Juvenile hormone acts through FoxO to promote Cdc2 and Orc5 transcription for polyploidy-dependent vitellogenesis

Zhongxia Wu, Qiongjie He, Baojuan Zeng, Haodan Zhou and Shutang Zhou
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MS TITLE: Juvenile hormone acts through FoxO to promote Cdc2 and Orc5 transcription for polyploidy-dependent vitellogenesis

AUTHORS: Zhongxia Wu, Qiongjie He, Haodan Zhou, and Shutang Zhou

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

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

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

Please attend to all of the reviewers' comments and ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion. I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

Summary: In this manuscript, Wu and colleagues provide clear and thorough data describing the molecular interactions employed by JH to control polyploidization in the fat body and ultimately vitellogenesis and oocyte maturation in Locusta migratoria. They show that JH promotes protein expression/stability of LCMT1, which methylates PP2A to suppress its phosphatase activity on FoxO. Moreover, they identify two targets of FoxO, Cdc2 and Orc5 that provide the cellular (polyploidization) and molecular (cell cycle progression and DNA replication) links between JH signaling and synchronous oocyte maturation.

Significance to the field: Prior to this work, the molecular mechanism of JH action in promoting oocyte maturation and polyploidization had been poorly understood. The authors have described how JH controls FoxO phosphorylation (by regulating the expression of LCMT1 which methylates PP2A so that it no longer dephosphorylates FoxO). In addition the authors have identified Cdc2 and Orc5 as targets of FoxO that control polyploidization in the fat body and thus, the ability of fat tissue to synthesize substantial quantities of Vg to support oocyte maturation.

Comments for the author

Summary
In this manuscript, Wu and colleagues provide clear and thorough data describing the molecular interactions employed by JH to control polyploidization in the fat body and ultimately vitellogenesis and oocyte maturation in *Locusta migratoria*. They show that JH promotes protein expression/stability of LCMT1, which methylates PP2A to suppress its phosphatase activity on FoxO. Moreover, they identify two targets of FoxO, Cdc2 and Orc5, that provide the cellular (polyploidization) and molecular (cell cycle progression and DNA replication) links between JH signaling and synchronous oocyte maturation.

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Line-by-line comments
Line 168: To be clearer about what is being evaluated add “in p-PP2A level” after “no apparent change was observed”.

Lines 168-170: Two points of clarification should be made regarding the data the authors used to arrive at the conclusion that “reduced levels of p-FoxO during vitellogenesis is unlikely an effect of Akt”. First, by correlating p-PP2A and p-Akt levels, the authors are testing if Akt leads to reduced levels of phosphorylated FoxO by inhibitory phosphorylation of PP2A. This is in contrast to Akt’s known role in phosphorylating FoxO. Did the authors consider briefly discussing the role of direct phosphorylation of FoxO by Akt? Second, p-FoxO levels decline after 3 days PAE (Fig. 1C) while p-Akt levels remain elevated 2 days PAE (Fig. 2A). In this context, if Akt directly phosphorylates FoxO, we would expect that p-FoxO levels would also remain elevated during this period. Thus, the data supports the authors’ conclusion but a sentence or two highlighting this connection would make it more obvious to the reader.

Lines 200-201: Are there any ideas on how JH is regulating LCMT1 protein level? Transcriptionally? Post-transcriptionally (translation)? Post-translationally (protein stability)? The authors could measure transcript levels for LCMT1 in fat body from precocene- and methoprene-treated females.

Line 221: Is this a true measure of egg production? Or is it more accurate to say vitellogenesis and/or oocyte maturation? Egg production could be measured by counting the number of eggs laid per cycle. If egg production is indeed influenced by knockdown of FoxO, then fewer eggs would be laid.

Conversely, if vitellogenesis/oocyte maturation but not egg production were influenced by FoxO knockdown, then equal numbers of small eggs would be produced.

Lines 227: It is slightly unclear what was done. Does this mean that expression of all 29 (or the remaining 24) genes was measured under conditions of FoxO knockdown? If so, this can be made clearer by stating that of the 29 genes upregulated by JH, only two, *Cdc2* and *Orc5* showed a reduction in transcript levels upon FoxO knockdown. Additionally, the data for all can be shown in a supplementary figure.

Line 229: Mention somewhere in this paragraph the mammalian/human orthologs for Cdc2 and Orc5.

Lines 247-253: For Figures 6F and 6G, lanes can be labeled and referred to in the main text to direct the reader to specific comparisons, making it easier to understand. For example, at the end of the sentence that starts on Line 247, the authors can write in parentheses “lanes 2” or “compare lanes 1 and 2”

Lines 260-263: Is the differences in ploidy shift between Cdc2 and Orc5 knockdown indicative of which factor may play a more important role in oocyte maturation? It does appear to correlate with severity of the oocyte size phenotype. Cdc2 knockdowns are less polyploid than Orc5 knockdowns and show a lower length*width index than Orc5. Relatedly, it is interesting that the FoxO knockdown oocyte length*width phenotype is slightly less severe than the individual Cdc2 or Orc5 knockdown phenotypes while the reduction in VgA and VgB protein level is the reverse (more severe in the FoxO knockdown than individual Cdc2 or Orc5 knockdowns).

Line 271: Regarding use of the phrase “egg production”, see comment for Line 221. Lines 330-333: Instead of repeating the results, summarize the finding.
Lines 333-334: Briefly explain how Cdc2 regulates the cell cycle (i.e. cell cycle progression).

Lines 337-338: This is a good place to bring up the difference in phenotypes for Cdc2 and Orc5 described in the comments for Lines 260-263.

Line 396: Instead of “orchestrate”, I think the authors mean to write “cooperate”

Comments on Figures
Figures 5B and 7B: Add labels within or below images for immunofluorescence markers. Figure 6A: If possible, horizontal labels would be easier to read.
Figure 6F and 6G: Add lane labels.

Reviewer 2

Advance summary and potential significance to field

This is a very interesting paper that explores the role that juvenile hormone (JH) plays in stimulating polyploidization of the fat body and vitellogenesis via the forkhead transcription factor FOXO, using the locust Locusta migratoria as a model organism. Specifically the authors show that JH-stimulated FoxO dephosphorylation act through LCMT1-mediated activation of PP2A. They also present evidence that FoxO activates the transcription of cell-division-cycle 2 (Cdc2) and origin-recognition-complex subunit 5 (Orc5), consequently stimulating fat-body cell polyploidization and Vg synthesis necessary to support oogenesis. Their data are convincing and generally support their model of the JH-regulation of fat body polyploidization and vitellogenesis through FOXO, which is a novel and exciting finding. This fits with a growing understanding of the way JH acts on physiology, and particularly how it works with other hormone signaling pathway for example the insulin-signaling pathway, which also targets FOXO. For this reason I think that the paper is of sufficient general interest to be published in Development.

Comments for the author

While the paper is very interesting, I have some questions/concerns about the data and the manuscript that the authors should address before publication:

1) The authors show that phosphorylated-FOXO levels initially rise in young adults but then declines, as JH titers increase (Fig 1C). They subsequently show that treatment with methoprene in 10 day-old adults in which p-FOXO levels are usually low is sufficient to initially stimulate FOXO phosphorylation, which then subsequently falls. They interpret these data as evidence that "short-term exposure of exogenous JH may activate signaling cascades triggering FoxO phosphorylation, whereas prolonged treatment of JH may stimulate FoxO dephosphorylation". This interpretation would be better supported if the authors showed that precocene treatment suppressed the peak in p-FOXO in early adults. This would therefore show that JH is not just sufficient to drive the p-FOXO pulse, but necessary during normal physiology.

3) Related to this, while the application of precocene does not affect the level of p-FOXO 10 days PAE, at the same time point, precocene appears to eliminate the methylation of PP2 (Fig 3C) and the expression of LCMT1 (Fig 4F). The authors also show that knockdown of both PP2 and LCMT1 is sufficient to increase p-FOXO levels 8 days PAE. Why is the knockdown of PP2 and LCMT1 sufficient to increase p-FOXO levels 8 days PAE, while eliminating them by suppressing JH through precocene treatment is insufficient to increase p-FOXO levels 10 days PAE? If these results are correct, it suggests that there is another mechanism, apart from JH, suppressing p-FOXO accumulation via LCMT1 and PP2 10 days PAE. The authors should address these inconsistencies in their data since they do not fit with the simple hypothesis that JH regulates p-FOXO levels via LCMT1 and PP2.

3) Why in Figure 1C does it appear that p-FOXO levels are fall to near zero 7 days PAE, and are zero at 10 days PAE, but control fat bodies show quite high levels of p-FOXO 8 days PAE (Figure 3C and 4F)? These suggest that there are either experiment-to-experiment variation in p-FOXO level or that p-FOXO levels fluctuate between 7 and 10 days PAE. Again, such temporal inconsistencies should be addressed by the authors.

4) The authors should include the data points, not just the means, in their bar graphs (see https://www.nature.com/articles/s41551-017-0079 for a compelling discussion of this), and must specify the meaning of the error bars and more details of the test used to compare the means (was the t-test pooled or unpooled?). Also, are the authors planning on releasing their data to Dryad or some other depository?

5) The authors show that changes in JH levels, and PP2 and LCMT1 activity can all change p-FOXO levels, and that JH levels affect PP2 methylation and LCMt1 levels, but they do not include an epistatic analysis confirming that JH acts through PP2 and LCMT1 to suppress p-FOXO levels. Specifically, they should show that the effect of the methoprene treatment on the dynamics of the p-FOXO (Figure 1D) levels are eliminated in adults with knockdown of PP2 and LCMT1.

6) Further, they do not show the effects of precocene treatment, or PP2 or LCMT1 knockdown on fat cell polyploidization, vitellogenesis and egg production. Such tests are necessary to show that the changes in the level of p-FOXO that they are observing are physiologically meaningful with regard to the phenotype they are interested in.

First revision

Author response to reviewers' comments

Response to reviewers' comments
Reviewer #1
Summary

In this manuscript, Wu and colleagues provide clear and thorough data describing the molecular interactions employed by JH to control polyploidization in the fat body and ultimately vitellogenesis and oocyte maturation in Locusta migratoria. They show that JH promotes protein expression/stability of LCMT1, which methylates PP2A to suppress its phosphatase activity on FoxO. Moreover, they identify two targets of FoxO, Cdc2 and Orc5, that provide the cellular (polyploidization) and molecular (cell cycle progression and DNA replication) links between JH signaling and synchronous oocyte maturation.
Significance to the field
Prior to this work, the molecular mechanism of JH action in promoting oocyte maturation and polyploidization had been poorly understood. The authors have described how JH controls FoxO phosphorylation (by regulating the expression of LCMT1 which methylates PP2A so that it no longer dephosphorylates FoxO). In addition, the authors have identified Cdc2 and Orc5 as targets of FoxO that control polyploidization in the fat body, and thus, the ability of fat tissue to synthesize substantial quantities of Vg to support oocyte maturation.
Line-by-line comments
Line 168: To be clearer about what is being evaluated add “in p-PP2A level” after “no apparent change was observed”.

We have made the suggested revision with thanks to the reviewer.

Lines 168-170: Two points of clarification should be made regarding the data the authors used to arrive at the conclusion that “reduced levels of p-FoxO during vitellogenesis is unlikely an effect of Akt”. First, by correlating p-PP2A and p-Akt levels, the authors are testing if Akt leads to reduced levels of phosphorylated FoxO by inhibitory phosphorylation of PP2A. This is in contrast to Akt’s known role in phosphorylating FoxO. Did the authors consider briefly discussing the role of direct phosphorylation of FoxO by Akt? Second, p-FoxO levels decline after 3 days PAE (Fig. 1C) while p-Akt levels remain elevated 2 days PAE (Fig. 2A). In this context, if Akt directly phosphorylates FoxO, we would expect that p-FoxO levels would also remain elevated during this period. Thus, the data supports the authors’ conclusion but a sentence or two highlighting this connection would make it more obvious to the reader.

We thank the reviewer for these comments. Following the reviewer’s suggestion, we have revised the descriptions. The points are now clearly clarified.

Lines 200-201: Are there any ideas on how JH is regulating LCMT1 protein level? Transcriptionally? Post-transcriptionally (translation)? Post-translationally (protein stability)? The authors could measure transcript levels for LCMT1 in fat body from precocene- and methoprene-treated females.

We measured the dynamics of LCMT1 expression in the fat body of adult females during 0-7 days PAE. No significant change was observed with LCMT1 mRNA levels in the previtellogenic and vitellogenic stages (Fig. S6A). We also measured LCMT1 mRNA levels in the fat body of adult females treated with precocene and those further treated with methoprene. Precocene treatment and additional methoprene application had no significant effect on LCMT1 mRNA expression (Fig. S6B and S6C). The data suggest that JH regulates LCMT1 expression at posttranscriptional level.

Line 221: Is this a true measure of egg production? Or is it more accurate to say vitellogenesis and/or oocyte maturation? Egg production could be measured by counting the number of eggs laid per cycle. If egg production is indeed influenced by knockdown of FoxO, then fewer eggs would be laid. Conversely, if vitellogenesis/oocyte maturation but not egg production were influenced by FoxO knockdown, then equal numbers of small eggs would be produced.

We have replaced “egg production” by “oocyte maturation” or “egg development” through the text. The reviewer is thanked for this comment.

Lines 227: It is slightly unclear what was done. Does this mean that expression of all 29 (or the remaining 24) genes was measured under conditions of FoxO knockdown? If so, this can be made clearer by stating that of the 29 genes upregulated by JH, only two, Cdc2 and Orc5 showed a reduction in transcript levels upon FoxO knockdown. Additionally, the data for all can be shown in a supplementary figure.

In the section of Introduction (paragraph 3), we introduce the background information regarding the identification and elucidation of genes involved in JH-dependent polyploidization during locust vitellogenesis. Briefly, JH upregulates 29 genes associated with fat body cell polyploidization, but only 6 genes are expressed in response to the JH receptor Met. To explore the involvement of FoxO in JH-dependent polyploidization, we examined the effect of FoxO RNAi on the expression of other 23 genes. Three genes including Cdc2, Orc5 and RFA2 showed a reduction in transcript levels upon FoxO knockdown. However, analysis of 3-kb upstream sequences revealed that the conserved FoxO response elements are present only in the promoters of Cdc2 and Orc5. Following the reviewer's suggestion, we have further clarified it in the revised text. The data of FoxO knockdown on the expression of those 23 genes are present in a new supplementary figure (Fig. S8).

Line 229: Mention somewhere in this paragraph the mammalian/human orthologs for Cdc2 and Orc5.

The mammalian/human ortholog for Cdc2 is Cyclin-dependent kinase 1 (Cdk1). Orc5 is universally used from yeast to mammals. We have mentioned Cdk1 in the revised text. Thanks go to the reviewer.

Lines 247-253: For Figures 6F and 6G, lanes can be labeled and referred to in the main text to direct the reader to specific comparisons, making it easier to understand. For example, at the end of the sentence that starts on Line 247, the authors can write in parentheses "lanes 2" or "compare lanes 1 and 2"

This has been done in the revision. The reviewer is thanked for thoughtful comments.

Lines 260-263: Is the differences in ploidy shift between Cdc2 and Orc5 knockdown indicative of which factor may play a more important role in oocyte maturation? It does appear to correlate with severity of the oocyte size phenotype. Cdc2 knockdowns are less polyploid than Orc5 knockdowns and show a lower length*width index than Orc5. Relatedly, it is interesting that the FoxO knockdown oocyte length*width phenotype is slightly less severe than the individual Cdc2 or Orc5 knockdown phenotypes while the reduction in VgA and VgB protein level is the reverse (more severe in the FoxO knockdown than individual Cdc2 or Orc5 knockdowns).

Cdc2 and Orc5 RNAi were conducted in parallel and with the same dsGFP controls. Cdc2 knockdown caused lower cell ploidy in the fat body than Orc5 knockdown (Fig. 7C). As well, Cdc2-depleted adult females had severer defects in Vg expression and oocyte maturation than Orc5-depleted individuals (Fig 7D-F). Thus, the differences in ploidy shift between Cdc2 and Orc5 knockdown is indicative of which factor may play a more important role in oocyte maturation.

Apart from Cdc2/Orc5 RNAi, FoxO RNAi was separately conducted using different groups of locusts. Because of experiment-to-experiment variation, the length*width index of the dsGFP controls for FoxO RNAi and Cdc2/Orc5 RNA was 8.0 and 5.8, respectively (Fig. 5F and 7F). The experimental locusts in FoxO RNAi appeared to be more developed than that in Cdc2/Orc5 RNAi.

Line 271: Regarding use of the phrase "egg production", see comment for Line 221.

As explained above, we have changed "egg production" to "oocyte maturation" or "egg development" through the text.

Lines 330-333: Instead of repeating the results, summarize the finding.

We have made the suggested revisions with thanks to the reviewer.

Lines 333-334: Briefly explain how Cdc2 regulates the cell cycle (i.e. cell cycle progression).

We have made the revision as followings: Cdc2 is a critical player in the regulation of cell cycle, not only promoting G2-M transition but also driving G1 progression and G1-S transition.

Lines 337-338: This is a good place to bring up the difference in phenotypes for Cdc2 and Orc5 described in the comments for Lines 260-263.

This has been done in the revision.

Line 396: Instead of “orchestrate”, I think the authors mean to write “cooperate”

We have changed “orchestrate” to “cooperate”.

Comments on Figures
Figures 5B and 7B: Add labels within or below images for immunofluorescence markers.

These have been done in revised figures.

Figure 6A: If possible, horizontal labels would be easier to read.

Following the reviewer’s comment #5, we have added the data of FoxO knockdown on the expression of 23 genes as a new figure (Fig. S8). As Fig. S8 includes the effect of FoxO knockdown on Cdc2 and Orc5 expression, the previous Fig 6A has been deleted.

Figure 6F and 6G: Add lane labels.

Lane labels have been added.

Reviewer #2
Advance Summary and Potential Significance to Field.
This is a very interesting paper that explores the role that juvenile hormone (JH) plays in stimulating polyploidization of the fat body and vitellogenesis via the forkhead transcription factor FOXO, using the locust Locusta migratoria as a model organism. Specifically, the authors show that JH-stimulated FoxO dephosphorylation act through LCMT1-mediated activation of PP2A. They also present evidence that FoxO activates the transcription of cell-division-cycle 2 (Cdc2) and origin-recognition-complex subunit 5 (Orc5), consequently stimulating fat-body cell polyploidization and Vg synthesis necessary to support oogenesis. Their data are convincing and generally support their model of the JH-regulation of fat body polyploidization and vitellogenesis through FOXO, which is a novel and exciting finding. This fits with a growing understanding of the way JH acts on physiology, and particularly how it works with other hormone signaling pathway for example the insulin-signaling pathway, which also targets FOXO. For this reason, I think that the paper is of sufficient general interest to be published in Development.

We thank Review #2 for the positive comments.

Comments for the Author.
While the paper is very interesting, I have some questions/concerns about the data and the manuscript that the authors should address before publication:
1) The authors show that phosphorylated-FOXO levels initially rise in young adults but then declines, as JH titers increase (Fig 1C). They subsequently show that treatment with methoprene in 10-day-old adults in which p-FOXO levels are usually low is sufficient to initially stimulate FOXO phosphorylation, which then subsequently falls. They interpret these data as evidence that “short-term exposure of exogenous JH may activate signaling cascades triggering FoxO phosphorylation, whereas prolonged treatment of JH may stimulate FoxO dephosphorylation”. This interpretation would be better supported if the authors showed that precocene treatment suppressed the peak in p-FOXO in early adults. This would therefore show that JH is not just sufficient to drive the p-FOXO pulse, but necessary during normal physiology.

This is a good comment from the referee. We performed experiments of prococene treatment at different time points. Shorter exposure of precocene failed to eliminate endogenous JH synthesis. We thus achieved JH-deprived adult females by precocene treatment on newly emerged adult females for 10 d, which covered the first gonadotrophic cycle. The reviewer’s point is well taken, we have modified the interpretation in the revised text.

2) Related to this, while the application of precocene does not affect the level of p-FOXO 10 days PAE, at the same time point, precocene appears to eliminate the methylation of PP2 (Fig 3C) and

the expression of LCMT1 (Fig 4F). The authors also show that knockdown of both PP2 and LCMT1 is sufficient to increase p-FOXO levels 8 days PAE. Why is the knockdown of PP2 and LCMT1 sufficient to increase p-FOXO levels 8 days PAE, while eliminating them by suppressing JH through precocene treatment is insufficient to increase p-FOXO levels 10 days PAE? If these results are correct, it suggests that there is another mechanism, apart from JH, suppressing p-FOXO accumulation via LCMT1 and PP2 10 days PAE. The authors should address these inconsistencies in their data since they do not fit with the simple hypothesis that JH regulates p-FOXO levels via LCMT1 and PP2.

We agree with the reviewer that other mechanism besides JH-LCMT1-PP2A axis might be involved in regulating FoxO dephosphorylation. Following the reviewer's suggestion, we have now discussed it in the revised text. Thanks go to the reviewer.

3) Why in Figure 1C does it appear that p-FOXO levels are fall to near zero 7 days PAE, and are zero at 10 days PAE, but control fat bodies show quite high levels of p-FOXO 8 days PAE (Figure 3C and 4F)? These suggest that there are either experiment-to-experiment variation in p-FOXO level or that p-FOXO levels fluctuate between 7 and 10 days PAE. Again, such temporal inconsistencies should be addressed by the authors.

Fig 3C and 4F show m-PP2A and LCMT1 abundance, respectively in response to precocene and methoprene treatment. We wonder that the reviewer might comment on p-FoxO levels in our RNAi experiments shown in Fig 2E and 4D. Certainly, there was experiment-to-experiment variation in p-FOXO levels. Also, the fluctuation of p-FOXO levels between 7 and 10 days PAE cannot be excluded. In addition, the exposure time of blots affect the band intensity, which contributed to the difference between western blot images in representative figures as well. In response to reviewer's comment, we have addressed it in the revision. We thank the reviewer for careful reading.

4) The authors should include the data points, not just the means, in their bar graphs (see https://www.nature.com/articles/s41551-017-0079 for a compelling discussion of this), and must specify the meaning of the error bars and more details of the test used to compare the means (was the t-test pooled or unpooled?). Also, are the authors planning on releasing their data to Dryad or some other depository?

This is an interesting suggestion. It seems that a limited number of published papers show the data points in their bar graphs. Following the reviewer's suggestion, we have modified the bar graphs to show data points. As data releasing is not required by the journal, all the original data in this paper are kept in files and ready to release upon requirement. Thanks go to the reviewer.

5) The authors show that changes in JH levels, and PP2 and LCMT1 activity can all change p-FOXO levels, and that JH levels affect PP2 methylation and LCMt1 levels, but they do not include an epistatic analysis confirming that JH acts through PP2 and LCMT1 to suppress p-FOXO levels. Specifically, they should show that the effect of the methoprene treatment on the dynamics of the p-FOXO (Figure 1D) levels are eliminated in adults with knockdown of PP2 and LCMT1.

We examined the effect of methoprene treatment on p-FoxO levels in the fat body of adult females previously subjected to PP2A or LCMT1 knockdown. As shown in Fig. S3E and S7E, methoprene-suppressed p-FoxO levels was blocked by depletion of PP2A or LCMT1.

6) Further, they do not show the effects of precocene treatment, or PP2 or LCMT1 knockdown on fat cell polyploidization, vitellogenesis and egg production. Such tests are necessary to show that the changes in the level of p-FOXO that they are observing are physiologically meaningful with regard to the phenotype they are interested in.

Our previous paper has reported that precocene treatment blocks fat body cell polyploidization, vitellogenesis and oocyte maturation (Guo et al., 2014). This has been described in the Discussion section with citation of this paper. We have examined the effect of PP2A or LCMT1 knockdown on vitellogenesis and egg development, which are shown in Fig. S3 and S7 and described in Result section. Because of COVID-19 pandemic, the cytometry core facility was shut down. Quantitative analysis of polyploidization by cytometry could not be carried out. Alternatively, we examined the morphology change of fat body cells after PP2A or LCMT1 knockdown, which demonstrates the reduction of ploidy (Fig. S3 and S7).

References
1. Guo, W., Wu, Z., Song, J., Jiang, F., Wang, Z., Deng, S., Walker, V. K. and Zhou, S. (2014). Juvenile hormone-receptor complex acts on mcm4 and mcm7 to promote polyploidy and vitellogenesis in the migratory locust. PLoS Genet 10, e1004702.

Second decision letter

MS ID#: DEVELOP/2019/184218

MS TITLE: Juvenile hormone acts through FoxO to promote Cdc2 and Orc5 transcription for polyploidy-dependent vitellogenesis
AUTHORS: Zhongxia Wu, Qiongjie He, Baojuan Zeng, Haodan Zhou, and Shutang Zhou
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. Where referee reports on this version are available, they are appended below. I do encourage you to look at and attend to the minor comments suggested by the reviewers on this revision.

Reviewer 1

Advance summary and potential significance to field

In this manuscript, Wu and colleagues provide clear and thorough data describing the molecular interactions employed by JH to control polyploidization in the fat body and ultimately vitellogenesis and oocyte maturation in Locusta migratoria. They show that JH promotes protein expression/stability of LCMT1, which methylates PP2A to suppress its phosphatase activity on FoxO. Moreover, they identify two targets of FoxO, Cdc2 and Orc5, that provide the cellular (polyploidization) and molecular (cell cycle progression and DNA replication) links between JH signaling and synchronous oocyte maturation. Prior to this work, the molecular mechanism of JH action in promoting oocyte maturation and polyploidization had been poorly understood. The authors have described how JH controls FoxO phosphorylation (by regulating the expression of LCMT1 which methylates PP2A so that it no longer dephosphorylates FoxO). In addition the authors have identified Cdc2 and Orc5 as targets of FoxO that control polyploidization in the fat body, and thus, the ability of fat tissue to synthesize substantial quantities of Vg to support oocyte maturation.

Comments for the author

Manuscript ID: DEVELOP-2020-188813v2-Zhou

Title: Juvenile hormone acts through FoxO to promote Cdc2 and Orc5 transcription for polyploidy-dependent vitellogenesis

Review of version 2
The authors have sufficiently addressed my comments and made appropriate revisions. A minor point, it was indicated that lane labels were added for Figures 6E and 6F, however, the revised figure does not show that particular modification. This change does not preclude acceptance for publication.

Summary
In this manuscript, Wu and colleagues provide clear and thorough data describing the molecular interactions employed by JH to control polyploidization in the fat body and ultimately vitellogenesis and oocyte maturation in *Locusta migratoria*. They show that JH promotes protein

expression/stability of LCMT1, which methylates PP2A to suppress its phosphatase activity on FoxO. Moreover, they identify two targets of FoxO, Cdc2 and Orc5, that provide the cellular (polyploidization) and molecular (cell cycle progression and DNA replication) links between JH signaling and synchronous oocyte maturation.

Significance to the field
Prior to this work, the molecular mechanism of JH action in promoting oocyte maturation and polyploidization had been poorly understood. The authors have described how JH controls FoxO phosphorylation (by regulating the expression of LCMT1 which methylates PP2A so that it no longer dephosphorylates FoxO). In addition the authors have identified Cdc2 and Orc5 as targets of FoxO that control polyploidization in the fat body, and thus, the ability of fat tissue to synthesize substantial quantities of Vg to support oocyte maturation.

Line-by-line comments
Line 168: To be clearer about what is being evaluated add “in p-PP2A level” after “no apparent change was observed”.

Lines 168-170: Two points of clarification should be made regarding the data the authors used to arrive at the conclusion that “reduced levels of p-FoxO during vitellogenesis is unlikely an effect of Akt”. First, by correlating p-PP2A and p-Akt levels, the authors are testing if Akt leads to reduced levels of phosphorylated FoxO by inhibitory phosphorylation of PP2A. This is in contrast to Akt’s known role in phosphorylating FoxO. Did the authors consider briefly discussing the role of direct phosphorylation of FoxO by Akt? Second, p-FoxO levels decline after 3 days PAE (Fig. 1C) while p-Akt levels remain elevated 2 days PAE (Fig. 2A). In this context, if Akt directly phosphorylates FoxO, we would expect that p-FoxO levels would also remain elevated during this period. Thus, the data supports the authors’ conclusion but a sentence or two highlighting this connection would make it more obvious to the reader.

Lines 200-201: Are there any ideas on how JH is regulating LCMT1 protein level? Transcriptionally? Post-transcriptionally (translation)? Post-translationally (protein stability)?

The authors could measure transcript levels for LCMT1 in fat body from precocene- and methoprene-treated females.

Line 221: Is this a true measure of egg production? Or is it more accurate to say vitellogenesis and/or oocyte maturation? Egg production could be measured by counting the number of eggs laid per cycle. If egg production is indeed influenced by knockdown of FoxO, then fewer eggs would be laid. Conversely, if vitellogenesis/oocyte maturation but not egg production were influenced by FoxO knockdown, then equal numbers of small eggs would be produced.

Lines 227: It is slightly unclear what was done. Does this mean that expression of all 29 (or the remaining 24) genes was measured under conditions of FoxO knockdown? If so, this can be made clearer by stating that of the 29 genes upregulated by JH, only two, *Cdc2* and *Orc5* showed a reduction in transcript levels upon FoxO knockdown. Additionally, the data for all can be shown in a supplementary figure.

Line 229: Mention somewhere in this paragraph the mammalian/human orthologs for Cdc2 and Orc5.

Lines 247-253: For Figures 6F and 6G, lanes can be labeled and referred to in the main text to direct the reader to specific comparisons, making it easier to understand. For example, at the end of the sentence that starts on Line 247, the authors can write in parentheses “lanes 2” or “compare lanes 1 and 2”

Lines 260-263: Is the differences in ploidy shift between Cdc2 and Orc5 knockdown indicative of which factor may play a more important role in oocyte maturation? It does appear to correlate with severity of the oocyte size phenotype. Cdc2 knockdowns are less polyploid than Orc5 knockdowns and show a lower length*width index than Orc5. Relatedly, it is interesting that the FoxO knockdown oocyte length*width phenotype is slightly less severe than the individual Cdc2 or Orc5 knockdown phenotypes while the reduction in VgA and VgB protein level is the reverse

(more severe in the FoxO knockdown than individual Cdc2 or Orc5 knockdowns).

Line 271: Regarding use of the phrase “egg production”, see comment for Line 221. Lines 330-333: Instead of repeating the results, summarize the finding.
Lines 333-334: Briefly explain how Cdc2 regulates the cell cycle (i.e. cell cycle progression).

Lines 337-338: This is a good place to bring up the difference in phenotypes for Cdc2 and Orc5 described in the comments for Lines 260-263.

Line 396: Instead of “orchestrate”, I think the authors mean to write “cooperate”

Comments on Figures
Figures 5B and 7B: Add labels within or below images for immunofluorescence markers.

Figure 6A: If possible, horizontal labels would be easier to read.

Figure 6F and 6G: Add lane labels.

preclude

Reviewer 2

Advance summary and potential significance to field

As I described in my initial review of this paper, I think that the paper contributes to our growing understanding of the way JH acts on physiology, and particularly how it works with other hormone signaling pathway for example the insulin-signaling pathway, which also targets FOXO. For this reason, I think that the paper is of sufficient general interest to be published in Development.

Comments for the author

The authors have done a very thorough job responding to my comments and I am now satisfied that the results support their conclusions. One comment that was not addressed fully is, however, my request that they indicate that the error bars are standard errors. Their note that "Values are represented by mean ± SE." is not sufficient. They should indicate that error bars are standard errors in the figure captions. Also, for the data in Figure 6C and 6D, why did the authors use a one-way ANOVA, as stated in the methoology? A two-way ANOVA would be more appropriate, and statistically more powerful. These are, however, minor points.