoroughly detailed analysis of a newly described process that involves the regulated turnover of histone proteins in response to physiological (starvation/quiescence) and/or developmental conditions. The turnover is dependent on specific ubiquitylation of lysine residues on many (replication dependent) H2B and H4 variants resulting in their subsequent removal and degradation. They have effectively used multiple approaches to provide a detailed understanding of the histone targets, the genes involved and how this might occur in specific cell types, including human cells. The writing was generally very good to excellent and both the logic and the interpretation of most of the experiments was solid.

We appreciate that the Referee is also positive for our study.

Although I was mostly impressed with the quality of this manuscript. I did have a few issues that may or may not be important for the authors to consider during their revision. Namely, it is not clear why they were examining the hypodermal lineage nor is it clear whether, in addition to starvation, this process is regulated in any other physiological or perhaps more relevant to the final experiment, during any other developmental contexts. The V cell lineage is already a bit particular due to the uncommon fates of the the daughter cells following the first division; one joins a syncytium (Vn.a) and the other (Vn.p) adopts stem cell-like properties. In Vn.a the histones are turned over while in Vn.p they are not. Does this happen in other asymmetrically dividing lineages where the cell fates of the daughters do not have the same dramatic outcomes. The QR divisions would suggest that this does not always happen, but I am not sure if QR.a and QR.p maintain similar fates. Perhaps examining the first Pn.p cell division or even the P cells themselves might complete this final experiment better and answer this question more thoroughly. The P cells are still hypodermal, but the outcomes are not as dramatically different as the V daughters.

Alternatively, perhaps examining an M cell division or another asymmetric division might enhance this part of the analysis.

We appreciate that the Referee raised such insightful questions and offered excellent ideas for us to explore further. This study focused on the V cell lineage, and we also believe that careful examination of other lineages can provide additional insights into the generality of the mechanisms that we proposed. The reason why this study chose to examine the V cell lineage because it presents a unique system in which one of the daughter cells, Vn.p cells, maintains stem cell-like properties whereas the other Vn.a cells undergo fusion with the hyp7 cell, thereby likely losing its original V-cell identity but adopting the hyp 7 cell fate. Such a draft cell fate change may correlate with the significant degradation of GFP::H2B in Vn.a after fusion with hyp7 cells.

We thank the Referee for pointing out QR.a and QR.p cells, and my lab have been working on them during the past decade. The Referee is correct: they both generate neurons and apoptotic cells. We did not notice any apparent changes for the H2B level during their development. We agree with the Referee that examining additional lineages can be also informative. Instead of examine each lineage, we performed the Western blot to carefully determine the levels of endogenous GFP::HIS-4 (a replication-dependent H2B) and HIS-41::GFP (the unique replication-independent H2B) in larvae and adult worms. Our new data reveal that GFP::HIS-4 is almost entirely degraded and replaced by HIS-41::GFP in adult animals (Fig. S8C-D). Although we did not depict the eventual state of transgenic GFP::H2B in the Q lineage and other tissues, these results suggest that H2B replacement and degradation occur in various tissues during development. Future systematic studies will investigate the mechanism in each cell lineage throughout development. We have now presented our new results in the revised Figure S8 and described them in the text on page 14, line 9:

"To examine whether histone degradation also occurs in other lineages during development, we performed the Western blot to determine the levels of endogenous GFP::HIS-4 (a replication-dependent H2B) and HIS-41::GFP (the unique replication-independent H2B) in larvae and adult worms. Our results reveal that GFP::HIS-4 is almost entirely degraded and replaced by HIS-41::GFP in adult animals (Fig. S8C-D), which suggests that H2B replacement and degradation occur in various tissues during development."

Keeping with this same concern, the SILAC experiment described on Page 12 and in Figure X is intriguing and the choice of using a confluent culture is quite appropriate, since replication should be halted and a quiescence program is initiated. However, with only one condition it is hard to know if the turnover that is observed is indeed regulated in a quiescence-dependent manner or whether it is simply homeostatic histone replacement. The authors very appropriately point out this caveat, however, It

would be more informative if the experiment was also repeated with sub-confluent cultures or a continuously growing population of cells as a kind of control, to better understand if this process is part of general cellular homeostasis or whether is regulated in a quiescence-dependent manner.

We appreciate the suggestion and the importance of control experiments. In response to the reviewer's comments, we have performed additional SILAC and LC-MS/MS assays using sub-confluent culture cells. The new results demonstrate that histone H2B turnover rate is faster in growing cells compared to quiescent cells (Fig. S7A). Notably, the histone variant H2A.v/z, which does not exhibit a cell-cycle-dependent expression pattern, shows a similar turnover rate in both sub-confluent and confluent culture cells (Fig. S7A). These results indicate that confluent culture conditions could minimize the effects of the cell cycle, allowing the histone turnover to reflect chromatin dynamics in a quiescence-dependent manner. We have presented new data in Figure S7 and described the results in the revised text on page 12, line 27:

"…minimizing the cell-cycle effects compared with sub-confluent culture cells…."

To complete this study it would also be informative to test if the orthologues of the genes that were identified in the initial genetic screen also contribute to the turnover of these histones in mammalian cells.

We thank the Referee for pointing out this experiment. We have performed Western blot assay to examine the relative of endogenous H2B in *wild-type*, *UBE2K-KO,* and *HECTD1-KO* cells. Consistent with our observations in *C. elegans*, the depletion of human UBE2K and HECTD1 increased the level of endogenous H2B (Fig. S7B). We have presented new data in Figure S7 and described the results in the revised text on page 13, line 7:

"By examining the level of endogenous H2B in wild-type, *UBE2K-KO*, and *HECDT1-KO* cells, we show that the depletion of UBE2K or HECDT1 increased the level of endogenous H2B compared to the WT controls (Fig. S7B)..."

On page 14 in the Discussion, the authors speculate that the levels of HIS-41 may comparatively high and this could explain the observed changes. This should be easily testable simply by measuring the levels of HIS-41 at different life stages of the growing animal and comparing to that of the other histones.

We thank the Referee for pointing out this experiment. We carefully examined the levels of endogenous GFP::HIS-4 (a replication-dependent H2B) and HIS-41::GFP (the unique replication-independent H2B) in larvae and adult worms. Our findings reveal that GFP::HIS-4 is almost entirely degraded and replaced by HIS-41::GFP in adult animals (Fig. S8C-D). We have presented new data in Figure S8 and described the results in the revised text on page 14, line 9:

"To examine whether histone degradation also occurs in other lineages during development, we performed the Western blot to determine the levels of endogenous GFP::HIS-4 (a replication-dependent H2B) and HIS-41::GFP (the unique replication-independent H2B) in larvae and adult worms. Our results reveal that GFP::HIS-4 is almost entirely degraded and replaced by HIS-41::GFP in adult animals (Fig. S8C-D), which suggests that H2B replacement and degradation occur in various tissues during development."

During my reading I am came across a number of small grammatical/typo errors that should be addressed to further improve the text of the manuscript. I have listed them in the lines below.

Thank the Referee for pointing out these errors.

in the Abstract- ...uncovered "mutants" defective in....
"chromatin", not chromatins (change this throughout the manuscript)

We have replaced "chromatins" with "chromatin" throughout the manuscript

Page 6- Progeny (not progenies); that retained (not retaining); conditions of starvation or during starvation (not starvation condition-this might be changed throughout the manuscript as well)

We have corrected these errors.

Page 7-has been far less explored (not was); remove "the" from transfected human HEK293T cells

We have corrected "was" and removed "the".

Page 8-DAF-2 is not a protein in this case is should be written as a gene daf-2; executed the dauer stage (not survived by entering..); all died on the third day following the shift to 25˚C (remove "exposed")

We have corrected these errors.

Page 9-the section concerning H4 K31 reduncancy is confusing. Could this section be re-worked to clearly specify why H4 woud be redundant in the first place.; 71 mutations that bear mutations in...

Thank the Referee for pointing out these questions. We have corrected the error on this page, and explained the rationale why H4 would be redundant before our genetic dissection of H4K31 on the revised page 10, line 1:

"…Considering that ubiquitination-mediated degradation of a protein complex may occur at multiple sites of its different subunits, we wondered whether other subunits in the histone octamer was ubiquitinated. Indeed, our MS experiments revealed that HIS-4 or HIS-41 bound core histones (H2A, H2B, H3, H4) carry additional ubiquitinated sites (Fig. S4B-C, bottom)..."

Page 10-the paragraph indicating that 172 of the identified genes that were in the downregulated subset were previously shown to be embryonic or larval lethal when compromised by RNAi could be written as such...

Thanks, we have written the sentence.

Page 13-Decorating histones at multiple locations around the histone core

Thanks, we have written the sentence.

Page 14-their abnormally high basic amino acid content

Thanks, we have written the sentence.

Reference:

Keall, R., Whitelaw, S., Pettitt, J., and Muller, B. (2007). Histone gene expression and histone mRNA 3' end structure in Caenorhabditis elegans. BMC Mol Biol *8*, 51.

Lin, K., Hsin, H., Libina, N., and Kenyon, C. (2001). Regulation of the Caenorhabditis elegans longevity protein DAF-16 by insulin/IGF-1 and germline signaling. Nat Genet *28*, 139-145.

Marzluff, W.F., Gongidi, P., Woods, K.R., Jin, J., and Maltais, L.J. (2002). The human and mouse replication-dependent histone genes. Genomics *80*, 487-498.

Marzluff, W.F., and Koreski, K.P. (2017). Birth and Death of Histone mRNAs. Trends Genet *33*, 745-759.

Pettitt, J., Crombie, C., Schumperli, D., and Muller, B. (2002). The Caenorhabditis elegans histone hairpin-binding protein is required for core histone gene expression and is essential for embryonic and postembryonic cell division. J Cell Sci *115*, 857-866.

1st Revision - Editorial Decision **22nd Jun 2023**

Dear Guangshuo,

Thank you for submitting a revised version of your manuscript. Your study has now been seen by all original referees. While two of the reviewers find that their previous concerns have been addressed and are now broadly in favour of the acceptance of the manuscript, reviewer #2 indicates that further clarification is needed to address two of their initially raised points. I would therefore invite you to clarify these remaining issues in the final minor revision.

There are also a few editorial points that have to be addressed before I can extend official acceptance of the manuscript:

1. Please correct the textual issues raised by referee #3.
2. Please submit a complete author checklist, which you can download from our author guidelines (https://wol-prod-cdn.literatumonline.com/pb-assets/embo-site/Author Checklist%20-%20EMBO%20J-1561436015657.xlsx). Please insert information in the checklist that is also reflected in the manuscript. The completed author checklist will also be included in the Review Process File (RPF), which will be published alongside your paper.
3. Our publisher has done their pre-publication check on your manuscript. I have attached the file here. Please take a look at the word file and the comments regarding the figure legends and respond to the issues. Please also use this version when you resubmit the revised version.
4. Please add the complete funding information in our online submission system - it has to be identical with the information provided in the final manuscript to ensure a machine-readable format upon publication.
5. Please rename "Conflict of interest" section into "Disclosure and competing interests statement" (further info: https://www.embopress.org/page/journal/14602075/authorguide#conflictsofinterest).
6. Please update the references according to The EMBO Journal style (up to 10 authors followed by et al.) Further information can be found here: https://www.embopress.org/page/journal/14602075/authorguide#referencesformat
7. If possible, the figure callouts should follow the alphabetic order in the manuscript text. Currently, Fig. 6B is called after Fig. 6A; Appendix Fig. S4B-C is called out after Appendix Fig. S4D, Appendix Fig. S5D is called out after Appendix Fig. S6, and Appendix Fig. S7B is mentioned after Appendix Fig. S7C.
8. Appendix Fig. S6 C-D is not mentioned in the manuscript.
9. CRediT has replaced the traditional author contributions section because it offers a systematic, machine-readable author contributions format that allows for more effective research assessment. Please remove the Authors Contributions from the manuscript and use the free text boxes beneath each contributing author's name in our online submission system to add specific details on the author's contribution. More information is available in our guide to authors.
10. Please upload Tables S1-S7 as Datasets and update the nomenclature to Dataset EV1, etc. Their legends need to be removed from the manuscript text file and included in their respective Excel sheets.
11. There are three Reagent tables in a separate Word file. They need to be either turned into simple tables and renamed Table 1-3 (https://www.embopress.org/page/journal/14602075/authorguide#tablesformat) or turned into a single Reagent table according to our template that can be found here: https://www.embopress.org/pb%2Dassets/embo-site/Reagents_Tools_Table_TEMPLATE.docx
12. Please rename the supplementary figures into Appendix Figure S1, etc. and update the nomenclature in the manuscript accordingly. Please assemble the Appendix figures and their legends in a single Appendix file prefaced with a short table of contents that includes the page numbers.
13. Please upload source data as one folder per main figure. Appendix Figure source data can be uploaded in a single folder, but also needs to be split in one file/folder per figure.
14. Please check if the source data fit to the respective figure panels. For example, the Western blot source data for Appendix figure S2F and S3B are identical and do not fit to the Appendix Fig, S2F panel. Source data for Appendix Figure S4E is labelled as for Appendix Fig S3B.
15. Source data for the following figure panels is missing: 1A, 1C, 1F, 2A, 3B, 3C, 8B.
16. Papers published in The EMBO Journal are accompanied online by a 'Synopsis' to enhance discoverability of the manuscript. It consists of A) a short (1-2 sentences) summary of the findings and their significance, B) 3-4 bullet points highlighting key results and C) a synopsis image that is 550x300-600 pixels large (width x height, jpeg or png format). You can either show a model or key data in the synopsis image. Please note that the image size is rather small and that text needs to be readable at the final size. Please send us this information together with the revised manuscript.

Please let me know if you have any questions regarding any of these points. You can use the link below to upload the revised files.

Thank you again for giving us the chance to consider your manuscript for The EMBO Journal. I look forward to receiving the final version.

With best wishes,

Ieva

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i.gailite@embojournal.org

Further information is available in our Guide For Authors: https://www.embopress.org/page/journal/14602075/authorguide

We realize that it is difficult to revise to a specific deadline. In the interest of protecting the conceptual advance provided by the work, we recommend a revision within 3 months (20th Sep 2023). Please discuss the revision progress ahead of this time with the editor if you require more time to complete the revisions.

Referee #1:

The authors have addressed all the points raised and have thus confirmed their results very well. The manuscript is now of great importance to the broad readership of EMBO J.

Referee #2:

Zhu et al address an important facet of chromatin regulation that has been overlooked. I find their molecular mechanistic insights to be convincing and significant. I see their efforts to connect those mechanisms to broader organismal significance as less satisfying. For example, the connections between insulin/IGF signaling, nutrient stress, and viability are murky, and the interpretations of the observations regarding H2B replacement during fed development could be clearer. However, I am convinced that H2B replacement is significant to developmental physiology, and I think this work makes important mechanistic insights. However, I am not satisfied with the response to two of my previous comments. I will quote the original comments and the response before stating my current comment.

1) My original comment: The authors refer to some histones as replication-dependent and others as replication-independent, and they cite Roberts, Sanicola et al 1987 (p-5; with mention of this again on p-7 with respect to his-4 and his-41 and possibly elsewhere). This is a very important distinction given their findings and overall model of histone turnover and replacement in fed vs. starved conditions. However, it isn't clear to me how this classification is drawn from the cited paper or what other, perhaps more recent data pertaining to transcription/mRNA abundance it is based on.

Author response: We apologize for not citing appropriate publications for clear explanation of the differences between the replication-dependent histone and replication-independent histone in C. elegans. The replication-dependent histone genes lack intron and encode a special type of mRNA that lack a poly(A) tail, instead ending with a stem-loop sequencing (Marzluff et al., 2002). The stem-loop structure is essential for all steps in the mRNA metabolism, including the rapid degradation of histone mRNA at the end of the S-phase (Marzluff and Koreski, 2017). However, the replication-independent histone genes encode polyadenylated mRNAs, which are not regulated by cell-cycle. Studies conducted by the Berndt Mu¨ller lab have classified histone genes and demonstrated the essential role of stem-loop binding protein in the expression of replication-dependent histone genes in C. elegans (Pettitt et al., 2002). Their subsequent systematic analysis of histone mRNA in C. elegans further confirmed that C. elegans replication-dependent histone mRNAs indeed lack a poly(A) tail and terminate three to six nucleotides after the hairpin structure (Keall et al., 2007). We have cited these publications in the revised text.

Additional comment: I appreciate this very clear explanation of criteria used to determine replication-dependent vs -independent histones. However, this sort of clear explanation is not provided in the manuscript, though I would have found it to be helpful as a reader. Instead, there is a single sentence in the revised version: "We noticed that the his-41 gene encodes the only DNA replication-independent H2B variant in C. elegans (Keall, Whitelaw et al., 2007, Marzluff, Gongidi et al., 2002, Pettitt, Crombie et al., 2002)." The problem is that Keall 2007, the paper that "systematically" analyzes each worm histone gene, does not appear to include his-41 (I'm looking at Additional File 1, which has the results for all histone genes). I don't understand how the authors could have simply "noticed" his-41 as replication-independent. Instead, it seems they must have looked for a stem-loop sequence, polyA signal, or introns on their own to reach this conclusion. If so, the specific evidence for the claim should be presented since it is not in literature already. Also, I would like to note that while Keall showed that the supposed replication-dependent histones have a 3' stem-loop sequence, they also showed that they have a polyA signal, surprisingly, and I would argue that they do not demonstrate that these histones are not also polyadenylated some of the time. In fact, all of these

histones are readily detected in polyA-based RNAseq experiments. Beyond merely looking for stem-loop sequences, polyA sites, and introns, it would be clarifying to look at RT-QPCR comparing oligo dT and random priming.

2) My original comment: On p-10, this finding is reported: "Previous RNAi experiments revealed that inhibiting 172 genes among those downregulated ones caused embryonic or larval lethality (Supplemental Table S6), which suggests that the aberrant transcription program may account for the double mutant phenotypes." Does this overlap reflect a statistically significant enrichment of genes with this phenotype in this gene set? The authors should perform a hypergeometric test to compute a p-value for this (as is done for GO term analysis).

Author response: We thank the reviewer for pointing out these questions. As suggested by the reviewer, we have performed GO term analysis, the new results show these genes are associated with endoplasmic reticulum, mitochondrion and protein synthesis, which may be also involved in embryo development. Our hypergeometric test to compute a p-value for this revealed that this overlap reflects a statistically significant enrichment of genes with this phenotype in this gene set. The new data and statistical analysis were presented in Supplemental Table S7. We described the new Table S7 in the revised text on page 11, line 14:

Additional comment: Unfortunately, my original comment was misunderstood. My question is whether 172 genes causing lethality out of 759 down-regulated genes is a statistically significant result. That is, if you took a random set of 759 genes out of those detected in your expression analysis (not the whole genome, since detectable genes are more likely to have a phenotype) and asked how many of them have this phenotype would it be significantly lower than 172? The hypergeometric test is the way to test this. I mentioned GO term enrichments because it is the same statistical test (gene set enrichment analysis), not because I thought it was important to look at GO terms for these 172 genes. The authors conclusion presumes that this is a significant enrichment of lethal genes among downregulated genes.

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The revised manuscript by Zhu et al. entitled "Global H2B degradation regulates insulin/IGF signaling-mediated nutrient stress" has addressed all my initial concerns and has built upon many of my comments and those of the other reviewers. The manuscript is quite interesting and, through a molecular genetic tour de force, they clearly delineate the importance of this process in various developmental contexts and/or physiological responses.
The language once again needs some minor work, which can be addressed by having the manuscript read and/or corrected by a native english speaker. I have listed below a number of minor issues that I came across and noted, but this list is not extensive and at some point I became less attentive and stopped. The authors should therefore make an earnest effort to identify the errors in the Text, Figure Legends, and in the Figures, to improve the grammar throughout.

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Page 3, last paragraph: sequestrates should be sequesters
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Figure 7A. in the schema-Lysis cells should be Lyse cells

Suppl Figures
Page 41-Legend Quantification not Quantifications; "could not detect" not detected

RESPONSE TO THE REVIEWERS

Referee #1:

The authors have addressed all the points raised and have thus confirmed their results very well. The manuscript is now of great importance to the broad readership of EMBO J.

We thank the Referee for the positive assessment of our revision.

Referee #2:

Zhu et al address an important facet of chromatin regulation that has been overlooked. I find their molecular mechanistic insights to be convincing and significant. I see their efforts to connect those mechanisms to broader organismal significance as less satisfying. For example, the connections between insulin/IGF signaling, nutrient stress, and viability are murky, and the interpretations of the observations regarding H2B replacement during fed development could be clearer. However, I am convinced that H2B replacement is significant to developmental physiology, and I think this work makes important mechanistic insights. However, I am not satisfied with the response to two of my previous comments. I will quote the original comments and the response before stating my current comment.

We appreciate that the Referee is also positive for our study and our revision. We acknowledge that our initial response may not have fully addressed all the questions raised. Our new data and analyses can address the remaining concerns.

1) My original comment: The authors refer to some histones as replication-dependent and others as replication-independent, and they cite Roberts, Sanicola et al 1987 (p-5; with mention of this again on p-7 with respect to his-4 and his-41 and possibly elsewhere). This is a very important distinction given their findings and overall model of histone turnover and replacement in fed vs. starved conditions. However, it isn't clear to me how this classification is drawn from the cited paper or what other, perhaps more recent data pertaining to transcription/mRNA abundance it is based on.

Author response: We apologize for not citing appropriate publications for clear explanation of the differences between the replication-dependent histone and replication-independent histone in C. elegans. The replication-dependent histone genes lack intron and encode a special type of mRNA that lack a poly(A) tail, instead ending with a stem-loop sequencing (Marzluff et al., 2002). The stem-loop structure is essential for all steps in the mRNA metabolism, including the rapid degradation of histone mRNA at the end of the S-phase (Marzluff and Koreski, 2017). However, the replication-independent histone genes encode polyadenylated mRNAs, which are not

regulated by cell-cycle. Studies conducted by the Berndt Müller lab have classified histone genes and demonstrated the essential role of stem-loop binding protein in the expression of replication-dependent histone genes in C. elegans (Pettitt et al., 2002). Their subsequent systematic analysis of histone mRNA in C. elegans further confirmed that C. elegans replication-dependent histone mRNAs indeed lack a poly(A) tail and terminate three to six nucleotides after the hairpin structure (Keall et al., 2007). We have cited these publications in the revised text.

Additional comment: I appreciate this very clear explanation of criteria used to determine replication-dependent vs -independent histones. However, this sort of clear explanation is not provided in the manuscript, though I would have found it to be helpful as a reader. Instead, there is a single sentence in the revised version: "We noticed that the his-41 gene encodes the only DNA replication-independent H2B variant in C. elegans (Keall, Whitelaw et al., 2007, Marzluff, Gongidi et al., 2002, Pettitt, Crombie et al., 2002)." The problem is that Keall 2007, the paper that "systematically" analyzes each worm histone gene, does not appear to include his-41 (I'm looking at Additional File 1, which has the results for all histone genes). I don't understand how the authors could have simply "noticed" his-41 as replication-independent. Instead, it seems they must have looked for a stem-loop sequence, polyA signal, or introns on their own to reach this conclusion. If so, the specific evidence for the claim should be presented since it is not in literature already. Also, I would like to note that while Keall showed that the supposed replication-dependent histones have a 3' stem-loop sequence, they also showed that they have a polyA signal, surprisingly, and I would argue that they do not demonstrate that these histones are not also polyadenylated some of the time. In fact, all of these histones are readily detected in polyA-based RNAseq experiments. Beyond merely looking for stem-loop sequences, polyA sites, and introns, it would be clarifying to look at RT-QPCR comparing oligo dT and random priming.

We agree with the reviewer. We understand the importance of providing clear experimental evidence to accurately classify histones in *C. elegans* as either replication-dependent or replication-independent. Indeed, the classification of histones in *C. elegans* lacks directly experimental evidence from the literature. We are thankful for the referee's suggestion to use oligo dT and random priming to distinguish between replication-dependent and replication-independent histones.

As suggested by the Referee, we extracted total RNA from GFP::HIS-4 or GFP::HIS-41 knock-in larval worms and synthesized the cDNAs. Based on quantitative reverse transcription PCR (qRT-PCR), we examined the relative expression levels of *gfp::his-4* mRNA by using the GFP-specific primer with either Oligo dT primers or random primers. The results showed that the relative expression level detected by random primers was eight times higher than that detected by Oligo dT primers (Appendix Fig. S1D). Employing the identical experimental approach and analysis methods, we investigated the relative expression of *his-41::gfp* mRNA in

HIS-41::GFP knock-in larval worms. We found that the relative expression level detected by random primers was only 0.7 times that of Oligo dT primers (Appendix Fig. S1D). These findings suggest that random primers are more suitable for detecting the relative expression of *his-4*, while Oligo dT primers are more appropriate for detecting the relative expression of HIS-41. This discrepancy may be attributed to the differences in their 3' UTR sequences, providing additional evidence for categorizing *his-4* as a replication-dependent histone and *his-41* as a replication-independent histone. We described these results in the new Appendix Fig. S1D and revised text on page #5, line #24:

"…The results of quantitative reverse transcription PCR (qRT-PCR) using random primers showed that the relative expression level of *gfp::his-4* mRNA was eight times higher compared to that detected by Oligo dT primers (Appendix Fig. S1D). Conversely, for *his-41::gfp* mRNA, the relative expression level detected by random primers was only 0.7 times that of Oligo dT primers (Appendix Fig. S1D). The discrepancy could be attributed to differences in their 3' UTR sequences, providing additional evidence for categorizing *his-4* as a replication-dependent histone and *his-41* as a replication-independent histone…."

2) My original comment: On p-10, this finding is reported: "Previous RNAi experiments revealed that inhibiting 172 genes among those downregulated ones caused embryonic or larval lethality (Supplemental Table S6), which suggests that the aberrant transcription program may account for the double mutant phenotypes." Does this overlap reflect a statistically significant enrichment of genes with this phenotype in this gene set? The authors should perform a hypergeometric test to compute a p-value for this (as is done for GO term analysis).

Author response: We thank the reviewer for pointing out these questions. As suggested by the reviewer, we have performed GO term analysis, the new results show these genes are associated with endoplasmic reticulum, mitochondrion and protein synthesis, which may be also involved in embryo development. Our hypergeometric test to compute a p-value for this revealed that this overlap reflects a statistically significant enrichment of genes with this phenotype in this gene set. The new data and statistical analysis were presented in Supplemental Table S7. We described the new Table S7 in the revised text on page 11, line 14:

Additional comment: Unfortunately, my original comment was misunderstood. My question is whether 172 genes causing lethality out of 759 down-regulated genes is a statistically significant result. That is, if you took a random set of 759 genes out of those detected in your expression analysis (not the whole genome, since detectable genes are more likely to have a phenotype) and asked how many of them have this phenotype would it be significantly lower than 172? The hypergeometric test is the way to test this. I mentioned GO term enrichments because it is the same statistical test (gene set enrichment analysis), not because I thought it was important to look at

GO terms for these 172 genes. The authors conclusion presumes that this is a significant enrichment of lethal genes among downregulated genes.

We apologize for misunderstanding this critique. We thank the Referee for explaining this issue. We have performed hypergeometric test and added the p-values to show that statistical significance in the revised Appendix Fig. S5D. We have now described the result in the revised text on page #11, line #28:

"…and this overlap reflects a statistically significant enrichment of genes with the phenotype (Appendix Fig. S5D and Dataset EV7)…."

Referee #3:

The revised manuscript by Zhu et al. entitled "Global H2B degradation regulates insulin/IGF signaling-mediated nutrient stress" has addressed all my initial concerns and has built upon many of my comments and those of the other reviewers. The manuscript is quite interesting and, through a molecular genetic tour de force, they clearly delineate the importance of this process in various developmental contexts and/or physiological responses.

We thank the Referee for the positive assessment of our study.

The language once again needs some minor work, which can be addressed by having the manuscript read and/or corrected by a native English speaker. I have listed below a number of minor issues that I came across and noted, but this list is not extensive and at some point I became less attentive and stopped. The authors should therefore make an earnest effort to identify the errors in the Text, Figure Legends, and in the Figures, to improve the grammar throughout.

We are grateful to the Referee for pointing out all these issues. In the latest version of the paper, we have made systematic grammatical corrections, which have significantly improved its readability.

Minor editorial issues:

Page 3, last paragraph: sequestrates should be sequesters

We have replaced "sequestrates" with "sequesters".

Page 5, last paragraph: "during these" is better than "dire conditions"

We have replaced "dire conditions" with "during these".

Page 6, first paragraph: remains abundant rather than maintains abundance

We have replaced “maintains abundance” with “remains abundant”.

Page 6, last paragraph: causing instead of rendering

We have replaced “rendering” with “causing”.

Page 7, second paragraph: remove the period and replace with a comma ", we examined..."
second paragraph: levels not amount ....were significantly increased

We have corrected these errors.

Page 13, second paragraph wild-type is an adjective; wild type is a noun

We thank for the Referee for pointing out this issue and have now have corrected these errors throughout the manuscript.

Page 13, last paragraph; remove "the" from "showed that the histone H2B turnover"...

We have removed the “the”.

Page 13, last paragraph; remove "the" from "the C. elegans larval development"...

We have removed the “the”.

Page 14, bottom first paragraph; remove "the" from "the epidermal cell differentiation"

We have removed the “the”.

Page 17, first paragraph; the last sentence is awkward and it is unclear what "the others may trigger" is actually referring to. This whole section could be rethought and rewritten to more effectively convey what the authors are trying to say.

We apologize for any confusion caused and deleted the last sentence.

Page 17, second paragraph; remove "the" from "the in vitro reconstitution..."

We have removed the “the”.

Page 17, last paragraph, last sentence; insert enzymes after E2 and E3

We have replaced “Quantifications” with “Quantification” throughout the manuscript. We have added the “enzymes”.

Page 18, second paragraph; remove "the" from "the proteasome degradation-associated..."

We have removed the “the”.

Page 22, nuclear and not "nucleus" co-immunoprecipitation lysis buffer???

We have replaced “nucleus” with “nuclear”.

Figure 1. Could mCherry be added to the label the appropriate figures. Also, in C, I assume that SK-TR are mutant variants? This is undefined in the legend and should be present to allow the reader to better understand the result/experiment in order to interpret the data independently.

We have added the label in the revised figure. We have presented the information of SK-TR in the figure legend on page #35, line #13:

“…HIS-41(SK-TR)::GFP indicates mutation of the serine 18 and lysine 27 residues of HIS-41 to threonine and arginine, respectively.”

Figure 2. Legend-RT-qPCR should be consistent with the text (either qRT-PCR (as described on page 5) or RT-qPCR). This could lead to some confusion.

We thank the reviewer for pointing out this issue. We have corrected the writing on this issue as qRT-PCR throughout the manuscript.

Figure 3 (Legend, page 37) Schematic of the purification (not purifiying)

We have corrected this error.

Figure 5D- Why is Ectopically written in pink only here?

We have converted the color of the font to black.

Figure 7A. in the schema-Lysis cells should be Lyse cells

We have replaced “Lysis” with “Lyse”.

Suppl Figures

Page 41-Legend Quantification not Quantifications; "could not detect" not detected

We have replaced “Quantifications” with “Quantification” throughout the manuscript.

2nd Revision - Editorial Decision **4th Aug 2023**

Dear Guangshuo,

Thank you for addressing the final points. I sincerely apologise for the delay in communicating the decision due to the high number of submissions we receive at the moment. I am now pleased to inform you that your manuscript has been accepted for publication.

Before we forward your manuscript to our publishers, there are a couple of points that need to be addressed:
1) In the final version we have lost the source data folder for Figure 8. I can recover the source data for the panel 8C from the previous version, but we would still need the files for figure 8B. You can forward these to me via email or a file sharing website.
2) I would like to propose some minor edits in the manuscript abstract (please see the attached file). I have also written a short blurb that will accompany the title of your manuscript in our online system. Please let me know if any corrections are needed:

Blurb:
Ubiquitin-proteasome system regulates histone H2B exchange in mammalian cells and in C. elegans during development and starvation.

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Finally, we would like to promote your manuscript among the Chinese readership. Therefore, we would like to invite you to prepare a short summary of the manuscript in Chinese (1500-2000 Chinese characters), which we will promote on the WeChat platform 'BioArt' with 440,000 followers.

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I have included below some general guidelines on how to prepare a summary and a link to recent examples for your reference. Please let me know if you have any questions about this.

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Best wishes,

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General WeChat Summary Guidelines

1. These summary articles are meant to be targeting general audience so please limit the use of specialized technical terms, acronyms and jargon.
2. A summary usually starts with brief background information of the reported work, which is followed by explaining the findings in some detail, and ends with a short review of the conclusions as well as the implications of the work and future directions for the research.
3. The summary should at least contain one graphical item, such as a scheme or a figure from the paper.
4. Please provide ONE SINGLE document containing all text and graphical materials, ideally as a Word.docx or .doc file. Please DO NOT provide the document as a .pdf file.
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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 manuscript.
Please note that a copy of this checklist will be published alongside your article.

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 χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
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Report the **clinical trial registration number** (at ClinicalTrials.gov or equivalent), where applicable.	Not Applicable	

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Include a statement about **sample size** estimate even if no statistical methods were used.	Yes	Materials and Methods, and Figure legends
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?	Yes	Materials and Methods
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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	Materials and Methods
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