The bHLH-PAS transcriptional complex Sim:Tgo plays active roles in late oogenesis to promote follicle maturation and ovulation

Rebecca Oramas, Elizabeth M. Knapp, Baosheng Zeng and Jianjun Sun
DOI: 10.1242/dev.201566

Editor: Swathi Arur

Review timeline
Original submission: 30 December 2022
Editorial decision: 14 February 2023
First revision received: 25 April 2023
Accepted: 12 May 2023

Original submission

First decision letter

MS ID#: DEVELOP/2022/201566

MS TITLE: The bHLH-PAS transcriptional complex Sim:Tgo plays active roles in late oogenesis to promote follicle maturation and ovulation

AUTHORS: Rebecca Oramas, Elizabeth M Knapp, Baosheng Zeng, and Jianjun Sun

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, and provide recommendations to further improve the clarity and rigor of the study. 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. If it would be helpful, you are welcome to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating your plans for addressing the referee’s comments, and we will look over this and provide further guidance.

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

1) As the investigators argue, there is much yet to be known about follicle maturation in any organism. This is an important developmental process and the work described here provides a

significant amount of novel insight into the regulation of Drosophila follicle maturation. This includes hormone regulation, the role of multiple regulatory proteins, including the Single-minded:Tgo master regulator, and Mmp2 and NOX, which are involved in follicle rupture. As the pathway of follicle maturation is better defined as to the proteins and genes involved, it becomes possible to observe and address the complexity of gene expression and its regulation. The more systems that this can be addressed in a sophisticated manner, the more likely that general rules and concepts will emerge that apply to many developmental and physiological pathways. The authors of this manuscript make a strong case for the value of their increasingly sophisticated view of Drosophila follicle maturation.

2) Overall, the data is convincing and the results and conclusions justified. The manuscript itself is well-written. I have only minor comments and some questions. The authors are highly knowledgeable about Sim and Tgo, as well as follicle maturation. Their solid understanding of the Sim and Tgo literature enhances the manuscript and interpretation of their data.

3) A significant part of the manuscript deals with showing that Tgo is the heterodimerization partner of Sim in regulating follicle cell transcription of Br-C, Cut, and Hnt. While this is unsurprising, it is worth demonstrating, as the results clearly do, since it would have been very interesting and novel if Sim did not require Tgo in this process or if Tgo was using additional bHLH-PAS proteins to carry out some aspect of follicle cell maturation.

4) There are interesting and surprising aspects of Tgo expression during follicle cell maturation. The upregulation of Tgo at s10A, followed by downregulation at s13, followed by upregulation at s14 is interesting, particularly since Ecdysone signaling is implicated in the regulation. One tends to think of Tgo levels as being static, but clearly not in follicle cells. This is a novel observation.

Comments for the author

Minor comments and questions the authors may wish to consider or address in the manuscript.

1) P. 6 and Fig 1. Do the authors have any thoughts on why Tgo is downregulated at s13, particularly since Sim is not present? Generally, it is thought that Drosophila Tgo has no function without a partner bHLH-PAS protein, so why bother to downregulate its expression.

2) Ecdysone signaling was shown to upregulate tgo expression at late s10A. Is ecdysone signaling also involved in the downregulation of tgo at s13 and upregulation at s14?

3) P. 7 line 11. States that “At stage 11, overexpression of EcRDN prevented induction of Sim, which further blocks Tgo translocation into the nucleus (Fig 1I).” However, Fig 1I only shows the effect of EcRDN on Tgo but not Sim. If the effect of EcR on Sim is shown elsewhere it should be referenced or data shown.

4) P. 8 line 10. How specific is Vm26Aa-Gal4 for follicle cells? The authors may want to comment on this since it is relevant for interpreting the data on egg laying.

5) P. 18. The authors are justified in noting that DDRCGTG may be a Sim:Tgo binding site in addition to the well-studied ACGTG site. They note that there exist DDRCGTG potential Sim:Tgo binding sites in the Nox gene region, Mmp2 enhancer and Oamb enhancer. However, given the work done showing in vivo-relevant binding of Sim:Tgo to ACGTG sequences, it would be of interest to know if any of those potential binding sites are ACGTG and which are the broader consensus sequence. I also wonder whether there is a consensus DDRCGTG sequence that might be relatively specific for Sim:Tgo binding sites on follicle-expressed genes.

6) P. 18 line 20. Typo. Change “ despite of the fact..” to “despite the fact..”.

7) Fig S1. Minor point, but why is the order of oogenesis: stage (A) 7, (B) 8, (C) 11, (D) 10A, (E) 12, (F) 14?
Shouldn’t stage 10A precede stage 11?

8) Fig S2A-D. The figure panels are believable, but the images that come later using a similar assay (Fig 4D,E,G,H; Fig 5 H-I') are more striking. I'm not sure all readers will readily grasp the differences between Fig S2A and Fig S2B-D. I wonder if it would be possible to either reference the later, more striking figure panels, or provide a little more detail about the differences in appearance between ruptured and unruptured follicles.

9) Fig S2 legend. Typo. Should be "Bright field image shown in blue" not "Bright field image show in blue".

10) Work by others revealed that the midline expression of sim, while seemingly simple, is quite complex consisting of a number of midline enhancers, including autoregulatory enhancers. One wonders whether follicle cell expression of sim will be equally complex and consist of multiple enhancers since it too has dynamic regulation. Regulation of midline gene expression by Sim also requires a number of coregulatory proteins that work on distinct enhancers in different ways. It will be interesting to identify coregulators of Sim:Tgo in follicle cell maturation and determine if any are in common with midline coregulators.

Reviewer 2

Advance summary and potential significance to field

In this manuscript, Rebecca Oramas and colleagues investigate the process by which ovulation is regulated using the fly egg chamber as model system. Following previous work, the authors identify that the bHLH-PAS transcription factor Tango acts as a cofactor with another bHLH-PAS protein Single-minded in order to promote ovulatory competency. They show that sim and tgo are expressed successively during follicular cell development from stage 10 to stage 14. They also show that their expression at the end of stage 14 is particularly important for ovulatory competence. They further show that tgo and sim control the expression of key genes for ovulation such as OAMB, Mmp2 and NOX. Finally, they show that these target genes are controlled by sim either independently or in conjunction with the transcription factor Hnt.

Comments for the author

Overall, this work provides new information on the ovulation process, which is much less studied, especially with regard to Drosophila oogenesis. Claims are well supported by the results. Results are clear, well presented and well controlled.
Could the authors at least comment on by which regulation process Tgo and sim are expressed successively during follicular cell development from stage 10 to stage 14 and especislly at late stage 14 that seems particularly critical for ovulatory competency.

Minor point:
The OA-induced follicle rupture experiment is a very nice assay to monitor ovulation, could the authors could better explain this experience and provide more details concerning the results, it is not easy to understand the results of the fig3D, S2B-D.

Reviewer 3

Advance summary and potential significance to field

This manuscript describes the role of Sim and its cofactor, Tgo, as a master regulator of the gene expression program that controls ovulation competency in Drosophila melanogaster. The authors provide a thorough characterization of how Sim:Tgo activity spatio-temporally controls genes important for promoting follicle cell differentiation and follicle rupture, ultimately leading to ovulation of mature oocytes. This study advances understanding of the molecular mechanism that underlies the complex process of ovulation.

Comments for the author

Summary
This manuscript describes the role of Sim and its cofactor, Tgo, as a master regulator of the gene expression program that controls ovulation competency in *Drosophila melanogaster*. The authors provide a thorough characterization of how Sim:Tgo activity spatio-temporally controls genes important for promoting follicle cell differentiation and follicle rupture, ultimately leading to ovulation of mature oocytes. This study advances understanding of the molecular mechanism that underlies the complex process of ovulation. Before being accepted for publication, some moderate and minor concerns, listed below, should be addressed.

Moderate concerns
Page 12, lines 1-18: While this section provides great evidence to support the use of the CRISPR-Cas9 system cell type-specific manipulation of gene expression in follicle cells, it seems misplaced in this manuscript. More specifically, the authors do not use the system to manipulate expression of their genes of interest.

Page 9, lines 15-16: knockdown seems patchy based on Fig 2K and SF2E so please comment on variability in knockdown efficiency within the Tgo RNAi samples – meaning, for each RNAi line, how variable is the knockdown (could be calculated as a percentage of cells per follicle); does this correlated to the short DA, rounder oocyte phenotype show in Fig 2E (i.e. do all sim RNAi stage 14s look normal?

Page 11, line 7: Since 47A04-Gal4 > sim RNAi did not affect mature follicle retention, update main text to indicate that. In fact, there is a differential impact of each driver on mature follicle retention – with 44E10 there is an approximate 4-fold increase, with CG13083 there is an approximate 2-fold increase, and with 47A04 there is no difference. This is counterintuitive since CG13083-Gal4 shows the earliest knockdown, and one might expect that driver to have the strongest phenotype. Please add and/or rework the text to help clarify.

Page 15, lines 13-15: Because of the generally diffuse NOX::GFP staining pattern, there should be an image of a younger follicle to show what “not detected” looks like for Fig 8A. Also, qualitatively it is very hard to see an upregulation at early stage 14. Quantification would make these observations easier to share.

Minor concerns
Page 6, Lines 11-12: Is Tgo expressed earlier? (Is it necessary to look at Tgo expression in earlier stages?; supplemental figure shows RNAi clones at stage 8, so it would be nice to mention what the expression looks like from 1-10
Page 6, Line 13: main text says 10B but figure shows 11 (add a 10B image or update main text) Page 9, line 9: maybe add “after three hours” at the end of this sentence to confirm that it’s the same timing, or if not, indicate how long it took to get such a low rupture rate

Page 11, lines 1-5: show pics of what constitutes “high, med, and low/none” sim expression
Page 12, lines 9-10: The authors did not mention the level of expression for UAS-Cas9-S, but that line was used, and data is shown Fig 5E.

Page 12, line 13: The authors may want to be more accurate by saying there is a trend of increased number of mature follicles but only a significant increase with the line that expresses Cas9 at the highest levels.

Page 17, line 2: Add the Knapp et al., 2020 reference just before figure notice.
Page 18, line 7: remove “s” from “upregulates”
Page 18, line 20: delete “of” after “despite”
Page 19, line 8: remove “y” from “homology”

First revision

Author response to reviewers' comments

Point-by-Point Response to reviewer's comments:

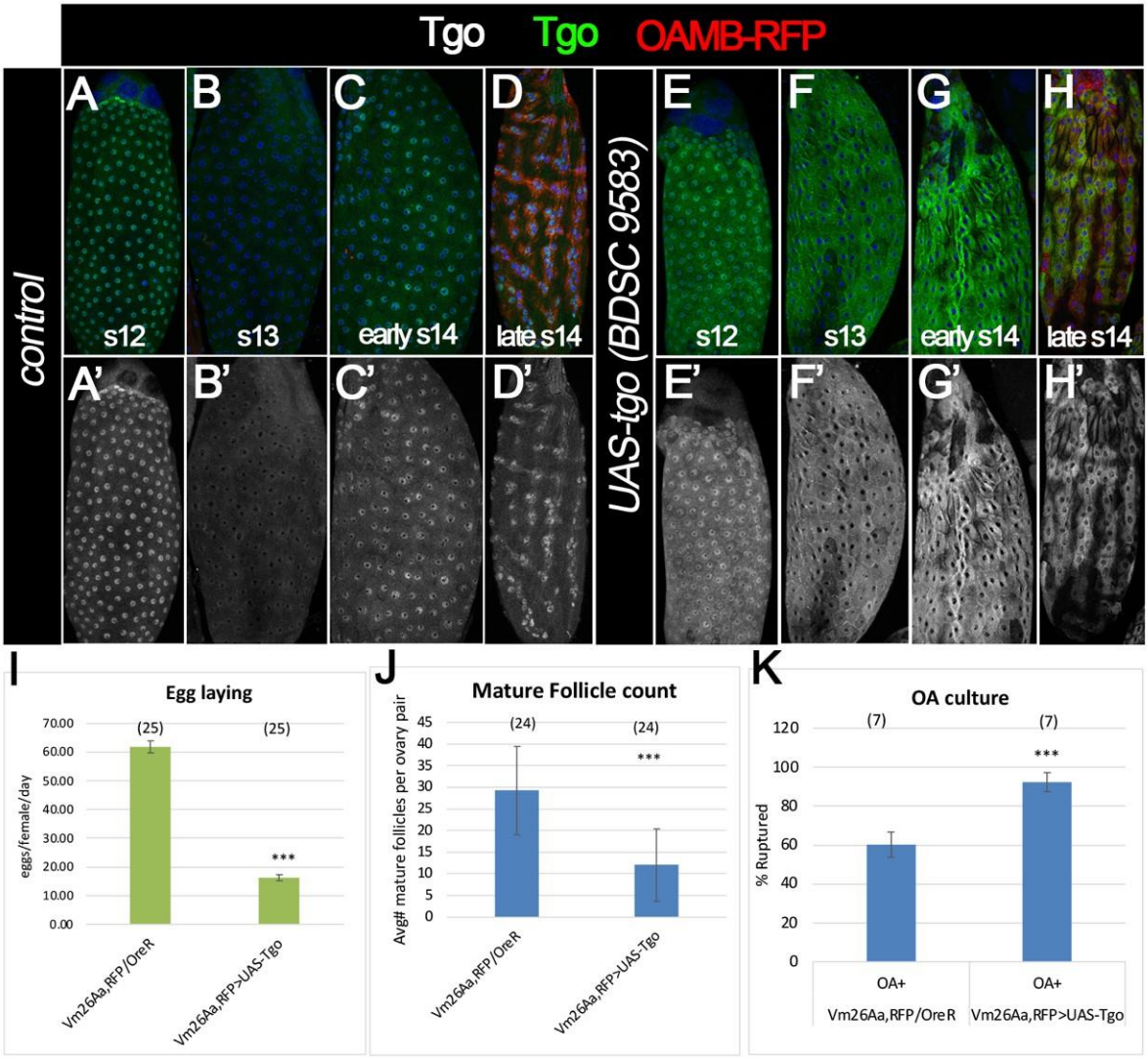
We appreciate the positive and constructive comments from reviewers. Here we listed our response to each reviewer's comments.

Reviewer 1 Comments for the Author...

1) P. 6 and Fig 1. Do the authors have any thoughts on why Tgo is downregulated at s13, particularly since Sim is not present? Generally, it is thought that Drosophila Tgo has no function without a partner bHLH-PAS protein, so why bother to downregulate its expression.

According to our analysis, we have clearly demonstrated the upregulation of Tgo at stage 10A and downregulation at stage 13. This downregulation is not due to the relocation of Tgo from the nucleus to the cytoplasm as the intensity measurement was the same (data not shown). It should be noted that there's still low levels of cytoplasmic Tgo expression at stage 13 according to our flip-out clone analysis (Fig S1).

It is unclear why Tgo expression level needs to be precisely controlled in follicle cells since Tgo has no function without a partner bHLH-PAS protein. To tackle this question, we used *Vm26Aa-Gal4* to drive *UAS-Tgo* expression in follicle cells from stages 10-14. First, we confirmed the Tgo overexpression using Tgo antibody. We can clearly see more cytoplasmic Tgo signal throughout late oogenesis with Tgo overexpression relative to controls (A-H, isolated Tgo signal in white A'-H'). Next, we evaluated the physiology of these females. To our surprise, *tgo*-overexpressing females laid significantly fewer eggs compared to the wild-type control (I). Through examining the ovaries after egg laying, we found that *tgo*-overexpressing females have significantly fewer mature follicles (J). It appeared that stage 12-13 follicles cannot advance into stage 14 follicles and become degenerated. We also measured the follicle rupture and showed that the fully matured follicles with *tgo*-overexpressing can still rupture in response to OA (K). Therefore, Tgo's expression levels need to be precisely controlled in follicle cells for the proper development of follicles. However, we don't know whether this defect is due to the upregulation of Tgo at stage 10-12, which causes toxicity, or at stage 13, which prevents normal downregulation of Tgo. In addition, more detailed analysis will be needed to tease out the oogenesis defect, which is outside the scope of this manuscript. Therefore, we decided not to include these experimental data in the current manuscript to avoid confusing the readers.



2) Ecdysone signaling was shown to upregulate tgo expression at late s10A. Is ecdysone signaling also involved in the downregulation of tgo at s13 and upregulation at s14?

As the reviewer suggested, we tested whether ecdysone signaling at stage 14 is responsible for the upregulation of Tgo as well as Sim. We used the same strategy as our previously published work (Knapp and Sun, 2017) to disrupt the ecdysone signaling by knocking down shade, the final enzyme converting ecdysone to active 20-hydroxyecdysone. We didn't observe any disruption of Sim or Tgo expression at stage 13 and 14, however we could still observe a similar follicle rupture defect to that previously reported (Knapp and Sun, 2017). Therefore, late ecdysone signaling at stage 14 is not responsible for the Sim and Tgo upregulation. We have added this data into a new supplemental figure (Fig S8).

3) P. 7 line 11. States that "At stage 11, overexpression of EcRDN prevented induction of Sim, which further blocks Tgo translocation into the nucleus (Fig 1I)." However, Fig 1I only shows the effect of EcRDN on Tgo but not Sim. If the effect of EcR on Sim is shown elsewhere it should be referenced or data shown.

Thank you for your suggestion. We didn't show the effect of EcR on Sim but rather inferred from our previous work that EcR is required for Ftz-F1 expression and Ftz-F1 is required for Sim expression. We have reworded the text as "At stage 11, we also did not observe Tgo nuclear localization in EcRDN-overexpressing follicle cells (Fig 1I). This is likely because EcRDN prevents Ftz-F1 expression, which is critical for Sim expression (Knapp et al., 2020)".

4) P. 8 line 10. How specific is Vm26Aa-Gal4 for follicle cells? The authors may want to comment on this since it is relevant for interpreting the data on egg laying.

The *Vm26Aa-Gal4* is highly specific in follicle cells and is not detected in any other tissues or developmental stages according to the published work (Peters et al., 2013), which is further confirmed by our observation. We modified the text as "*Vm26Aa-Gal4*, a Gal4 driver expressed in follicle cells starting at stage 10 and expressed in no other developmental context or tissues besides the female ovary (Peters et al., 2013)".

5) P. 18. The authors are justified in noting that DDRCGTG may be a Sim:Tgo binding site in addition to the well-studied ACGTG site. They note that there exist DDRCGTG potential Sim:Tgo binding sites in the Nox gene region, Mmp2 enhancer and Oamb enhancer. However, given the work done showing in vivo-relevant binding of Sim:Tgo to ACGTG sequences, it would be of interest to know if any of those potential binding sites are ACGTG and which are the broader consensus sequence. I also wonder whether there is a consensus DDRCGTG sequence that might be relatively specific for Sim:Tgo binding sites on follicle-expressed genes.

DDRCGTG motif consists of both ACGTG and GCGTG sequences. According to the sequence analysis, it seems that GCGTG is more dominant than ACGTG at the promoter/enhancer regions of Nox, Mmp2, and Oamb genes. We added this information to the discussion and additional supplemental table (Table S2). However, all these analyses are pure bioinformatic analysis, we don't know whether Sim will bind to any of these sites in the follicle cells. It is our future direction to profile the Sim binding sites in follicle cells using CUT&RUN or ChIP-seq, which is beyond the scope of this manuscript.

6) P. 18 line 20. Typo. Change " despite of the fact.." to "despite the fact..".
Corrected. Thank you.

7) Fig S1. Minor point, but why is the order of oogenesis: stage (A) 7, (B) 8, (C) 11, (D) 10A, (E) 12, (F) 14? Shouldn't stage 10A precede stage 11?

The organization of Fig S1 is not based on the stage but rather based on the two different RNAi lines. Instead of showing all the stages for both RNAi lines, we used the closely related stages for both RNAi lines. We happened to have stage 10A for tgo^{RNAi2} and stage 11 for $_{tgo}RNAi1$.

8) Fig S2A-D. The figure panels are believable, but the images that come later using a similar assay (Fig 4D,E,G,H; Fig 5 H-I') are more striking. I'm not sure all readers will readily grasp the differences between Fig S2A and Fig S2B-D. I wonder if it would be possible to either reference the later, more striking figure panels, or provide a little more detail about the differences in appearance between ruptured and unruptured follicles.

Thank you for your suggestion. We have modified Figure S2 to highlight the differences in appearance between ruptured and unruptured follicles. We have generated insets showing an example of a ruptured follicle (without follicle cell nuclei showed by DAPI signal) next to an unruptured follicle (with follicle cell nuclei) to highlight the key differences. In addition, we also increased the image contrast to improve the visualization.

9) Fig S2 legend. Typo. Should be "Bright field image shown in blue" not "Bright field image show in blue".
Corrected. Thank you.

10) Work by others revealed that the midline expression of sim, while seemingly simple, is quite complex consisting of a number of midline enhancers, including autoregulatory enhancers. One wonders whether follicle cell expression of sim will be equally complex and consist of multiple enhancers since it too has dynamic regulation. Regulation of midline gene expression by Sim also requires a number of coregulatory proteins that work on distinct enhancers in different ways. It will be interesting to identify coregulators of Sim:Tgo in follicle cell maturation and determine if any are in common with midline coregulators.

Thank you for your suggestion. The only isoform expressed in follicle cells is sim-RC (FBtr0334613) according to our previous RNAseq data (Knapp et al. 2020). This is not the same isoform of sim expressed in embryos using early promoter (FBtr0082710; sim-RA) or late promoter (FBtr0082711; sim-RB). Furthermore, previous experiments shown in the author response to reviewers' comments in Knapp et al., 2020 assessed sim3.7-Gal4 (BDSC#26784) expression in the ovary and observed that this Gal4 could not drive any expression in the ovary. This makes sense as the 3.7kb fragment of sim in this Gal4 construct does not contain the Ftz-f1 binding site identified in the CUT&RUN experiment by Knapp et al., 2020 and its associated transcript FBtr0082711 (*sim-RB*) is not expressed in follicle cells. Therefore, the isoform expressed in the follicle cells is unique and unlikely using the same enhancers used in other developmental stages.

We agree with reviewer that it will be interesting to identify the coregulators of Sim:Tgo in follicle cell maturation in the future. We are in the process of identifying the enhancers for Mmp2 expression, which is likely regulated by multiple factors including Sim:Tgo due to its spaciotemporal expression pattern. Future work will be able to illustrate the transcription factors that bind to the Mmp2 enhancers, some of which may be a coregulators of Sim:Tgo if this complex directly regulates Mmp2 expression.

Reviewer 2 Comments for the Author...

1) Could the authors at least comment on by which regulation process Tgo and Sim are expressed successively during follicular cell development from stage 10 to stage 14 and especially at late stage 14 that seems particularly critical for ovulatory competency.

We have clearly demonstrated that ecdysone signaling regulates both Tgo and Sim expression at stage 10; however, it is currently unclear what signals mediate the downregulation of Tgo and Sim at stage 13 and re-upregulation at stage 14. As reviewer 1 suggested (See comment 2), we tested whether ecdysone signaling at stage 14 also responds to the regulation of Sim and Tgo. Unfortunately, it does not affect Sim and Tgo expression at mature follicle cells (shown in Figure S8).

2) The OA-induced follicle rupture experiment is a very nice assay to monitor ovulation, could the authors could better explain this experience and provide more details concerning the results, it is not easy to understand the results of the fig3D, S2B-D.

Thanks for your comments. We have modified Figure S2A-D to highlight the differences in appearance between ruptured and unruptured follicles (see response to comment 8 of reviewer 1 above).

Reviewer 3 Comments for the Author…

1) Page 12, lines 1-18: While this section provides great evidence to support the use of the CRISPR-Cas9 system cell type-specific manipulation of gene expression in follicle cells, it seems misplaced in this manuscript. More specifically, the authors do not use the system to manipulate expression of their genes of interest.

We partly agree with reviewer's comment. Due to the technical challenges of the genetical manipulation in the mechanistic investigation, we were unable to use the CRISPR-Cas9 system to manipulate the gene of interest and study the Oamb, Mmp2, Nox::GFP expression along with RNA interference. However, since *sim-RNAi* has no predicted off-target and the reproductive phenotype is validated by both RNAi and CRISPR-Cas9 system, our mechanistic investigation is solid and conclusive. On the other hand, we felt it is appropriate to keep the CRISPR-Cas9 data right after the RNAi data. It is additive and may not contribute to the overall conclusion of this manuscript, but at least it demonstrated that tissue-specific CRISPR-Cas9 can be used in follicle cells to knock out genes. We are open to reviewer's suggestion to relocate the data or remove the data.

2) Page 9, lines 15-16: knockdown seems patchy based on Fig 2K and SF2E so please comment on variability in knockdown efficiency within the Tgo RNAi samples – meaning, for each RNAi line, how variable is the knockdown (could be calculated as a percentage of cells per follicle); does this correlated to the short DA, rounder oocyte phenotype show in Fig 2E (i.e. do all sim RNAi stage 14s

look normal?

To address the reviewers comment we repeated our Tgo antibody staining, quantified the percentage of mature follicles with short vs. long dorsal appendages, and the percentage of follicle cells with vs. without Tgo expression in stage 10 and stage 12 follicles of control, *sim*- knockdown, and *tgo*-knockdown females. Our results suggest that Tgo expression is more strongly affected in stage 10-12 follicles upon overexpression of tgo^{RNAi1} and tgo^{RNAi2}, compared to overexpression of sim^{RNAi}, as expected. Overall, Tgo depletion in stages 10-12 was slightly stronger with tgo^{RNAi1} than tgo^{RNAi2}, however, both lines were very efficient at depleting Tgo at these follicle stages. We found that the *tgo*-knockdown efficiency at stage 10-12 correlates well with the dorsal appendage defects observed. We added these data in Fig S2 (Fig S2E-F).

3) Page 11, line 7: Since 47A04-Gal4 > sim RNAi did not affect mature follicle retention, update main text to indicate that. In fact, there is a differential impact of each driver on mature follicle retention – with 44E10 there is an approximate 4-fold increase, with CG13083 there is an approximate 2-fold increase, and with 47A04 there is no difference. This is counterintuitive since CG13083-Gal4 shows the earliest knockdown, and one might expect that driver to have the strongest phenotype. Please add and/or rework the text to help clarify.

The mature follicle retention after egg laying is pretty variable. We typically do not rely on these data to make a conclusion about an ovulation defect. Instead, we rely on these data to make sure there's no oogenesis defects. In other words, as long as there's no significant reduction in mature follicles, we consider there's no oogenesis defect. We then rely on the ovulation time to determine whether there's an ovulation defect. Therefore, the slight difference in fold change between different Gal4 lines could be due to the internal variation, particularly the number of mature follicles is slightly higher in controls with *CG13083-Gal4* and *47A04-Gal4* than with *44E10-Gal4*. To reflect this nature, we reword our text as following "Regardless of the Gal4 driver used, *sim* depletion with either of these Gal4 lines led to a significant decrease in egg laying (Fig 4A, S5A, S5J) without defects in stage-14 follicle formation (Fig 4B, S5B, S5K). In fact, both *44E10-Gal4* and *CG13083-Gal4* led to mature follicle retention (Fig 4B and S5K), a strong indication of ovulation defect. In addition, *sim* knockdown with all the Gal4 lines led to an increase in ovulation time (Fig 4C, S5C, S5L, Table S1)". For more explanation about interpreting mature follicle counting, reviewer is referred to **Beard, A., Oramas, R. and Sun, J**. (2023). Assessing Ovulation in Drosophila melanogaster. In Drosophila Oogenesis: Methods and Protocols (ed. Giedt, M. S.) and Tootle, T. L.), pp. 253–276. New York, NY: Springer US.

4) Page 15, lines 13-15: Because of the generally diffuse NOX::GFP staining pattern, there should be an image of a younger follicle to show what "not detected" looks like for Fig 8A. Also, qualitatively it is very hard to see an upregulation at early stage 14. Quantification would make these observations easier to share.

As reviewers suggested, we added a stage-12 egg chamber showing no NOX::GFP expression in follicle cells in Fig 8. We also replaced stage-13 and early stage 14 images to clearly illustrate our finding that NOX::GFP expression begins in stage-13 but upregulates by early stage 14. All images are taken from the same slide and using the same image acquisition parameter to accurate comparison. We also quantify the intensity of NOX::GFP in different stages (added in Fig S7), which is consistent with our conclusion.

5) Page 6, Lines 11-12: Is Tgo expressed earlier? (Is it necessary to look at Tgo expression in earlier stages?; supplemental figure shows RNAi clones at stage 8, so it would be nice to mention what the expression looks like from 1-10

According to our clonal analysis, we found that Tgo is faintly detected in follicle cells from stage 7 to 9 and upregulated at stage 10A. We didn't observe detectable Tgo before stage 7. We mentioned this in the first paragraph of the result as "Tgo is faintly detected in the cytoplasm of follicle cells in mid-stage egg chambers (stages 7-9) and upregulated at late stage 10A (Fig 1A and S1A-B)".

6) Page 6, Line 13: main text says 10B but figure shows 11 (add a 10B image or update main text)
Figure 1A-E is taken from the same slide at the same time so that it can be accurately showing the

different expression level. Therefore, it will be hard for us to replace one single panel with stage 10B egg chamber (we just did have stage 10B in the acquired images). We refer the reader to Figure 1F, which is showing the stage 10B egg chamber with Tgo nuclear localization.

7) Page 9, line 9: maybe add "after three hours" at the end of this sentence to confirm that it's the same timing, or if not, indicate how long it took to get such a low rupture rate
Corrected. Thank you.

8) Page 11, lines 1-5: show pics of what constitutes "high, med, and low/none" sim expression
As reviewers suggested, we labeled current panels within the Figure S4 as high, med, and low/no so reader can reference as an example of these expression levels.

9) Pge 12, lines 9-10: The authors did not mention the level of expression for UAS-Cas9-S, but that line was used, and data is shown Fig 5E.
Corrected. thank you.

10) Page 12, line 13: The authors may want to be more accurate by saying there is a trend of increased number of mature follicles but only a significant increase with the line that expresses Cas9 at the highest levels.
Corrected. Thank you.

11) Page 17, line 2: Add the Knapp et al., 2020 reference just before figure notice.
Corrected. Thank you.

12) Page 18, line 7: remove "s" from "upregulates"
Corrected. Thank you.

13) Page 18, line 20: delete "of" after "despite"
Corrected. Thank you.

14) Page 19, line 8: remove "y" from "homology"
Corrected. Thank you.

Second decision letter

MS ID#: DEVELOP/2022/201566

MS TITLE: The bHLH-PAS transcriptional complex Sim:Tgo plays active roles in late oogenesis to promote follicle maturation and ovulation

AUTHORS: Rebecca Oramas, Elizabeth M Knapp, Baosheng Zeng, and Jianjun Sun
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 2

Advance summary and potential significance to field

In this manuscript, Rebecca Oramas and colleagues investigate the process by which ovulation is regulated using the fly egg chamber as model system. Following previous work, the authors identify that the bHLH-PAS transcription factor Tango acts as a cofactor with another bHLH-PAS protein Single-minded in order to promote ovulatory competency. They show that sim and tgo are expressed successively during follicular cell development from stage 10 to stage 14. They also show

that their expression at the end of stage 14 is particularly important for ovulatory competence. They further show that tgo and sim control the expression of key genes for ovulation such as OAMB, Mmp2 and NOX. Finally, they show that these target genes are controlled by sim either independently or in conjunction with the transcription factor Hnt.

Overall, this work provides new information on the ovulation process, which is much less studied, especially with regard to Drosophila oogenesis. Claims are well supported by the results. Results are clear, well presented and well controlled.

Comments for the author

In this revised manuscript, Rebecca Oramas and colleagues have substantially improved their manuscript and modified accordingly the figures. The authors provided detailed information to the comments and questions submitted by the reviewers. The manuscript has improved since the last submission and I would recommend its publication in Development.  

Reviewer 3

Advance summary and potential significance to field

This manuscript describes the role of Sim and its cofactor, Tgo, as a master regulator of the gene expression program that controls ovulation competency in Drosophila melanogaster. The authors provide a thorough characterization of how Sim:Tgo activity spatio-temporally controls genes important for promoting follicle cell differentiation and follicle rupture, ultimately leading to ovulation of mature oocytes. This study advances understanding of the molecular mechanism that underlies the complex process of ovulation.

Comments for the author

The reviewers have addressed all of the suggested revisions appropriately