Sequestration to lipid droplets promotes histone availability by preventing turnover of excess histones

Roxan A. Stephenson, Jonathon M. Thomalla, Lili Chen, Petra Kolkhof, Roger P. White, Mathias Beller and Michael A. Welte
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MS TITLE: Sequestration to lipid droplets promotes histone availability by preventing turnover of excess histones

AUTHORS: Roxan Amanda Stephenson, Jonathon M Thomalla, Lili Chen, Petra Kolkhof, Mathias Beller, and Michael Andres Welte

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

The overall evaluation is positive and we would like to publish a revised manuscript in Development, provided that the referees' comments can be satisfactorily addressed. Please attend to all of the reviewers' comments in your revised manuscript and detail them in your point-by-point response. If you do not agree with any of their criticisms or suggestions explain clearly why this is so.

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

Reviewer 1

Advance summary and potential significance to field

Here, the authors characterize the regulation of histone protein levels during the late stages of Drosophila oogenesis. Stemming from their previous work using embryos, the authors observe Jabba-mediated interactions between histone proteins and lipid droplets (LDs). Jabba appears to

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prevent the degradation of histone proteins as oocytes mature. Modest increases of labeled histones upon MG132 treatment suggest that loss of histones in the absence of Jabba depends on the proteasome. H2Av levels in stage 14 oocytes scale with Jabba protein levels, suggesting that Jabba may physically protect H2Av from degradation.
Transgenic analysis shows that deleting a stretch of positive amino acids from Jabba prevents its binding to and protection of histones in vivo.

Comments for the author

In general, the presented data are solid and support the general conclusions of the authors. The manuscript is well-written and the logic of the experimental design and interpretations of the data are clearly explained.

Previous work from the Welte lab has shown that LDs and Jabba help to buffer histone levels in early embryos. One potential weakness of the paper is that the novelty of the results is reduced by these previous studies. Nevertheless, this current work extends our understanding and shows that (1) LD mediated transport plays only a minor role in setting the levels of histones in the oocyte, (2) a majority of histone proteins are synthesized in the oocyte, and (3) Jabba prevents degradation of these newly synthesized histones during the final stages of oogenesis. I believe this work will be of interest to the readers of Development and is appropriate for the journal. However, several points should be addressed before publication.

One major conclusion of the paper is that Jabba prevents the degradation of histones, but the authors do not provide compelling insights into the mechanism. The authors present some data that the proteasome may be responsible for this degradation, as has been observed in other contexts. However, in "data not shown", the authors find no evidence that histone proteins are ubiquitinated in Jabba mutants.

Moreover, MG132 treatment only leads to a modest increase in mean fluorescence intensity of tagged histone proteins in a Jabba mutant background. Here, the authors should assay a control ubiquitinated protein to better evaluate the degree to which they are inhibiting proteasome activity under their experimental conditions. Does the modest increase in histone levels directly track with an incremental repression of proteasome activity?

Along the same lines, the authors state that "initial attempts to inhibit proteasome function genetically with temperature-sensitive mutants (Neuburger et al., 2006; Smyth & Belote, 1999) proved unsuccessful as we failed to identify conditions that supported development to stage 14 and increased H2Av levels."

Perhaps the authors could try to use temperature shifts on females that carry stored stage 14 eggs (see Elife 2019 Nov 22;8:e49455.doi: 10.7554/eLife.49455) to avoid problems with oocyte development. Stage 14 eggs appear to continue to synthesize histone proteins, so genetically inhibiting proteasome activity in stored mutant eggs may yield more robust results.
If further functional assays fail to reveal a major role for proteasome activity in regulating histone levels within the oocyte, the authors should re-state their claims regarding the importance of this specific mechanism. Alternative mechanisms could be considered experimentally, or at the very least discussed further in the text.

Minor: The fluorescence in Figure 7A' is difficult to see. The authors should consider pseudo-coloring the blue Jabba signal green.

Reviewer 2

Advance summary and potential significance to field

Many animal zygotes rely on a large supply of maternally provided histone to support genome duplication in the absence of transcription during early development. In this manuscript, Stephenson et al. investigate the interesting question of how histone proteins are produced and stored during Drosophila oogenesis. The Welte lab has shown previously that in the early embryo

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the Jabba protein functions as a storage depot for maternal histones by binding to and sequestering H2a and H2b proteins in cytoplasmic lipid droplets. Here they examine the role of Jabba during oogenesis using primarily quantitative microscopic approaches and conclude that Jabba functions in the accumulation of H2a and H2b proteins in the oocyte by protecting these proteins from proteosome-mediated degradation. They further show that this function of Jabba requires Jabba binding both to H2 type histones and to lipid droplets. They also demonstrate that much of H2 type histones in the oocyte is synthesized in the oocyte rather than being transported via Jabba from the nurse cells, addressing a longstanding developmental question of exactly where maternal histones originate in Drosophila. All of the experiments are well performed and quantified, and the data are clearly presented and support the conclusions.

Comments for the author

Minor points:
In Figure 3B and 3C I suggest indicating H2Av-RFP as for H2B-mEOS3.2 in Figure 3D.
Figure 5 Supplemental Figure 1 panel B should be in the main Figure 5. Those data are the most convincing.

Figure 7A' fluorescent western is a little difficult to see as presented; brightness and/or contrast needs to increase.

A brand new paper published after submission of this manuscript (PMID: 33408246) describes histone gene expression during Drosophila oogenesis. Perhaps the authors could include this in their discussion of histone biosynthesis during oogenesis if they feel it's appropriate.

This line in the Discussion doesn't seem entirely accurate: "Each of the NC nuclei have been estimated to contain about 500 times more DNA than diploid nuclei (King, 1970); the 900 follicle cells (Fadiga & Nystul, 2019) contain DNA levels ranging from 8- 16 times more DNA than a diploid nucleus (Mulligan & Rasch, 1985)." e.g. The follicle cells reach a ploidy of 16C, with is 8 times the level of DNA in a 2C, diploid nucleus, not 16 times as stated.
Is the LD associated H2Av pool in the early stage nurse cells supporting nurse cell endoreplication? This might fit with the measurements that very little of this pool is transferred to the oocyte and most of the oocyte H2Av is synthesized within the oocyte.

Reviewer 3

Advance summary and potential significance to field

This manuscript by Stephenson and colleagues explores the mechanism by which Drosophila oocytes accumulate vast quantities of histones during oogenesis, and a role for Jabba and lipid droplets (LDs) in regulating this accumulation by preventing histone degradation. This group has previously described the role of Jabba and LDs in regulating histones in early Drosophila embryos (e.g., Johnson et al., eLife 2018), but the focus on mechanisms of LD/Jabba transport from nurse cells (NCs) to the oocyte is new in this study. The authors begin by characterizing the increase in H2Av levels during oogenesis using quantitative fluorescence microscopy. The dependence of this increase on Jabba is supported by elegant experiments quantifying H2Av levels after knocking out or manipulating the gene dose of Jabba. Rigorous assessment of mRNA levels and polysome-bound mRNA between WT and Jabba knockouts rule out a role for Jabba in transcriptional or translational regulation of histones. Furthermore, experiments demonstrating the requirements of both the LD and histone binding domains of Jabba for H2Av accumulation in the oocyte were beautiful and further validated the necessity of Jabba-dependent LD sequestration for histone accumulation.

Comments for the author

Major points:
There are two areas in which additional experiments will significantly strengthen the conclusions of the authors:
1. The authors rule out the contribution of LD/Jabba-mediated histone transport from NCs to the oocyte by showing no change in LD levels between stages 12 and 14. They attempt to

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approximate the contribution of LD-mediated transport indirectly by comparing H2Av-RFP levels between WT and Jabba knock-out. However they do not directly assess what fraction of histone protein in the oocyte originated from the NCs at each stage. The authors could take advantage of their H2B-mEos3.2 line (or their H2Av-Dendra2 line from the Johnson et al. 2018 paper) and quantitatively determine how much histone is coming from the NCs. This would involve photoconverting the NC pool of histone protein and quantifying the accumulation of this NC-derived pool relative to the total pool in the oocyte via live-cell imaging.
2. The authors argue that, in the absence of Jabba, histones are degraded by the proteasome, yet they see only a modest increase in H2Av upon MG132 treatment. The small effect of proteasomal inhibition on H2Av levels in oocytes could be due to partial inhibition as the authors propose, or H2Av could be degraded by another mechanism. In some cases, LD proteins can be degraded by the lysosome (see Schulze et al. 2020 in PNAS). Therefore, it is possible that LD-bound histones are degraded through this pathway. A similar experiment as shown in Figure 5 should be performed with pharmacological inhibition of lysosomal acidification. This is the most important point to address.

Minor points:
1. It would benefit the reader if an LD marker were included in Figure 2A to better demonstrate the relationship between H2Av and LD levels at each stage. Furthermore, presenting the data in Figure 8A at a higher magnification with an LD marker would more conclusively show that deletion of residues 192-321 ablates LD binding.
In sum, this is an excellent paper that advances the field’s understanding of the role of lipid droplets during development. Additional experiments will strengthen the model proposed by the authors and improve an already strong piece of work.

First revision

Author response to reviewers' comments

Response to Reviewers

We thank the reviewers for their careful and very positive reviews. We were pleased that the reviewers had high praise, using comments such as “elegant experiments”, “rigorous assessment”, and “beautiful [experiments]”, “well written“ and felt that “All of the experiments are well performed and quantified, and the data are clearly presented and support the conclusions” and that “this is an excellent paper that advances the field’s understanding of the role of lipid droplets during development.”

The main concern raised by reviewers #1 and #3 is that the effect of proteasome inhibition is only modest. We believe that the argument for a major effect of the proteasome is much stronger but was not obvious, in part because the way we presented the data obscured the effect size. Our reanalysis of the data shows that the proteasome inhibitor MG132 inhibits 42% of H2Av turnover in *Jabba* mutants, even at concentrations that very likely only partially inhibit the proteasome. This means that the proteasome makes a major contribution to H2Av turnover. We are thankful to the reviewers that their criticism prompted us to determine the quantitative estimate of this effect. We explain the details of our reanalysis in the response to reviewer #1.

Reviewer #1

Concern:
One major conclusion of the paper is that Jabba prevents the degradation of histones, but the authors do not provide compelling insights into the mechanism. The authors present some data that the proteasome may be responsible for this degradation, as has been observed in other contexts. However, in “data not shown”, the authors find no evidence that histone proteins are ubiquitinated in Jabba mutants.

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Response:
We realize now that our statement about lack of ubiquitination was misleading, for two reasons. First, the reviewer seemed to interpret this statement that it throws doubt on the involvement of the proteasome. That is not the case because certain proteins are indeed known to be turned over by the proteasome without being ubiquitinated. Examples are discussed in the references in the relevant paragraph in the text. Second, we were mistaken about the weight of our observation. It turns out that even for proteins known to be turned over solely via ubiquitin-dependent degradation, the accumulation of modified, ubiquitinated bands is relatively minor, and it is the major, non-modified band that heavily accumulates. Thus, our observation that no ubiquitinated bands were detected cannot be interpreted. We have deleted this observation from the text and apologize that this statement caused confusion.

Concern:
Moreover, MG132 treatment only leads to a modest increase in mean fluorescence intensity of tagged histone proteins in a Jabba mutant background.

Response:

As mentioned in our introduction to the Response to reviewers, our reanalysis of our data shows the proteasome inhibitor MG132 inhibits 42% of H2Av turnover, even at concentrations that very likely only partially inhibit the proteasome. So the contribution of the proteasome is not modest, but large. This effect size was previously masked because of the way we had presented the data.

To arrive at our conclusion, we re-analyzed the data in Fig. 5B/Fig.5- Supplemental Fig.1. We have included these data as Fig.5-Supplemental Fig. 2. This analysis is detailed for the 2.5 µg/ml MG132 concentration in panel A of that figure; panel B shows the outcome of this analysis for all concentrations. The analysis in panel A is as follows: In the presence of the drug, H2Av levels in the wild type rise by ~1839 AU from stage 12 to stage 14. A similar rise in Jabba egg chambers would lead to a level of B = ~3,481 AU (=1642 in stage 12 + 1839).
Without drug, levels in Jabba fall instead to ~606 AU = D. Thus, if the drug had no effect on turnover, we would expect histone levels close to D (0% effect); if it completely stopped turnover, we might see the same rise in the wild type, and thus observe B (100 % effect). The observed value is C, which represents 42% of the maximal possible effect. In panel B, the effect is indeed 0% for no drug and then steadily rises to 42% at 2.5 µg/ml and then falls again, likely because the drug starts interfering with development.

There, we conclude that that our experiment show that the proteasome is at least responsible for 42% of the histone turnover in *Jabba* mutants, and quite likely more, given the limitations of the experimental set up. We believe this analysis supports the conclusion that Jabba indeed prevents turnover of histones by some mechanism and that a major contributor to turnover is a proteasome-dependent pathway. We have also included in the discussion that the other 58% could, in principle, be due to a different pathway, such as lysosomal degradation.

We have clarified in the discussion that our experiments only mean that the proteasome makes a considerable contribution and that we cannot rule out that other pathways are also involved.

Concern:

Here, the authors should assay a control ubiquitinated protein to better evaluate the degree to which they are inhibiting proteasome activity under their experimental conditions. Does the modest increase in histone levels directly track with an incremental repression of proteasome activity?

Response:
This is in principle a good experiment, but it is not clear what test protein to use. It would have to be one that is known to be only degraded by the proteasome pathway from stage 12 to 14. Establishing this baseline is already a complicated, non-trivial task.

In addition, the only person able to do these experiments is Roxan Stephenson, and no one else

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in the laboratory is equipped to get the assay up and running quickly. These experiments rely on the tricky in vitro egg maturation assay.
Roxan has started a postdoc at NIH. As a resident of Maryland, traveling back to Rochester would until recently have required extensive quarantining for both legs of the trip and thus a major time away from work. A potential visit to the University of Rochester was further complicated by the University's visitor restriction policies due to COVID-19, which are not yet fully lifted. Thus, in the time we had for revisions, travel restrictions between Maryland and New York State and restriction on visitors to the University of Rochester made a trip by Roxan practically impossible.

But as described above, we believe our reanalysis of the effect size of the MG132 treatment make such experiments no longer necessary, especially since we now explicitly acknowledge that other mechanisms might contribute to turnover.

Concern:
Along the same lines, the authors state that "initial attempts to inhibit proteasome function genetically with temperature-sensitive mutants (Neuburger et al., 2006; Smyth & Belote, 1999) proved unsuccessful as we failed to identify conditions that supported development to stage 14 and increased H2Av levels."
Perhaps the authors could try to use temperature shifts on females that carry stored stage 14 eggs (see Elife 2019 Nov 22;8:e49455.doi: 10.7554/eLife.49455) to avoid problems with oocyte development. Stage 14 eggs appear to continue to synthesize histone proteins, so genetically inhibiting proteasome activity in stored mutant eggs may yield more robust results.

Response:
We impaired the proteasome using a mutant form of the proteasome subunit ß2, also called *DTS7*. We generated flies expressing these temperature-sensitive mutants in both WT and *Jabba*$^{-/-}$ backgrounds, either endogenously or using the Gal4/UAS system. We performed temperature shifts using the oocyte aging strategy discussed in Greenblatt et al., 2019 and collected stage 14 egg chambers from these flies to measure histone levels by western analysis. When *DTS7* was expressed in a *Jabba*$^{-/-}$ background, H2Av levels were increased at 29°C relative to 25°C. While the difference between *Jabba*$^{-/-}$*; UAS-DTS7* at 29°C versus 25°C is statistically significant, the difference between *Jabba*$^{-/-}$*; DTS7* at 29°C relative to 25°C is trending towards significance. Even though the magnitude of the effect is hard to estimate based on the variability in the wild type control, these data do provide independent evidence that the proteasome is involved in the turnover of H2Av in *Jabba* mutants.

Concern:
If further functional assays fail to reveal a major role for proteasome activity in regulating histone levels within the oocyte, the authors should re-state their claims regarding the importance of this specific mechanism. Alternative mechanisms could be considered experimentally, or at the very least discussed further in the text.

Response:
We believe our new quantitative analysis of our previous data does already demonstrate a major role for proteasome activity. We have included in the discussion that the remaining 58% of turnover could be due to non-proteasomal mechanisms. As discussed above, we have also performed experiments with temperature-sensitive proteasome mutants, where we find that inhibiting proteasome activity increases H2Av levels. These observations support our conclusions that in the absence of Jabba H2Av is turned over by the proteasome.

Concern:
The fluorescence in Figure 7A' is difficult to see. The authors should consider pseudo-coloring the blue Jabba signal green.

Response:
We have pseudo-colored the blue Jabba signal in green.

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Reviewer #2

Concern:
In Figure 3B and 3C I suggest indicating H2Av-RFP as for H2B-mEOS3.2 in Figure 3D.

Response:
We have made the requested change.

Concern:
Figure 5 Supplemental Figure 1 panel B should be in the main Figure 5. Those data are the most convincing.

Response:
We have made the requested change.

Concern:
Figure 7A' fluorescent western is a little difficult to see as presented; brightness and/or contrast needs to increase.

Response:
We have made the requested change.

Concern:
A new paper published after submission of this manuscript (PMID: 33408246) describes histone gene expression during Drosophila oogenesis. Perhaps the authors could include this in their discussion of histone biosynthesis during oogenesis if they feel it's appropriate.

Response:
We have made requested change.

Concern:
This line in the Discussion doesn't seem entirely accurate: "Each of the NC nuclei have been estimated to contain about 500 times more DNA than diploid nuclei (King, 1970); the 900 follicle cells (Fadiga & Nystul, 2019) contain DNA levels ranging from 8-16 times more DNA than a diploid nucleus (Mulligan & Rasch, 1985)." e.g. The follicle cells reach a ploidy of 16C, with is 8 times the level of DNA in a 2C, diploid nucleus, not 16 times as stated.

Response:
We have made the requested change.

Concern:
Is the LD associated H2Av pool in the early-stage nurse cells supporting nurse cell endoreplication? This might fit with the measurements that very little of this pool is transferred to the oocyte and most of the oocyte H2Av is synthesized within the oocyte.

Response:

This is an interesting idea! We have included this as speculation in the discussion.

Reviewer #3:

Concern:
1. The authors rule out the contribution of LD/Jabba-mediated histone transport from NCs to the oocyte by showing no change in LD levels between stages 12 and 14. They attempt to approximate the contribution of LD-mediated transport indirectly by comparing H2Av-RFP levels between WT and Jabba knock-out. However, they do not directly assess what fraction of histone protein in the oocyte originated from the NCs at each stage. The authors could take advantage of their H2B-mEos3.2 line (or their H2Av-Dendra2 line from the Johnson et al. 2018 paper) and quantitatively determine how much histone is coming from the NCs. This would involve photoconverting the NC pool of histone protein and quantifying the accumulation of this NC-

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derived pool relative to the total pool in the oocyte via live-cell imaging.

Response:
The proposed experiment would not address the contribution of LD-mediated transport specifically, because photoconversion would happen in both cytosol and on lipid droplets. Thus, it would measure overall histone transport from nurse cells, something for which our current analysis already provides an upper bound. In addition, because of the huge volume of the nurse cell cytoplasm, it is technically challenging to convert a majority (or a major fraction) of the LD-associated H2Av in the nurse cells. Given that the egg chamber is very thick, much of the volume is not readily accessible for photoconversion. Finally, it is unclear whether a fraction of the histone pool in nurse cell nuclei exchanges with the cytoplasmic histone pool before transfer into the ooplasm. While we agree that it would be beneficial to know exactly how much histone is transferred, this is beyond the scope of our manuscript.

Concern:
2. The authors argue that, in the absence of Jabba, histones are degraded by the proteasome, yet they see only a modest increase in H2Av upon MG132 treatment. The small effect of proteasomal inhibition on H2Av levels in oocytes could be due to partial inhibition as the authors propose, or H2Av could be degraded by another mechanism. In some cases, LD proteins can be degraded by the lysosome (see Schulze et al. 2020 in PNAS). Therefore, it is possible that LD-bound histones are degraded through this pathway. A similar experiment as shown in Figure 5 should be performed with pharmacological inhibition of lysosomal acidification. This is the most important point to address.

Response: As discussed in the cover letter and the response to reviewer #1 who had raised similar concerns, we believe that our re-analysis shows that the MG132 effect is not modest, but substantial. The reviewer makes a valuable point that the LD-histones may be degraded by the lysosome as in Schulze *et al*., 2020. However, due to COVID restrictions, we were unable to perform the lysosome experiment within a reasonable timeframe (see also response to Reviewer #1). While we acknowledge the lysosome as an alternative possibility, we see no evidence of lipid turnover (as implied in the Schulze paper): *Jabba* mutant embryos have normal lipid droplet content (Li *et al*., 2012).

Concern:
1. It would benefit the reader if an LD marker were included in Figure 2A to better demonstrate the relationship between H2Av and LD levels at each stage.

Response: We stained ovaries expressing H2Av-RFP with the LD dye BODIPY and repeated the experiment in Figure 2A to better demonstrate the relationship between H2Av and LDs at each stage. These data are provided in the new Fig.2- Supplemental Figure 1.

Concern:
Furthermore, presenting the data in Figure 8A at a higher magnification with an LD marker would more conclusively show that deletion of residues 192-321 ablates LD binding.

Response
We generated images at higher magnification and provide them in the new Fig. 8-Supplemental Fig. 1.

Second decision letter

MS ID#: DEVELOP/2020/199381

MS TITLE: Sequestration to lipid droplets promotes histone availability by preventing turnover of excess histones

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AUTHORS: Roxan Amanda Stephenson, Jonathon M Thomalla, Lili Chen, Petra Kolkhof, Roger P White, Mathias Beller, and Michael Andres Welte
ARTICLE TYPE: Research Article

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

Reviewer 1

Advance summary and potential significance to field

Here, the authors characterize the regulation of histone protein levels during the late stages of Drosophila oogenesis. Stemming from their previous work using embryos, the authors observe Jabba-mediated interactions between histone proteins and lipid droplets (LDs). Jabba appears to prevent the degradation of histone proteins as oocytes mature. Increases of labeled histones upon MG132 treatment suggest that loss of histones in the absence of Jabba depends on the proteasome. H2Av levels in stage 14 oocytes scale with Jabba protein levels, suggesting that Jabba may physically protect H2Av from degradation. Transgenic analysis shows that deleting a stretch of positive amino acids from Jabba prevents its binding to and protection of histones in vivo.
In the first submission, there were shared concerns regarding the mechanism proposed to regulate H2Av levels in the absence of Jabba. However, reanalysis and new experiments performed by the authors have addressed these critiques.

Comments for the author

The authors have addressed my previous comments and concerns. I find the paper acceptable for publication.

Reviewer 2

Advance summary and potential significance to field

This manuscript provides advances in our understanding of how histone proteins are deposited into eggs in order to support early development.

Comments for the author

I find the response to all reviews adequate and support publication in Development.

Reviewer 3

Advance summary and potential significance to field

This manuscript by Stephenson and colleagues explores the mechanism by which Drosophila oocytes accumulate vast quantities of histones during oogenesis, and a role for Jabba and lipid droplets (LDs) in regulating this accumulation by preventing histone degradation. This group has previously described the role of Jabba and LDs in regulating histones in early Drosophila embryos (e.g., Johnson et al., eLife 2018), but the focus on mechanisms of LD/Jabba transport from nurse cells (NCs) to the oocyte is new in this study. The authors begin by characterizing the increase in H2Av levels during oogenesis using quantitative fluorescence microscopy. The dependence of this increase on Jabba is supported by elegant experiments quantifying H2Av levels after knocking out or manipulating the gene dose of Jabba. Rigorous assessment of mRNA levels and polysome-bound mRNA between WT and Jabba knockouts rule out a role for Jabba in transcriptional or translational regulation of histones. Furthermore, experiments demonstrating the requirements of both the LD and histone binding domains of Jabba for H2Av accumulation in the oocyte were beautiful and further validated the necessity of Jabba-dependent LD sequestration for histone accumulation.

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Comments for the author

The authors have thoughtfully responded to our comments. We support publication of this manuscript in Development. The authors are congratulated on completing an interesting and high-quality study.

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