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Follicle cell contact maintains main body axis polarity in the Drosophila melanogaster oocyte

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Revision 0

Review #1

1. Evidence, reproducibility and clarity:

Evidence, reproducibility and clarity (Required)

Summary

The formation of mutually exclusive domains of Partition defective (Par) proteins works as a foundation for establishment of cell polarity in a variety of cells. The Drosophila oocyte is a well-known model system to study mechanisms of the asymmetric distribution Par proteins. At stage 6/7 of oogenesis, an unknown signal the posterior follicle cells (PFC) induces the recruitment of the Par-1 kinase to the posterior cortex of the oocyte and the concomitant exclusion of aPKC/Par-6 from this region. By contrast, Bazooka (Par-3) remains at the posterior with Par-1 and only disappears from the posterior at early stage 9. Millas et al. investigate the nature of the PFC signal and whether PFC continue to play a role in keeping Bazooka away from the posterior after the original signal is received by the oocyte. They do so by following the distribution of Bazooka and other Par proteins in living oocytes after pulling away or ablating the PFC at various stages of oogenesis.

Major comments

1. Quality of live imaging
Judging from the appearance of the polar follicle cells and the size of the follicle cells, the authors constantly have an issue with maintaining a steady focal plane during live imaging in most movies (Figure 2 and video1; Figure 3 and video 2; Figure 4 and video 4; Figure 5 and video 5, FigureS5 and video 6). The conclusions of the paper are based on measuring changes in fluorescence intensity at the oocyte posterior over time, and this will be undermined by a varying focal plane. Considering the bullet shape of the oocyte, imaging the posterior at different focal planes could also cause artefacts. Supplementary Fig 3D-E and video 3 (a control experiment) are examples where the focal plane did not drift.
2. Mechanical contact of PFC with the oocyte cortex causes the posterior exclusion of Bazooka and maintains oocyte polarity
By physically pulling PFC away from the oocyte at stage 10b (Figures 1-2) the authors observed that in some oocytes Bazooka re-localises to posterior and concluded that it is a mechanical contact between PFC and the oocyte cortex that keeps Bazooka away from the posterior. Although this is an interesting observation per se, this is after the polarity of the oocyte has been defined (stages 6-9) and the posterior determinant, oskar mRNA has been localised. Could the authors do the same experiment at stages 6-9 to directly address whether the distance between the PFC and the oocyte cortex actually matters, considering that Bazooka remains at the posterior up to early 9 when the PFC and the oocyte are still at close contact?

The conclusion that the signal between PFC and the oocyte could be mechanical is only one of potential interpretations of the experiment. It still could be a short range/ non-diffusible biochemical signal that is sensitive to the distance between the PFC and the oocyte membrane. The authors do not provide any evidence for or against either interpretation.
3. Figure 5B is supposed to demonstrate that local loss of Par-1 at the posterior causes the re-growth of microtubules from this region. However, the data provided are not convincing. The accumulation of red vesicles at the posterior cortex 150 min post ablation does not look like a specific signal for Jupiter-mCherry-marked microtubules. Similar vesicles start to be visible in the neighbouring follicle cells at the same time.

Minor comments

1. In Figure 4A-C, it is not clear what area has been ablated
2. The authors should provide a simple 1-6 numbering for Video files

2. Significance:

Significance (Required)

The observation that the PFC are required to maintain oocyte polarity at stage 9 is significant, but not very surprising, given the recent observation by Doerflinger et al that the posterior localisation of Par-1 requires continuous myosin activation, demonstrating that the antagonism between anterior and posterior Par proteins is not sufficient to maintain polarity once established. The authors must improve the quality of the live imaging to support this conclusion.

The conclusion that phosphorylation of Bazooka by Par-1 is not sufficient to exclude Bazooka from the posterior cortex is not novel (see Doerflinger et al 2010, 2022).

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

Estimated time to Complete Revisions (Required)

(Decision Recommendation)

Cannot tell / Not applicable

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Yes

Review #2

1. Evidence, reproducibility and clarity:

Evidence, reproducibility and clarity (Required)

This manuscript examines an important and unsolved question concerning the establishment of the polarity axis in the Drosophila oocyte, namely how the follicle cells located at the posterior of the egg chamber trigger a signal to the oocyte for its subsequent polarization. To address this question the authors, center their studies on the localization of PAR proteins which are distributed along the antero-posterior axis. They more specifically focus on the mutual exclusion of Par1 and Bazooka/Par3 (Baz)at the posterior of the oocyte.
The signaling event from the posterior follicle cells toward the oocyte is an essential process however it remains unsolved despite numerous screening and genetic manipulation approaches, leaving open the possibility that classical signaling involving a diffusible ligand emitted by follicular cells with its receptor located at the plasma membrane of the oocyte, would not be applied here.

Here the authors are using original biophysical approaches to address whether signaling between the follicular cells and the oocyte would involve mechanical features.

The authors focus at the dual exclusion between Baz and Par1 between the stages 10 and 11. To specifically follow these two proteins in the oocyte without being disrupted by their expression in the follicle cells, they used the Gal4/UASp system to express Baz and Par1.
They found that Baz accumulate again at the posterior of the oocyte at stage 10B following the loss of contact between the posterior follicle cells (PFCs) and the oocyte whereas Par1 is gradually lost at that position. By using a glass micropipette to aspirate and pull on the PFCs they observed a premature Baz accumulation at the oocyte posterior. Then, to spatially improve the targeted area in the PFCs, the authors use a pulsed UV lazer, and show that PFCs are required to locally maintain posterior exclusion of Baz. Using a similar setup, they show that similarly Par1 is eliminated at the oocyte cell cortex region that had been in contact with ablated PFCs. However, Par1, with a kinetic slower to the one of Baz, is never disappearing before Baz appearance. Although difficult to distinguish, the authors report that the disappearance of Par1 is locally connected by an increase in microtubules (see major points). Finally, upon PFCs ablation, the authors show that the posterior reappearance of Baz is followed by the appearance of aPKC. However, the reappearance of PKC is slower than the removal of Par1, suggesting that in this case Par1 is not removed by PKC.

The particularly interesting results of this work show that cellular contacts between PFCs and the oocyte are necessary to maintain Baz exclusion and Par1 localization. Furthermore, the ablation

results suggest that individual PFCs are required to maintain local posterior exclusion of Baz. Overall it is an interesting observation, and most of the data are presented in a clean organized manner.

Major comments

1. The authors concentrate their studies on the distribution of Par3 and Par1 at the posterior part of the oocyte, mainly at stage 10 according to the images in the figures and movies.
The involvement of Par3 and Par1 on polarized transport to the posterior pole of the oocyte has been well characterized previously between stages 7 and 9.
The results of the authors are very interesting but they do not show that beyond the return of Baz and the disappearance of Par1 at the developmental stage they are looking at, the antero-posterior polarization and more particularly the localization of oskar in the posterior is affected. This is an important point as the authors propose that follicle cell contact maintains main body axis polarity. This would be possible by monitoring the impact of PFC ablation on the maintenance of oskar localization by tracking osk RNA with the MCP-MS2 system, or also by visualizing the staufen protein with a stau-GFP transgene.
2. The authors use the Jupiter protein fused to the cherry protein to track MTs. This is perfectly fine to highlight the cytoplasm in the oocyte and to outline the cell-cell contacts between the PFCs and the oocyte. However, with Jupiter-cherry the microtubules are not clearly detected in the oocyte in the data presented.This is a problem because the authors want to make an important point with the potential reappearance of microtubules in the oocyte while Par1 has disappeared in the vicinity of the destroyed PFCs. (Fig5).
The authors should use another microtubule reporter that allows better detection of microtubules in the oocyte, Jupiter-GFP, EB1-GFP, Ensconsin MT binding domain (EMTB)-RFP.

Minor comments:

1. The stage of the oocyte is not always indicated, this is particularly the case with the Fig2 with the pulling experiment with a glass micropipette.
2. With the Fig 3E, to highlight the fact that the intensity of Baz increases very quickly after the removal of PFCs (1 mn) the authors should include an insert with a shorter time scale.
The authors could also comment on the difference in velocity in baz reappearance when the ablation of PFCs includes or not polar cells.
3. In the discussion line 240, this is not myosin II but myosin V which anchored oskar mRNA at the posterior.
4. For the suppl figure 5, the n is not mentioned in the legend

2. Significance:

Significance (Required)

Nature and significance of the advance and work in the context of existing literature

This manuscript examines an important and unsolved question concerning the establishment of the polarity axis in the Drosophila oocyte, namely how the follicle cells located at the posterior of the egg chamber trigger a signal to the oocyte for its subsequent polarization (Gonzalez-Reyes et al ; Nature 1995 ;doi: 10.1038/375654a0) and (Roth et al; 1995; Cell; doi: 10.1016/0092-8674(95)90016-0).
To address this question the authors, center their studies on the localization of PAR proteins which are distributed along the antero-posterior axis. They more specifically focus on the mutual exclusion of Par1 and Bazooka/Par3 (Baz) at the posterior of the oocyte.
The signaling event from the posterior follicle cells toward the oocyte is an essential process however it remains unsolved despite numerous screening and genetic manipulation approaches, leaving open the possibility that classical signaling involving a diffusible ligand emitted by follicular cells with its receptor located at the plasma membrane of the oocyte, would not be applied here.
We still know little about the modalities of this signaling between the follicular cells and the oocyte necessary for the polarization of the latter. We know that the first sign of anteroposterior polarization in the oocyte is posteriorly the recruitment of Par1 and subsequently the elimination of Baz. However, we do not know the nature of this signaling. Furthermore, we do not know whether this signaling must be maintained in order to maintain the polarization of the oocyte and more particularly to maintain the localization of oskar RNA, the posterior determinant of the oocyte,
Here the authors are using original biophysical approaches to address whether signaling between the follicular cells and the oocyte would involve mechanical features.
Important results of this work show that cell contacts between PFCs and the oocyte are necessary to maintain baz exclusion and Par1 localisation. Furthermore, the ablation results suggest that individual PFCs are required to maintain local posterior exclusion of Bazooka.

Audience:
These results will be of interest to those interested in the relationship between cell signaling and polarization in particular in a developmental context.

Reviewer's area of expertise:
Cell polarity, microtubule-associated transport, oocyte development in Drosophila.

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

Estimated time to Complete Revisions (Required)

(Decision Recommendation)

Between 1 and 3 months

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Yes

Review #3

1. Evidence, reproducibility and clarity:

Evidence, reproducibility and clarity (Required)

The Drosophila oocyte is a classical model to study establishment of cell polarity, and it is known for decades how Bicoid and Oskar define the anterior-posterior axis of the embryo. However, Bicoid and Oskar are not conserved so that these findings cannot be generalized. The situation is different for the Par proteins, which have been identified in C. elegans. They are not only conserved but also their mode of of action seems to be preserved. 20 years ago it was a very surprising finding that the Par proteins contribute to establishment of polarity in the Drosophila oocyte. A fascinating and simple mutual inhibition model emerged over the years, in which the same molecular mechanisms establish cell polarity in the C. elegans one-cell embryo and in the Drosophila oocyte: Anteriorly localised Par-3/Bazooka recruits aPKC kinase, which excludes Par-1 by phosphorylation, whereas posteriorly localised Par-1 kinase excludes Par-3/Bazooka by phosphorylation. The manuscript by Milas et al. challenges this model by closely analysing Par localisation in living Drosophila oocytes. The authors provide strong evidence that the kinetics of Par-1 and Bazooka localisation are not consistent with the model.

Milas et al. first describe a morphological difference between the anterior-lateral and posterior cortex of the oocyte by showing that only the posterior cortex is tightly connected to the overlaying epithelium. This morphological difference correlates with the localisation of Par-1, which is restricted to the posterior, while Bazooka localises only to those regions of the cortex, where there is a gap between the oocyte and the epithelium. This gap expands towards the posterior cortex during stage 10A and encloses it at stage 11. Unexpectedly, Par-1 and Bazooka localisations overlap at the posterior cortex when the gap expands, which contradicts the mutual inhibition model. The authors hypothesised that the close contact of epithelium with the oocyte might influence Par-1/Bazooka localisation. To test this they mechanically detached the epithelium from the oocyte and also ablated groups of epithelial cells. These manipulations resulted in posterior spreading of Bazooka protein within 30-60 minutes. Interestingly, the

authors found that in those regions of the posterior cortex, where cells have been ablated, Par-1 and Bazooka colocalise for 30 minutes, which is difficult to reconcile with a model in which Par-1 excludes Bazooka by phosphorylation. The authors also show that Par-1 finally disappeared form the regions where epithelial cells have been ablated. However, aPKC, the kinase that is supposed the exclude Par-1 by phosphorylation, appeared only after Par-1, which argues against the idea that aPKC prevents Par-1 localisation. In summary, the described localisation kinetics are in conflict with the current model, in which direct phosphorylation activities of Par-1 and aPKC orchestrate the mutual exclusive Par domains in the Drosophila oocyte. The data suggest that the mechanisms underlying mutual inhibition are more complex than thought and involve contact with posterior epithelial cells.

The microscopy used by the authors is state of the art, the data are of high quality and the quantitative analysis is convincing. The results are surprising but conclusive since the experiments were performed and presented in a professional way. This combination makes the manuscript very interesting.

Major points:

1. The finding that the posterior cortex is in close contact to the epithelium, while there is a gap between the remaining oocyte cortex and the epithelium is very interesting, and should be quantified and characterised more precisely. When does the gap form and how exactly does it spread posteriorly? Is it possible to distinguish the gap from the attachment zone by using markers for the ECM (e.g. viking-GFP) or adhesion proteins (e.g. Integrin)?
2. The authors suggest that direct contact between the epithelium and the oocyte is required to exclude Bazooka from the posterior oocyte cortex. The polar cells of the follicular epithelium have almost no contact to the oocyte. One would expect that if only the polar cells are ablated, this would not lead to posterior spreading of Bazooka. Such a control experiment could support the author´s model.

Minor points:

1. There are repeatedly double negations which make the text difficult to understand (e.g. "Bazooka exclusion was lost...." (line 104) or "Par-1 does not delocalise from the posterior pole prior to accumulation of Bazooka" (line 163). I see that this follows the logic of the published molecular mechanisms but for the sake of comprehensibility, the authors should try to formulate the results in a positive way (at least in a repeating sentence).
2. Based on the kinetics of Par-1 localisation the authors the conclude that Par-1 binds to diffusible binding sites at the oocyte cortex, which are modulated by posterior epithelial cells. This is one possible explanation for their results but other interpretations are equally possible. Since the authors provide no further evidence for the existence of Par-1 binding sites their interpretation should be formulated more carefully.
3. The authors should mention that they use the Par-1 isoform (N1S) which fully rescues the par-1 mutant phenotype (see Doerflinger et. al, Curr Biol, 2006). What is known about the rescuing activity of the Bazooka transgenes that were used in the manuscript?
4. In principle it is possible that the posterior spreading of Bazooka (after follicle cell detachment

or ablation) is caused by premature ooplasmic steaming. However, the movies show that this is not the case. This should be stated in the text.

2. Significance:

Significance (Required)

The Drosophila oocyte is a classical model to address the fundamental biological question of how cell polarity is established. The current model of mutual Par protein inhibition is a critical part of our understanding of cell polarisation, and was proposed to be conserved between flies and worms. In the case of Drosophila this model mainly relies on a combination of genetic and biochemical data. Milas et al. tested this model by using in vivo imaging, and found that the kinetics of Par localisation do not correspond to the existing model. This suggests that central aspects of the proposed mechanisms controlling mutual Par inhibition in the Drosophila oocyte are not conserved or not fully understood. The work makes therefore a surprising and important contribution to the understanding of cell polarity.

I work for many years on Drosophila oogenesis and my main interest switched from cell polarity to membrane trafficking.

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

Estimated time to Complete Revisions (Required)

(Decision Recommendation)

Between 1 and 3 months

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Yes

[All red references to figures, videos or text lines are referring to the revised manuscript]

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

Summary

The formation of mutually exclusive domains of Partition defective (Par) proteins works as a foundation for establishment of cell polarity in a variety of cells. The Drosophila oocyte is a well-known model system to study mechanisms of the asymmetric distribution Par proteins. At stage 6/7 of oogenesis, an unknown signal the posterior follicle cells (PFC) induces the recruitment of the Par-1 kinase to the posterior cortex of the oocyte and the concomitant exclusion of aPKC/Par-6 from this region. By contrast, Bazooka (Par-3) remains at the posterior with Par-1 and only disappears from the posterior at early stage 9. Milas et al. investigate the nature of the PFC signal and whether PFC continue to play a role in keeping Bazooka away from the posterior after the original signal is received by the oocyte. They do so by following the distribution of Bazooka and other Par proteins in living oocytes after pulling away or ablating the PFC at various stages of oogenesis.

Major comments

1. Quality of live imaging
Judging from the appearance of the polar follicle cells and the size of the follicle cells, the authors constantly have an issue with maintaining a steady focal plane during live imaging in most movies (Figure 2 and video1; Figure 3 and video 2; Figure 4 and video 4; Figure 5 and video 5, FigureS5 and video 6). The conclusions of the paper are based on measuring changes in fluorescence intensity at the oocyte posterior over time, and this will be undermined by a varying focal plane. Considering the bullet shape of the oocyte, imaging the posterior at different focal planes could also cause artefacts. Supplementary Fig 3D-E and video 3 (a control experiment) are examples where the focal plane did not drift.

Response: We appreciate the concerns of this reviewer regarding our imaging modality, and we agree that a stable focus is important for intensity analysis in space and time. However, the intensity quantifications were performed on z-projections of the 3D volumes, and if potential drifts are not excessive, the signal integration is expected to be reliable. In addition, we measured the intensity in the sum of 6 z-slices, which reduces the fluctuations in intensity that occur due to potential z-drift. In our original manuscript we have also not included image data with large drifts. That said, we wanted to improve the image quality in general, in the hope to address this reviewer's concerns more broadly, and made the following additional analysis, experiments and changes:

1) We have checked all the data presented in our original manuscript for focus stability. There was no focus drift in Video 1 (Fig. 2); the small movements are caused by the positional control of the pipette holder. Indeed, video 2 (and the image series in Fig. 3) contains manually induced corrections in the focusing. We were able to correct this z-drift because we are imaging 73 z-planes and only using the sum of 6 z-slices to measure and present the data. We show z-corrected data in new Video 2 and Fig. 3. Note that, when performing corrections, we made sure that the region in which the measurements are done (posterior of the egg chamber) remains in focus. This approach sometimes results in movements at the anterior of the egg chamber due to changes in the sample adherence to the glass surface. There was no focus drift in Video 4 (which is the new Video 5 and new Suppl Fig. 3). The small movements at the anterior side of the egg chamber in this video are also due to changes in the sample adherence. Video 5 (Fig. 5) and 6 (Fig. S5) did not have focus drift,

however, we have replaced this data with new data, showing the same phenomenon (for reasons outlined below).

2) We have consulted experts in the field on how to improve the sample preparation. Consequently, we have repeated the key experiments using a modification in the sample preparation, as described in Gaspar and Yu et al., JCB, 2014. We dissected egg chambers in Schneider's medium supplemented with insulin and FBS, and covered the sample with halocarbon oil to prevent drying out. The consequence is that the egg chambers are surrounded by a thin layer of aqueous solution, which not only improves imaging, but also sample stability and survival. Of note, laser ablation became even more effective under these preparation conditions. We have added this information to the Methods (lines 500–506)

By repeating all ablation experiments with this new sample preparation protocol, we have obtained better sample stability for intensity analysis and, most importantly, we can confirm our previous findings fully. New Fig. 4–6 show time lapse images and intensity timelines under the new preparation modality, confirming that PFCs are continuously needed to maintain Par polarity and body axis determination. Thus, we do not doubt our interpretation of the originally acquired data.

2. Mechanical contact of PFC with the oocyte cortex causes the posterior exclusion of Bazooka and maintains oocyte polarity

By physically pulling PFC away from the oocyte at stage 10b (Figures 1-2) the authors observed that in some oocytes Bazooka re-localises to posterior and concluded that it is a mechanical contact between PFC and the oocyte cortex that keeps Bazooka away from the posterior. Although this is an interesting observation per se, this is after the polarity of the oocyte has been defined (stages 6-9) and the posterior determinant, oskar mRNA has been localised. Could the authors do the same experiment at stages 6-9 to directly address whether the distance between the PFC and the oocyte cortex actually matters, considering that Bazooka remains at the posterior up to early 9 when the PFC and the oocyte are still at close contact?

Response: As this reviewer highlights, data from other groups have shown that Bazooka remains at the posterior up to stage 9. This precludes any interpretation of a mechanical manipulation experiment at stages 6–9. Since Bazooka is already localized, and mechanical detachment causes re-localization, we do not expect any changes. We also want to emphasize that the mechanical manipulation experiment merely indicated that the PFC-oocyte contact is important for polarity maintenance. As we show in the new version of the manuscript, the presence of PFCs is also crucial for the maintenance of *osk* mRNA localization (new Fig. 5).

The conclusion that the signal between PFC and the oocyte could be mechanical is only one of potential interpretations of the experiment. It still could be a short range/ non-diffusible biochemical signal that is sensitive to the distance between the PFC and the oocyte membrane. The authors do not provide any evidence for or against either interpretation.

Response: Our laser ablation experiments provide evidence that the re-localization of Bazooka is constrained to the region facing the ablated cells. Hence, PFC contact is single (PFC) cell-precise. This argues for a biochemical signal that is "short-range" and cannot diffuse laterally within the membrane or inside the extracellular space beyond the distance of a single follicle cell. Or it is a non-diffusible signal molecule, which is essentially anchored at the cell cortex, and binding to its target is a mechanical contact process. Our data provides evidence for polarity maintenance requiring tight cell-cell (mechanical) contact, which provides the local process of the cell-to-cell signal transduction that then modulates Par binding / unbinding at the oocyte cortex. We may not call it "mechanical signal" but simply "contact". However, the important

observation is that transduction through contact is single PFC-precise. To prevent confusion about the terminology in the original version, we made sure that in the revised version we avoid the term “mechanical signal” (e.g. lines 291–292).

3. Figure 5B is supposed to demonstrate that local loss of Par-1 at the posterior causes the re-growth of microtubules from this region. However, the data provided are not convincing. The accumulation of red vesicles at the posterior cortex 150 min post ablation does not look like a specific signal for Jupiter-mCherry-marked microtubules. Similar vesicles start to be visible in the neighbouring follicle cells at the same time.

Response: We provide new data with improved image quality (Fig. 5D) acquired from flies expressing Jupiter::GFP, *oskar*-mCherry. Images show a local increase in Jupiter::GFP signal, which necessitates microtubule polymerization (due to property of Jupiter binding only microtubule lattice). This signal is also higher than the autofluorescence of vesicles in the oocyte.
On a different but important note, our new data presented in Fig. 5 also suggests that the loss of *osk* posterior localization after PFC ablation can occur before microtubules start to polymerize at the posterior (due to loss of Par-1, which inhibits MT polymerization). Thus, the presence of microtubules growing from the posterior end and pointing into the oocyte cytoplasm may not be the primary reason for *osk* delocalization, e.g. by kinesin-1 mediated transport (see interpretations in Zimyanin et al 2008). This was quite surprising yet very insightful for understanding polarity maintenance.

Minor comments

1. In Figure 4A-C, it is not clear what area has been ablated

Response: We have added a dashed circle to show where the ablation was performed.

2. The authors should provide a simple 1-6 numbering for Video files

Response: In the new version the videos have been renumbered in a simple fashion.

Reviewer #1 (Significance (Required)):

Significance

The observation that the PFC are required to maintain oocyte polarity at stage 9 is significant, but not very surprising, given the recent observation by Doerflinger et al that the posterior localization of Par-1 requires continuous myosin activation, demonstrating that the antagonism between anterior and posterior Par proteins is not sufficient to maintain polarity once established. The authors must improve the quality of the live imaging to support this conclusion.

Response: We have noted that, very recently, Doerflinger et al (2022) provided evidence for polarity loss in stage 9-10 oocytes, provided that myosin regulatory light chain (MRLC) is phosphorylated and myosin is activated. They show that continued MRLC dephosphorylation inside the oocyte is required to maintain polarity. Strictly speaking, one cannot conclude the need for a persistent PFC “signaling” from their observation as they show the need for a persistent intracellular process of the oocyte. In our present study, we show that maintenance of Par and mRNA polarity requires persistent PFC contact at a single cell resolution. Hence, we show that PFCs play an ongoing role in polarity maintenance. More excitingly, one could now conclude that PFC contact, and the signal transduction across cells, leads to MRLC dephosphorylation, and design experiments to test that.

The conclusion that phosphorylation of Bazooka by Par-1 is not sufficient to exclude Bazooka from the posterior cortex is not novel (see Doerflinger et al 2010, 2022).

> Response: We thank this reviewer for pointing out earlier studies that show Par-1 is not sufficient. We are aware of these studies, which show that in some stages Par-1 and Bazooka colocalize at the posterior. We suspect that this reviewer has noticed our conclusion on lines 108–109 and 175–176 which, we admit, should have been formulated and worded more precise. We now say that after PFC ablation Par-1 is not sufficient to prevent Bazooka re-localization – the response to an acute perturbation – which has not been tested so far. We also modified the text to say that during stage 10B to 11 transition Bazooka re-localization is not prevented by Par-1 (lines 111–112).

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

This manuscript examines an important and unsolved question concerning the establishment of the polarity axis in the Drosophila oocyte, namely how the follicle cells located at the posterior of the egg chamber trigger a signal to the oocyte for its subsequent polarization. To address this question the authors center their studies on the localization of PAR proteins which are distributed along the antero-posterior axis. They more specifically focus on the mutual exclusion of Par1 and Bazooka/Par3 (Baz) at the posterior of the oocyte.
The signaling event from the posterior follicle cells toward the oocyte is an essential process however it remains unsolved despite numerous screening and genetic manipulation approaches, leaving open the possibility that classical signaling involving a diffusible ligand emitted by follicular cells with its receptor located at the plasma membrane of the oocyte, would not be applied here.

Here the authors are using original biophysical approaches to address whether signaling between the follicular cells and the oocyte would involve mechanical features.

> Response: We thank this reviewer for recognizing the relevance of our scientific question and the originality of our experimental approach.

The authors focus at the dual exclusion between Baz and Par1 between the stages 10 and 11. To specifically follow these two proteins in the oocyte without being disrupted by their expression in the follicle cells, they used the Gal4/UASp system to express Baz and Par1.
They found that Baz accumulate again at the posterior of the oocyte at stage 10B following the loss of contact between the posterior follicle cells (PFCs) and the oocyte whereas Par1 is gradually lost at that position. By using a glass micropipette to aspirate and pull on the PFCs they observed a premature Baz accumulation at the oocyte posterior. Then, to spatially improve the targeted area in the PFCs, the authors use a pulsed UV lazer, and show that PFCs are required to locally maintain posterior exclusion of Baz. Using a similar setup, they show that similarly Par1 is eliminated at the oocyte cell cortex region that had been in contact with ablated PFCs. However, Par1, with a kinetic slower to the one of Baz, is never disappearing before Baz appearance. Although difficult to distinguish, the authors report that the disappearance of Par1 is locally connected by an increase in microtubules (see major points). Finally, upon PFCs ablation, the authors show that the posterior reappearance of Baz is followed by the appearance of aPKC. However, the reappearance of PKC is slower than the removal of Par1, suggesting that in this case Par1 is not removed by PKC.

The particularly interesting results of this work show that cellular contacts between PFCs and the oocyte are necessary to maintain Baz exclusion and Par1 localization. Furthermore, the ablation

results suggest that individual PFCs are required to maintain local posterior exclusion of Baz. Overall it is an interesting observation, and most of the data are presented in a clean organized manner.

Response: Again, we thank this reviewer for the positive feedback, highlighting the originality and relevance, and for recognizing the overall quality of our data.

Major comments

1- The authors concentrate their studies on the distribution of Par3 and Par1 at the posterior part of the oocyte, mainly at stage 10 according to the images in the figures and movies.
The involvement of Par3 and Par1 on polarized transport to the posterior pole of the oocyte has been well characterized previously between stages 7 and 9.
The results of the authors are very interesting but they do not show that beyond the return of Baz and the disappearance of Par1 at the developmental stage they are looking at, the antero-posterior polarization and more particularly the localization of oskar in the posterior is affected. This is an important point as the authors propose that follicle cell contact maintains main body axis polarity. This would be possible by monitoring the impact of PFC ablation on the maintenance of oskar localization by tracking osk RNA with the MCP-MS2 system, or also by visualizing the staufen protein with a stau-GFP transgene.

Response: We are grateful to this reviewer for pointing out this weakness. We have invested considerable time in acquiring new data of osk mRNA localization changes following PFC ablation. Adopting the MCP-MS2 system for *osk*, we now present this data in Fig. 5, which confirms the relevance of PFC mediated oocyte polarity maintenance at the level of the *osk* transcript. After ablation, posterior *osk* localization is rapidly lost, and the decay occurs after Par-1 has started to disappear. Furthermore, although the time of appearance of microtubules at the posterior is quite variable, we can say that *osk* loss at the posterior occurs before or, at most, at a similar time but never after microtubule polymerization. We believe that this addition of data has strengthened our overall conclusion and thank the reviewer for the suggestion.

2- The authors use the Jupiter protein fused to the cherry protein to track MTs. This is perfectly fine to highlight the cytoplasm in the oocyte and to outline the cell-cell contacts between the PFCs and the oocyte. However, with Jupiter-cherry the microtubules are not clearly detected in the oocyte in the data presented. This is a problem because the authors want to make an important point with the potential reappearance of microtubules in the oocyte while Par1 has disappeared in the vicinity of the destroyed PFCs. (Fig5).
The authors should use another microtubule reporter that allows better detection of microtubules in the oocyte, Jupiter-GFP, EB1-GFP, Ensconsin MT binding domain (EMTB)-RFP.

Response: We agree that the original images of microtubule growth at the posterior were at the limit of the detection limit. As suggested by the reviewer, we used Jupiter-GFP as a reporter for microtubule lattice formation (growth). In these experiments (Fig. 5D), we observe Jupiter signal accumulation at the posterior well beyond the background and the autofluorescence of vesicles (see criticism by Reviewer 1). Although the time of reappearance of microtubules is variable, it happens likely after the disappearance of *osk* mRNA (see Fig. 5A–B versus D).

Minor comments:

1- The stage of the oocyte is not always indicated, this is particularly the case with the Fig2 with the pulling experiment with a glass micropipette.

Response: In the new version of the manuscript, we mention the stage of the oocyte for all the figures or data sets. We have also introduced a fair amount of stage distinction for previous and additional data in the revised manuscript (e.g. Fig. 5).

2- With the Fig 3E, to highlight the fact that the intensity of Baz increases very quickly after the removal of PFCs (1 min) the authors should include an insert with a shorter time scale.
The authors could also comment on the difference in velocity in baz reappearance when the ablation of PFCs includes or not polar cells.

Response: We have added an inset to Fig 3E where we show the initial phase of Bazooka accumulation, and we mention the slight difference in kinetics between the cases where polar cells were left intact and those where they were ablated (lines 148–149).

3- In the discussion line 240, this is not myosin II but myosin V which anchored oskar mRNA at the posterior.

Response: We thank the reviewer for noticing this error, which we corrected (line 265).

4- For the suppl figure 5, the n is not mentioned in the legend

Response: We have added this information, now in Fig. 6. This remark prompted us to reassure that all figure legends mention n.

Reviewer #2 (Significance (Required)):

Nature and significance of the advance and work in the context of existing literature

This manuscript examines an important and unsolved question concerning the establishment of the polarity axis in the Drosophila oocyte, namely how the follicle cells located at the posterior of the egg chamber trigger a signal to the oocyte for its subsequent polarization (Gonzalez-Reyes et al ; Nature 1995 ;doi: 10.1038/375654a0) and (Roth et al; 1995; Cell; doi: 10.1016/0092-8674(95)90016-0).
To address this question the authors, center their studies on the localization of PAR proteins which are distributed along the antero-posterior axis. They more specifically focus on the mutual exclusion of Par1 and Bazooka/Par3 (Baz) at the posterior of the oocyte.
The signaling event from the posterior follicle cells toward the oocyte is an essential process however it remains unsolved despite numerous screening and genetic manipulation approaches, leaving open the possibility that classical signaling involving a diffusible ligand emitted by follicular cells with its receptor located at the plasma membrane of the oocyte, would not be applied here.
We still know little about the modalities of this signaling between the follicular cells and the oocyte necessary for the polarization of the latter. We know that the first sign of anteroposterior polarization in the oocyte is posteriorly the recruitment of Par1 and subsequently the elimination of Baz.
However, we do not know the nature of this signaling. Furthermore, we do not know whether this signaling must be maintained in order to maintain the polarization of the oocyte and more particularly to maintain the localization of oskar RNA, the posterior determinant of the oocyte,
Here the authors are using original biophysical approaches to address whether signaling between the follicular cells and the oocyte would involve mechanical features.
Important results of this work show that cell contacts between PFCs and the oocyte are necessary to maintain baz exclusion and Par1 localisation. Furthermore, the ablation results suggest that individual PFCs are required to maintain local posterior exclusion of Bazooka.

Response: We appreciate the overall positive feedback from this reviewer.

Audience:
These results will be of interest to those interested in the relationship between cell signaling and polarization in particular in a developmental context.

Reviewer's area of expertise:
Cell polarity, microtubule-associated transport, oocyte development in Drosophila.

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

The Drosophila oocyte is a classical model to study establishment of cell polarity, and it is known for decades how Bicoid and Oskar define the anterior-posterior axis of the embryo. However, Bicoid and Oskar are not conserved so that these findings cannot be generalized. The situation is different for the Par proteins, which have been identified in C. elegans. They are not only conserved but also their mode of action seems to be preserved. 20 years ago it was a very surprising finding that the Par proteins contribute to establishment of polarity in the Drosophila oocyte. A fascinating and simple mutual inhibition model emerged over the years, in which the same molecular mechanisms establish cell polarity in the C. elegans one-cell embryo and in the Drosophila oocyte: Anteriorly localised Par-3/Bazooka recruits aPKC kinase, which excludes Par-1 by phosphorylation, whereas posteriorly localised Par-1 kinase excludes Par-3/Bazooka by phosphorylation. The manuscript by Milas et al. challenges this model by closely analysing Par localisation in living Drosophila oocytes. The authors provide strong evidence that the kinetics of Par-1 and Bazooka localisation are not consistent with the model.

Milas et al. first describe a morphological difference between the anterior-lateral and posterior cortex of the oocyte by showing that only the posterior cortex is tightly connected to the overlaying epithelium. This morphological difference correlates with the localisation of Par-1, which is restricted to the posterior, while Bazooka localises only to those regions of the cortex, where there is a gap between the oocyte and the epithelium. This gap expands towards the posterior cortex during stage 10A and encloses it at stage 11. Unexpectedly, Par-1 and Bazooka localisations overlap at the posterior cortex when the gap expands, which contradicts the mutual inhibition model. The authors hypothesised that the close contact of epithelium with the oocyte might influence Par-1/Bazooka localisation. To test this they mechanically detached the epithelium from the oocyte and also ablated groups of epithelial cells. These manipulations resulted in posterior spreading of Bazooka protein within 30-60 minutes. Interestingly, the authors found that in those regions of the posterior cortex, where cells have been ablated, Par-1 and Bazooka colocalise for 30 minutes, which is difficult to reconcile with a model in which Par-1 excludes Bazooka by phosphorylation. The authors also show that Par-1 finally disappeared from the regions where epithelial cells have been ablated. However, aPKC, the kinase that is supposed the exclude Par-1 by phosphorylation, appeared only after Par-1, which argues against the idea that aPKC prevents Par-1 localisation. In summary, the described localisation kinetics are in conflict with the current model, in which direct phosphorylation activities of Par-1 and aPKC orchestrate the mutual exclusive Par domains in the Drosophila oocyte. The data suggest that the mechanisms underlying mutual inhibition are more complex than thought and involve contact with posterior epithelial cells.

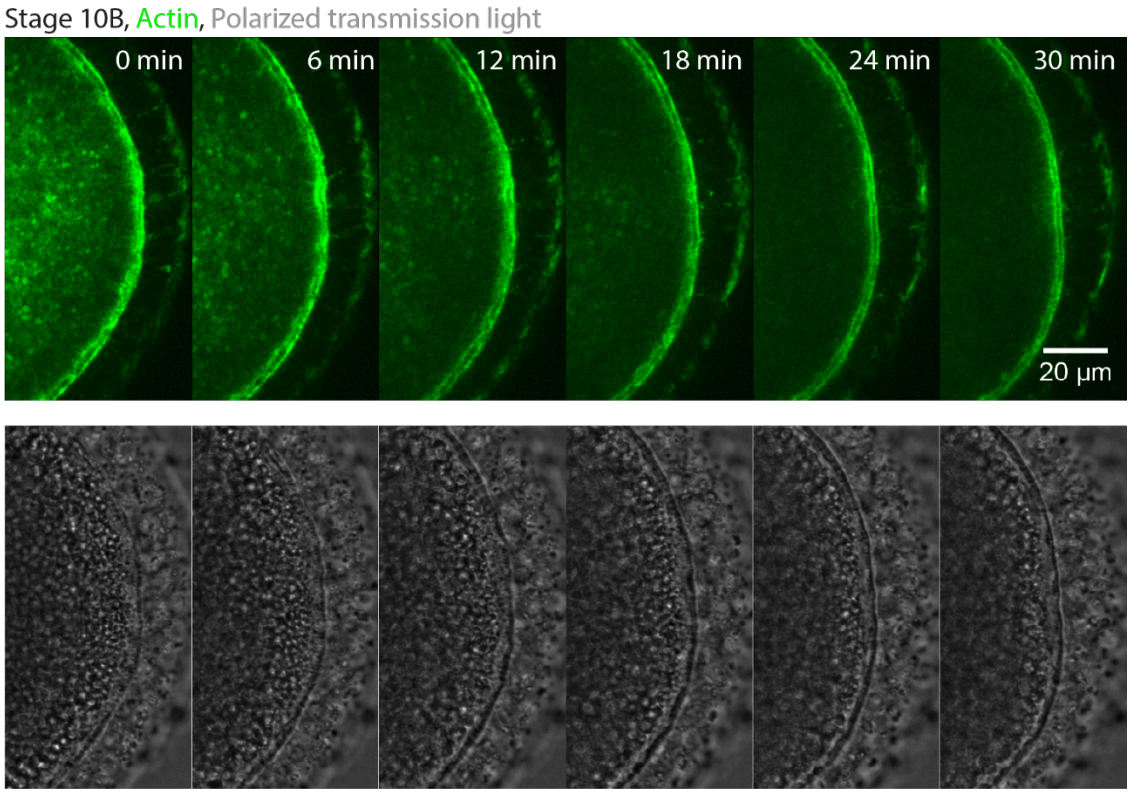
The microscopy used by the authors is state of the art, the data are of high quality and the quantitative analysis is convincing. The results are surprising but conclusive since the experiments were performed and presented in a professional way. This combination makes the manuscript very interesting.

> Response: We thank this reviewer for recognizing state of art, quality, conclusiveness, and professionalism of our work.

Major points:
1) The finding that the posterior cortex is in close contact to the epithelium, while there is a gap between the remaining oocyte cortex and the epithelium is very interesting, and should be quantified and characterised more precisely. When does the gap form and how exactly does it spread posteriorly? Is it possible to distinguish the gap from the attachment zone by using markers for the ECM (e.g. viking-GFP) or adhesion proteins (e.g. Integrin)?

Response: We have a small number of images suggesting that stage 8 oocytes never exhibit an *inter*cellular gap between oocyte and follicle cells. At this stage, Bazooka is localized along the entire oocyte cortex, including at the posterior end. We now show a quantification of presence of intercellular gap in stages 9–11 oocytes (in updated Fig. 1D). This data suggests that the gap starts to appear at the lateral oocyte cortex in stage 9, and only thereafter Bazooka is excluded from the posterior (we never see Bazooka exclusion before lateral gap formation). We mention these pieces of information briefly in the revised manuscript (lines 97–98).

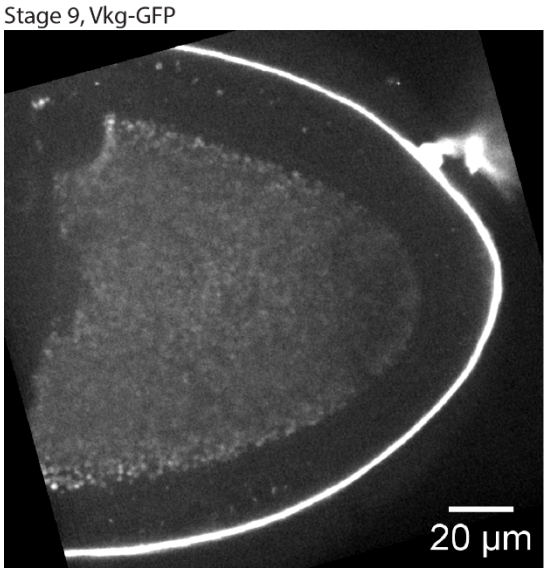
The appearance of the intercellular gap all around the oocyte, and the disappearance specifically at the posterior, is difficult to document with live imaging as it occurs sometime between stage 8 and 9. The reformation of the gap at the posterior is similarly time-randomized, and the gap formation occurs within 10–20 min. Considering the duration of these stages, and the sample lifetime for acquisition, we could not collect many datasets that would document these events. However, by chance of things we acquired one time-lapse video that shows the re-appearance of the gap at the posterior in stage 10B (Fig 1. In this letter)



Letter Figure 1: Time-lapse images of the posterior end of a stage 10B egg chamber, showing the actin reporter Utrophin-GFP (top) and polarized transmission light (bottom). In this example,

the cell-cell interphase between oocyte and PFCs markedly changes so that two distinct GFP signal lines (top) and a darker line (bottom) form in the time course of ~20 min.

Furthermore, as suggested by this reviewer, we have obtained flies expressing the ECM component reporter vkg-GFP (FlyTrap cc00791) and looked at egg chambers in various stages but could not confirm any distinction of follicle cell attachment laterally versus posteriorly from this reporter. The image in Figure 2 of this letter shows an example egg chamber from this line; the GFP signal is localized at the basal side of follicle cells, but no clear signal appear on the apical side nor is there a distinct posterior versus lateral localization (the autofluorescence of yolk in the oocyte provides a sense for the exposure and contrast in the image). Thus, this particular reporter did not reveal characteristics at the posterior or the lateral oocyte-follicle cell interface.



Letter Figure. 2: Confocal image (projection of 6 z-slices volume) of an egg chamber where vkg-GFP (collagen IV subunit) is expressed, a component of the extracellular matrix. The signal is mostly basal of follicle cells, and we did not detect any difference localization at the apical side (follicle cell-oocyte interface).

Regarding the study of cell-cell adhesion, we are convinced that this is at the heart of signal transfer from intercellular contact. We also believe that this requires a more thorough analysis and screening of various adhesion and ECM molecules, but this goes beyond the scope of the present study, which is the first demonstration for the requirement of contact.

2) The authors suggest that direct contact between the epithelium and the oocyte is required to exclude Bazooka from the posterior oocyte cortex. The polar cells of the follicular epithelium have almost no contact to the oocyte. One would expect that if only the polar cells are ablated, this would not lead to posterior spreading of Bazooka. Such a control experiment could support the author´s model.

Response: This is an interesting suggestion as alternative working hypothesis, but very challenging to test experimentally. It assumes that signal transduction by PFC-oocyte contact exhibits a threshold so that ablating those cells with very small cell-cell contact surfaces would not alter Par protein localization in the oocyte. It also suggests that when only 2–3 non-polar

follicle cells are ablated Bazooka will still localize at their location exclusively. However, we emphasize here that it is technically very challenging to ablate just two follicle cells or just the polar cells; this is because we do not have a good resolution of ablation in the z-direction. Although we do see the response of PFC ablation being cell size-precise – so that the boundary of Bazooka re-localization is between two PFCs – we cannot ablate just one single PFC.

Minor points:

1) There are repeatedly double negations which make the text difficult to understand (e.g. "Bazooka exclusion was lost...." (line 104) or "Par-1 does not delocalise from the posterior pole prior to accumulation of Bazooka" (line 163). I see that this follows the logic of the published molecular mechanisms but for the sake of comprehensibility, the authors should try to formulate the results in a positive way (at least in a repeating sentence).

Response: We thank the reviewer for noticing the potential negative of these formulation for readability. Indeed, we wanted to follow the logic of the published Par protein interaction and localization, but it may add confusion. We have decided to correct a selection of sentence and formulated them as positive statements (lines 105–107 and 168), while we left a few unchanged because they refer to the preceding sentence (e.g. lines 91–92).

2) Based on the kinetics of Par-1 localisation the authors conclude that Par-1 binds to diffusible binding sites at the oocyte cortex, which are modulated by posterior epithelial cells. This is one possible explanation for their results but other interpretations are equally possible. Since the authors provide no further evidence for the existence of Par-1 binding sites their interpretation should be formulated more carefully.

Response: We have re-formulated this section and introduced it cautiously as a possible explanation (lines 167 and 194). We agree that we do not provide formal evidence for diffusible binding sites for Par-1. However, another explanation we conceive formally as possible is that the binding affinity between Par-1 and its binding site exhibits a spatial gradient (as shown in Fig. 5C of the original submission). For that to occur, the binding affinity at any cortex location would have to depend in some (linear or non-linear) way on the affinity in its neighborhood; this would render a spatial gradient in affinity at any time point. Affinity would also have to change in time according to the spatial gradient. Essentially, this would lead to a “diffusion equation for the biochemical affinity”. The molecular events leading to such a space-time coupling of biochemical affinity are not easy to discern. Therefore, we prefer the first model where binding sites (i.e. molecules) are diffusing within the cortex.

3) The authors should mention that they use the Par-1 isoform (N1S) which fully rescues the par-1 mutant phenotype (see Doerflinger et. al, Curr Biol, 2006). What is known about the rescuing activity of the Bazooka transgenes that were used in the manuscript?

Response: Yes, we use the Par-1 isoform (provided by St. Johnston’s lab) that rescues the *par-1* mutant, and we now explicitly mention this in the Methods section. The rescuing activity of the Bazooka transgene used in this study was shown in Benton and St Johnston, 2003b (doi:10.1016/S0960-9822(03)00508-6). We have also added this explicitly in the Methods section (lines 478–479)

4) In principle it is possible that the posterior spreading of Bazooka (after follicle cell detachment or ablation) is caused by premature ooplasmic steaming. However, the movies show that this is not the case. This should be stated in the text.

Response: We mention that there is no cytoplasmic streaming in the context of Par1 removal (original manuscript, line 194). However, we agree that we should probably mention this piece of information earlier in the paper and have modified the text accordingly (line 154).

Reviewer #3 (Significance (Required)):

The Drosophila oocyte is a classical model to address the fundamental biological question of how cell polarity is established. The current model of mutual Par protein inhibition is a critical part of our understanding of cell polarisation, and was proposed to be conserved between flies and worms. In the case of Drosophila this model mainly relies on a combination of genetic and biochemical data. Milas et al. tested this model by using in vivo imaging, and found that the kinetics of Par localisation do not correspond to the existing model. This suggests that central aspects of the proposed mechanisms controlling mutual Par inhibition in the Drosophila oocyte are not conserved or not fully understood. The work makes therefore a surprising and important contribution to the understanding of cell polarity.
Response: We thank this reviewer for recognizing the significance of our work.

I work for many years on Drosophila oogenesis and my main interest switched from cell polarity to membrane trafficking.

October 28, 2022

RE: JCB Manuscript #202209052T

Dr. Ivo A Telley
Instituto Gulbenkian de Ciência
Rua da Quinta Grande 6
Oeiras 2780-156
Portugal

Dear Dr. Telley:

Thank you for submitting your revised manuscript entitled "Follicle cell contact maintains main body axis polarity in the Drosophila melanogaster oocyte". We would be happy to publish your paper in JCB pending final revisions necessary to meet our formatting guidelines (see details below).

To avoid unnecessary delays in the acceptance and publication of your paper, please read the following information carefully.

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3) Figure formatting: Scale bars must be present on all microscopy images, including inset magnifications. Molecular weight or nucleic acid size markers must be included on all gel electrophoresis.

4) Statistical analysis: Error bars on graphic representations of numerical data must be clearly described in the figure legend. The number of independent data points (n) represented in a graph must be indicated in the legend. Statistical methods should be explained in full in the materials and methods. For figures presenting pooled data the statistical measure should be defined in the figure legends. Please also be sure to indicate the statistical tests used in each of your experiments (either in the figure legend itself or in a separate methods section) as well as the parameters of the test (for example, if you ran a t-test, please indicate if it was one- or two-sided, etc.). Also, if you used parametric tests, please indicate if the data distribution was tested for normality (and if so, how). If not, you must state something to the effect that "Data distribution was assumed to be normal but this was not formally tested."

5) Abstract and title: The abstract should be no longer than 160 words and should communicate the significance of the paper for a general audience. The title should be less than 100 characters including spaces. Make the title concise but accessible to a general readership.

6) Materials and methods: Should be comprehensive and not simply reference a previous publication for details on how an experiment was performed. Please provide full descriptions in the text for readers who may not have access to referenced manuscripts.
** Please remove the reference cited for the laser ablation setup and provide a description.

7) Please be sure to provide the sequences for all of your primers/oligos and RNAi constructs in the materials and methods. You must also indicate in the methods the source, species, and catalog numbers (where appropriate) for all of your antibodies. Please also indicate the acquisition and quantification methods for immunoblotting/western blots.

8) Microscope image acquisition: The following information must be provided about the acquisition and processing of images:
a. Make and model of microscope
b. Type, magnification, and numerical aperture of the objective lenses
c. Temperature
d. Imaging medium
e. Fluorochromes
f. Camera make and model

g. Acquisition software
h. Any software used for image processing subsequent to data acquisition. Please include details and types of operations involved (e.g., type of deconvolution, 3D reconstitutions, surface or volume rendering, gamma adjustments, etc.).

9) References: There is no limit to the number of references cited in a manuscript. References should be cited parenthetically in the text by author and year of publication. Abbreviate the names of journals according to PubMed.

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11) eTOC summary: A ~40-50-word summary that describes the context and significance of the findings for a general readership should be included on the title page. The statement should be written in the present tense and refer to the work in the third person.

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14) A separate author contribution section following the Acknowledgments. All authors should be mentioned and designated by their full names. We encourage use of the CRediT nomenclature.

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Thank you for this interesting contribution, we look forward to publishing your paper in Journal of Cell Biology.

Sincerely,

Ian Macara, Ph.D.
Editor
The Journal of Cell Biology

Tim Fessenden, Ph.D.
Scientific Editor
Journal of Cell Biology

Reviewer #1 (Comments to the Authors (Required)):

In this revised version, Ana Milas and colleagues have significantly improved their manuscript and modified the figures accordingly.
The authors have provided detailed information to the comments and questions submitted by the reviewers. In particular, they now show that maintenance of the posterior localisation of oskar mRNA is lost during ablation of posterior follicular cells.
The manuscript is much improved since the last submission and I recommend its publication in Journal of Cell Biology

Reviewer #2 (Comments to the Authors (Required)):

I was already convinced by the first version of the manuscript. The quality of the revised version is further enhanced especially by the addition of the experiment sowing disrupted oskar mRNA localisation after ablation of the posterior follicle cells, which was requested by another reviewer. My own questions were also addressed in a satisfying way.

1st Revision - Authors' Response to Reviewers: November 2, 2022

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Authors: We appreciate the positive evaluation.

Reviewer #2 (Comments to the Authors (Required)):

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Authors: We appreciate the positive evaluation.