Drosophila CTP synthase regulates collective cell migration by controlling the polarized endocytic cycle

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MS TITLE: Drosophila CTP synthase regulates collective cell migration by controlling the polarized endocytic cycle

AUTHORS: Pei-Yu Wang, Archan Chakraborty, Anna C.-C. Jang, Chau-Ting Yeh, Mei-Ling Cheng, Jau-Song Yu, and Li-Mei Pai

Dear Li-Mei,

I have now received all the referees' reports on the above manuscript, and have reached a decision. I am sorry to say that the outcome is not a positive one. The referees' comments are appended below, or you can access them online: please go to Development's submission site and click on the 'Manuscripts with Decisions' queue in the Author Area.

As you will see, the referees raise some significant concerns about your paper, and are not strongly in favour of publication. Two of them in particular raise concerns about the strength of the evidence. Having looked at the manuscript myself, I tend to agree with their views, and I must therefore, reject your paper.

I do realise this is disappointing news, but Development receives many more papers than we can publish, and we can only accept manuscripts that receive strong support from referees.

I do hope you find the comments of the referees helpful, and that this decision will not dissuade you from considering Development for publication of your future work. Many thanks for sending your manuscript to Development.

Yours sincerely

Thomas

Thomas Lecuit
Handling Editor
Development

Reviewer 1

Advance Summary and Potential Significance to Field

This manuscript by Wang et al. involves the CTP synthase in the collective migration of border cells in Drosophila. It proposes that CTP regulates the polarization of PIP2 and PI3P and that it regulates vesicular trafficking to ensure that RTK activity is polarized. While the observation is novel and would be interesting to the cell biology community, most of the data are non-conclusive as they are poorly controlled, as they rely on weak and difficult to interpret genetic interactions and as phenotypes are subtle and, again, difficult to interpret. As such, this manuscript seems way too preliminary in its current form. Below are some of the most obvious problems that were identified. It seems to me that the manuscript requires major improvements and extended phenotypical analysis that are beyond a regular revision cycle. As such, I strongly recommend the rejection of the manuscript at this stage.

Comments for the author

Major points:
1) No rescue experiments are provided. This study heavily relies on dsRNA lines and none are validated. As a consequence, confidence in the data provided is extremely weak.
2) Most of the controls are not adequate. The authors should compare the expression of dsRNA against a specific gene with a control dsRNA against a non-expressed protein (GFP, mCherry, LacZ, etc) and not the expression of LacZ as a control. As a general rule, the controls should be as close to the genotype tested as possible and they should be identical from one experiment to another.
3) All the genetic interactions rely on subtle increase of subtle phenotypes. Although they might be statistically significant, it does not mean that it is biologically significant. Indeed, by repeating an experiment enough times, it is possible to render any data statistically relevant. Some effects are so subtle that, although statistically relevant, I have a hard time to find them relevant. See for example Fig.5D. Furthermore, increase of phenotypes can be due to indirect effects that might not always be direct genetic interactions, in particular, as mentioned, when the phenotypes are subtle.
4) The loss of function phenotypes of CTP synthase should dramatically affect PIP2 synthesis. However, in the image provided, the sole phenotype observed with the PIP2-probe is a reduction (and not a loss as stated by the authors) of a front enrichment. Total PIP2 and overall distribution seems normal. This raises several questions: 1) is CTP really depleted, 2) is the probe really specific to PIP2 or does it also bind to other PIPs, 3) where would the probe localize in absence of PIP2. Therefore, I am even not convinced that the front accumulation is an accumulation of PIP2, which is a central point. Furthermore, although aPKC is normal, the reduction of the front accumulation might be

due to an effect on polarity or the polarization of trafficking in the BC cluster (see below) and might have nothing to do with local production of PIP2.
5) I am also not convinced that the loss-of-function of CTPsyn is complete. The mutant used d06966 expresses wt levels of the isoform A of CTPsyn (see Azzam and Liu, PLoS Gen., 2013). This needs to be clearly stated in the manuscript (and Azzam and Liu should be quoted). Furthermore, it is used in heterozygous conditions (so the other copy is intact) and the dsRNA is co-expressed. There is no data showing the expression of any transcript (qPCR at the entire animal level for example) or of the protein (no western blot, immunofluorescence) and there is no rescue experiment to demonstrate that the phenotype is due to the loss-of-function. This is key as the entire manuscript rely on this genetic background being a loss-of-function.
6) How does the authors explain that cluster with a single cell expressing CTPsyn would be sufficient for the migration. Are clusters with a single cell expressing RTKs sufficient for migration ? This increases my concern that the mutant is not a relevant loss-of-function for BC migration.
7) What about the other mutants described in Azzam and Liu ? Were they tested ? Why are the authors depleting with Slbo-Gal4 while c306-Gal4 is expressing earlier in BC and hence usually leads to stronger phenotypes.
8) The genetic interaction that links to PIP2 synthesis (Pis, PI4KIIIa, Sktl) are not entirely convincing. In particular, the fact that Sktl, which should be key for the production of PIP2 does not genetically interact is extremely puzzling. My understanding is that PIP2 might also be produced via other intermediate (PI5P), but enzymes of this pathway are not tested. This is also not debated in the discussion.
9) Fig.5 is puzzling as it mixes expression of dominant negatives, dsRNA and possibly (if the text and the figure legend is correct) the overexpression of Rab7. Why using Rab5DN and not its dsRNA. Or why using Rab11 dsRNA and not the DN? If Rab7 is indeed an overexpression of the GFP form, then this experience is meaningless as in general overexpression of Rab does not lead to increased activity. Anyways the authors cannot conclude that CTPsyn is not involved in trafficking to the late endocytic pathway, as a negative result means nothing here. Actually, even if the genetic interactions of this figure would be convincing (they do not convince me), this would not demonstrate any role of CTPsyn in endocytosis, it would simply suggest that it genetically interacts with constituent of the trafficking pathways. The fact that DE-Cad is normal also suggests that trafficking is not entirely affected.
10) In BC, Rab5 blocks Rac activation and Rab11 regulates Moesin activity, Rac activity and protrusion restriction, Cdc42 and Misshapen localization, among other things (Ramel et al., Colombié et al., Plutoni et al.). Ideally, the author should look at pMoe, RacFRET, Cdc42 and Msn distribution. At least, they should analyze protrusions numbers and distribution.
11) Along these lines, most of the phenotypes described would be consistent with an overall loss of polarization (that may or may not be due to trafficking defaults). Indeed, a loss of overall polarization would lead to a change of distribution of actin, of endocytic compartments and possibly of the PIP2 probe. It may also lead to genetic interaction with other pathways involved in BC migration (such as trafficking). The data that aPKC is normal is based on a single image. Like many other data presented in the manuscript (not listed here), this should be quantified precisely and ideally, other markers should be studied (Dlg, Par3 or Par6).
12) Why is the FYVE probe still on vesicles when CTPsyn is perturbed. As for the PIP2 probe, it should be soluble if PIPs synthesis is dramatically affected. It seems that the phenotype is a loss of endocytic polarization and not of PI3P polarization. Again, this question the pertinence of the loss of function background used here. (Also Rab5 is not a FYVE domain protein, l.227)

13) Several data are not relevant. For example, the DON inhibitor phenotype might be extremely indirect. The PI3K-CAAX experiment is meaningless as no phenotype does not mean no involvement (a validated loss-of-function would be more interesting).

Minor points:
1) There are multiple problems of labelling in the figures. See above for Fig.5. Fig.7: labels are misplaced on the lower part. Fig.3: for dsRNA, overexpression is not the right working.
2) The author should choose between PI4,5P2 and PIP2.
3) Several data need to be quantified, including the distribution of CTPsyn in figure 1.
4) In most of the figures, the morphology of control vs loss of function clusters is significantly different, with control clusters forming robust protrusions. The difference of morphology could explain some of the phenotypes, in particular the polarization of PIP2 probe.
5) The PIP2 probe also distributes at the border cell - nurse cell interphase. This should be described in the text.
6) Statistical analyses should be more precisely described (please, systematically indicate what is compared to what).
7) The model in Fig.7 is useless, as it does not explain the role of CTPsyn at all.

Reviewer 2

Advance Summary and Potential Significance to Field

In this manuscript, the authors identify a novel role of cytidine triphosphate synthase (CTPsyn) in border cell migration during Drosophila oogenesis. The authors use genetic perturbations along with fixed sample imaging to show that border cell migration is impaired in the CTPsyn mutant background. This defect is further pronounced when several proteins involved in the endocytic process are perturbed genetically in the mutant background. The authors then provide evidence through imaging techniques for the role of CTPsyn in establishing polarized distributions of endocytic components and phosphorylated RTKs.

It has been shown that there is a polarized endocytic cycle that regulates border cell migration (Assaker et al., 2010). What was most interesting in this paper was that it appears the mechanism to localize PI(4,5)P2 synthesis involves not just localized PI kinase(s), but localization of an enzyme that is further upstream in the biosynthesis pathway. This will be of broad interest to the field, because we do not commonly think of this as a regulatory point in the direction of PIP synthesis. I found the manuscript to be well written and the conclusions to be largely supported by the data.

Comments for the author

Comments:

1) The authors describe border cell movement, but do not define what border cells are and their role in oogenesis. It could be informative to add a short sentence to give some context for the purpose of this process, such as consequence(s) of defective border cell migration, for readers unfamiliar with the system.
2) The authors show that PIP2 localization and activated RTKs are less polarized in the CTPsyn mutant background, yet surprisingly, the actin network appears polarized in these border cells (Fig. 2 and 6), even though PIP2/RTKs are a known regulators of actin. It

would be insightful if the authors could discuss further whether leading edge actin levels and dynamics are affected in the CTPsyn mutant background.
3) The authors show that CTPsyn depletion affects the polarized distribution of PIP2. But, are PIP2 levels lower in CTPsyn mutant border cells? Can the authors assess whether the total levels of membrane-bound PHPLC are unchanged or use another method to assess PIP2 levels?

Minor comments:

1) If the formatting allows, it would be helpful for microscopy figures to be bigger. It's hard to read the text/labels on some figures, such as 4B and 6A.
2) It would be useful to note why the CTPsyn d06966/+ heterozygote is used. Is the homozygous mutant lethal?
3) Line 64-65: This sentence "During this process, the extracellular signals are ligands of RTK as guidance cues" is missing a word or two
4) Line 68: Should it be "migratory border cell cluster" instead of "migration border cell cluster?"
5) Line 111: There isn't a citation for how the asymmetric distribution of CTPsyn is needed for actin polymerization. Was this shown previously?

Reviewer 3

Advance Summary and Potential Significance to Field

Wang et al. report on the function of a biosynthetic enzyme, CTP synthase, that is required for phosphatidyl (PI) synthesis in the context of collective cell migration of border cells during fly oogenesis. They show that CTP synthase is required for the efficient migration of border cells, and that PIP2 asymmetries in the migrating border cells are decreased after CTPsynth function is compromised. They then go on to examine endosomal, and possibly recycling endosomal, function in permitting polarized RTK signaling required for border cell migration. The first part of the manuscript is largely done well, although the phenotypes are often on the weaker side, but addressing CTP function in 3 different ways is thorough. The second part of the manuscript becomes more scattershot, leaping between PIP2, PI3P, and PI4P as well as a variety of different compartments and rationales. There are also concerns about data quality, data omissions, and tests for statistical significance.

Comments for the author

1) This is a sloppy manuscript, and needs significant refinement. For example, in Line 111 the authors state, "Moreover, this asymmetrical distribution of CTPsyn is required for actin polymerization", but two paragraphs later they state that F-actin is unaffected after disrupting CTP synthase function, "Interestingly, the pattern of actin did not show significant alteration" (Line 140). F-actin is not well-shown or quantified, and should be shown in detail and quantified in a revision. Another example, remarkably both the abstract and the methods mention ER-enrichment with a Sec61 marker, and yet this isn't presented in the text or figures (I don't think I could've missed this, but apologies if so…). And another example, the main text says that a Rab5DN, a Rab7-GFP, and a Rab11 shRNA were used to address function - comparable functional analyses should be performed. The Rab field has moved away from using dominant negative approaches, so it should be explained why a Rab5 DN is used, while shRNAs are used for other Rabs. There is also little difference between the Rab7 and Rab11 results (compare Fig. 5C to 5D), so drawing strong conclusions from this data should be avoided. The authors are encouraged to

submit a more carefully reviewed manuscript in the future - this took considerable time to figure out these inconsistencies.

2) More detail is needed on the image quantitation - are the shown images identically imaged and leveled? In some panels, it appears that overall levels could be affected rather than asymmetries. These data need to be carefully stated and controlled for. In Fig. 6A it particularly looks like there is less overall Rab11.

3) One major concern is if the authors have identified the relevant PIP species that is affecting border cell migration - PIP3, PIP2, PI3P, and PI4P are all examined and/or implicated. For example, PIP2 localization and function is examined thoroughly, while PIP3 localization is not (but function is addressed). PI4P and PI3P are also both implicated, but not rigorously examined. It would be helpful if similar criteria was used in the evaluation of each of these species (although it is understood there are varying reagents available for both). for example, is a PIP3 sensor (available from Bloomington) also asymmetrically enriched? Are the changes in PIP distributions actually changes in distribution or overall levels (above point #2) - this should be carefully examined. Given the emphasis on PIP2 function, it is odd that Sktl does not genetically interact with CTPsyth. This also goes to the writing of the manuscript - there is a strong emphasis on plasma membrane PIP2 in the first part of the manuscript, but mechanistically what this accomplishes for border cell migration is not completely clear - it seems most likely (from the manuscript data) that the primary function of PIP2 enrichment is to establish a gradient of downstream PIPs (PI3P, PI4P).

4) The Methods section is very brief and should be more detailed. For example, when statistical analysis is used the method should be stated in each figure legend (is missing in some). Also, the method of statistical analysis should be justified. The student's t-test must be applied to normally distributed data (so normality should be stated in Methods), and is (to my knowledge) not appropriate for applying to categorical data such as the bins on cell migration (non-parametric analyses such as Mann-Whitney should be applied).

5) Lines 209-212 suggest the primary role of PIP2 in endocytosis is to recruit the PI phosphatases - this is incorrect, most people in the field would say PIP2 is deeply implicated in recruiting the correct coat and adaptor proteins for endocytosis, and then the PI phosphatases terminate this recruitment. This goes to a deeper issue in the presentation of the logic of the paper. There is endocytosis, which is the uptake of material from the cell surface, and then there is endosomal function (and potentially the recycling function the authors seem interested in). These are different, but linked processes, and this needs to be carefully delineated in the manuscript.

6) Fig. S3 - please quantify PIP2 distribution.

7) Is there evidence for CTP synthase function outside of the ER? Or do the authors believe the CTP synthase localization is in the ER but juxtaposed near the PM? Their own schematic (Fig. 3A) shows synthesis in the ER, though they notably do not include CTP synthase in the schematic. Again, the abstract mentions ER enrichment, but I do not see this data in either the main text or figures.

Minor notes:

There are a variety of data presentation styles - in some cases scatter plots are used, in other cases bar graphs of different types. It would be better if the authors decided on one style (in general, box and whisker or other more informational plots are the better choice).

It is understandable, but there are a number of typos and/or awkward grammar:
- Lines 51-53, not sure what the "despite" statement refers to
- Line 54-55, "investigated how molecules are deposited in a polarized manner", this sounds like ECM deposition, but really it's how asymmetric distributions of proteins are established.
- Line 91, "therefore" doesn't make sense here
- Line 126, "protein segments fused with GFP" doesn't make sense
- Line 181, don't capitalize eukaryotic

Author response to reviewers' comments

Point-by-point Response to the reviewers' comments

Reviewer 1 Advance Summary and Potential Significance to Field:

This manuscript by Wang et al. involves the CTP synthase in the collective migration of border cells in Drosophila. It proposes that CTP regulates the polarization of PIP2 and PI3P and that it regulates vesicular trafficking to ensure that RTK activity is polarized. While the observation is novel and would be interesting to the cell biology community, most of the data are non- conclusive as they are poorly controlled, as they rely on weak and difficult to interpret genetic interactions and as phenotypes are subtle and, again, difficult to interpret. As such, this manuscript seems way too preliminary in its current form. Below are some of the most obvious problems that were identified. It seems to me that the manuscript requires major improvements and extended phenotypical analysis that are beyond a regular revision cycle. As such, I strongly recommend the rejection of the manuscript at this stage.

Reviewer 1 Comments for the Author:
Major points:
1) No rescue experiments are provided. This study heavily relies on dsRNA lines and none are validated. As a consequence, confidence in the data provided is extremely weak.
Response: To clear demonstrate the role of CTPsyn, we agree that a clonal analysis of CTPsyn mutant in border cell migration is required for this study, therefore, we have now provided the migration delay results of border cell clusters with more than one mutant cell of CTPsyn^{d06966} (Fig. 2G-I). Indeed, only 4% of border cell cluster completed the migration while 96% showing delay phenotypes. Previously, the generation of mutant clone was driven by Slbo-GAL4 and only few egg chambers with mutant clones were analyzed. To increase the efficiency of mutant clones generation we have applied e22C-GAL4 to drive the flipase and 25 individual egg chambers containing more than one mutant cell in the border cell cluster were examined in details (Barth et al., 2012). We have now included the western blot of ovary and immunostaining of CTPsyn in follicle cells with expression of CTPsyn RNAi line in germline and somatic cells, respectively, to confirm the knockdown effects on protein levels (Fig. S1B-D). Taken together, these results clearly demonstrated the reduction of CTPsyn caused border cell migration delay.

2) Most of the controls are not adequate. The authors should compare the expression of dsRNA against a specific gene with a control dsRNA against a non-expressed protein (GFP, mCherry, LacZ, etc) and not the expression of LacZ as a control. As a general rule, the controls should be as close to the genotype tested as possible and they should be identical from one experiment to another.
Response: We have added the result of migration defects of control RNAi which is provided by VDRC stock center (#60000), and a mild defect was detected on this control RNAi in heterozygote CTPsyn^{d06966} mutant background (about 78% border cell cluster showed complete migration) while the CTPsyn RNAi showed a strong phenotype in mutant background (only around 38% with complete migration) (Fig. 2B). This control RNAi was used in experiments comparing knockdown effects of CTPsyn RNAi by different Gal4 lines (Fig. S1E). The different effects between control RNAi and CTPsyn RNAi in border cell migration were statistically significant.

3) All the genetic interactions rely on subtle increase of subtle phenotypes. Although they might be statistically significant, it does not mean that it is biologically significant. Indeed, by repeating an experiment enough times, it is possible to render any data statistically relevant. Some effects are so subtle that, although statistically relevant, I have a hard time to find them relevant. See for example Fig.5D. Furthermore, increase of phenotypes can be due to indirect effects that might not always be direct genetic interactions, in particular, as mentioned, when the phenotypes are subtle.

Response: We agree that genetic interaction results can only suggest potential interactions between different genes. The CTPsyn mutant allele d06966 and Rab11 RNAi did not show strong genetic interaction (Fig. S4), however, we observed that the asymmetric distribution of Rab11 signals was altered in border cell clusters expressing CTPsyn RNAi (Fig. 5D-E), and the differences were quantified and statistically significant (Fig. 5F). The CTPsyn RNAi effect was confirmed by the reduction of CTPsyn protein levels on western blot and of filament structure which is correlated to the levels of CTPsyn protein in Fig S1 A-D.

4) The loss of function phenotypes of CTP synthase should dramatically affect PIP2 synthesis. However, in the image provided, the sole phenotype observed with the PIP2-probe is a reduction (and not a loss as stated by the authors) of a front enrichment. Total PIP2 and overall distribution seems normal. This raises several questions: 1) is CTP really depleted, 2) is the probe really specific to PIP2 or does it also bind to other PIPs, 3) where would the probe localize in absence of PIP2. Therefore, I am even not convinced that the front accumulation is an accumulation of PIP2, which is a central point. Furthermore, although aPKC is normal, the reduction of the front accumulation might be due to an effect on polarity or the polarization of trafficking in the BC cluster (see below) and might have nothing to do with local production of PIP2.

Response: As to the three questions reviewer raised, our understanding is as following: **1)** CTPsyn RNAi line used in this study was confirmed by its effects on reduced protein levels detected by western blot (Fig. S1D). **2)** The probe used here PHPLCδ binds with a very high affinity to PI(4, 5)P2 and its derivative InsP3 (inositol 1,4,5-trisphosphate) (Varnai et al., 2002).
3) Up to date, there is no reported results about the probe localization in the absence of PIP2. Indeed, we also think the total levels of PIP2 did not show significant difference (now quantified in Fig.S3A), but the asymmetric distribution was reduced (Fig. 3). As to the why PIP2 is enriched at leading edge is not clearly understood currently, one possibility is that the local enrichment was generated by more active CTPsyn activity regionally, for example CTPsyn associated ER might locate proximity to the leading edge. Unfortunately, it is very difficult to clearly show a co-local enrichment of PIP2 and CTPsyn at the front of border cell cluster by our current facility. To examine overall cell polarity, we performed CTPsyn RNAi knockdown experiments to observe aPKC and Dlg expression in migration delayed border cell clusters. Indeed, no obvious reduction or mislocalization of aPKC and Dlg compared to wild-type border cell clusters. Furthermore, E-cadherin and armadillo localization were normal as well. Even though, we cannot exclude the possibility that polarized trafficking is affected by CTPsyn, which may lead to the loss of polarized phospho-tyrosine distribution, there is no study showing CTPsyn could regulate trafficking. On contrast, the role of CTPsyn in PI production was reported in previous publications. We now discuss both possibilities in the discussion, line 298.

5) I am also not convinced that the loss-of-function of CTPsyn is complete. The mutant used d06966 expresses wt levels of the isoform A of CTPsyn (see Azzam and Liu, PLoS Gen., 2013). This needs to be clearly stated in the manuscript (and Azzam and Liu should be quoted). Furthermore, it is used in heterozygous conditions (so the other copy is intact) and the dsRNA is co-expressed. There is no data showing the expression of any transcript (qPCR at the entire animal level for example) or of the protein (no western blot, immunofluorescence) and there is no rescue experiment to demonstrate that the phenotype is due to the loss-of-function. This is key as the entire manuscript rely on this genetic background being a loss-of-function.

Response: We thank Reviewer for the reminding and we now have explained the mutant allele information in the text on page 5, line 128. To show the RNAi effect, now we provide the western blot result to show the reduction of protein levels. To clearly demonstrate depletion of CTPsyn would cause border cell migration defects, we performed the CTPsyn^{d06966} mutant clonal analysis and showed a strong delay phenotype (Fig. 2, and S2).

6) How does the authors explain that cluster with a single cell expressing CTPsyn would be sufficient for the migration. Are clusters with a single cell expressing RTKs sufficient for migration? This increases my concern that the mutant is not a relevant loss-of-function for BC migration.
Response: Previously, we used Slbo-GAL4 to make mutant clone and only few egg chambers with mutant clones were analyzed. We now have done an extensive analysis on clonal mutation using a stronger Gal4 line, e22C-GAL4 to generate mutant clones and 25 individual egg chambers containing more than one mutant cell in the border cell cluster were examined in details. Indeed, only 4% of border cell cluster completed the migration while 96% showing delay phenotypes (Fig. 2).

7) What about the other mutants described in Azzam and Liu? Were they tested? Why are the authors depleting with Slbo-Gal4 while c306-Gal4 is expressing earlier in BC and hence usually leads to stronger phenotypes?
Response: Since the CTPsyn^{d06966} mutant clone showed clear phenotypes in border cell migration, we have not tested the other mutant alleles. We thank Reviewer's suggestion and have now added the C306-GAL4 /CTPsyn RNAi results in Fig. S1, and indeed C306 generated a stronger phenotype than Slbo-GAL4.

8) The genetic interaction that links to PIP2 synthesis (Pis, PI4KIIIa, Sktl) are not entirely convincing. In particular, the fact that Sktl, which should be key for the production of PIP2 does not genetically interact is extremely puzzling. My understanding is that PIP2 might also be produced via other intermediate (PI5P), but enzymes of this pathway are not tested. This is also not debated in the discussion.
Response: It is true that PI(4,5)P2 can be converted from PI5P, but the enzyme mediating this reaction is not found in *Drosophila* yet (Fig. 4A). Therefore, we do not have the opportunity to examine its effects. Now we have included this in the discussion as Reviewer suggested. Sktl is the closest one to PI(4,5)P2, and knockdown of itself actually has a stronger effect in border cell migration than other biosynthetic enzymes of this pathway . As to the lack of genetic interaction between CTPsyn and Sktl, one possible reason is that CTPsyn functioning in the generation of PIP2 is 5 steps upstream and the reduction of CTPsyn might be compensated by enzymes functioning between these steps.

9) Fig.5 is puzzling as it mixes expression of dominant negatives, dsRNA and possibly (if the text and the figure legend is correct) the overexpression of Rab7. Why using Rab5DN and not its dsRNA. Or why using Rab11 dsRNA and not the DN? If Rab7 is indeed an overexpression of the GFP form, then this experience is meaningless as in general overexpression of Rab does not lead to increased activity. Anyways the authors cannot conclude that CTPsyn is not involved in trafficking to the late endocytic pathway, as a negative result means nothing here. Actually, even if the genetic interactions of this figure would be convincing (they do not convince me), this would not demonstrate any role of CTPsyn in endocytosis, it would simply suggest that it genetically interacts with constituent of the trafficking pathways. The fact that DE-Cad is normal also suggests that trafficking is not entirely affected.
Response: We apologize that there were typo mistakes in previous version, and DN-Rab7 was used but not Rab7GFP. To avoid the complications of different genotypes, we have now only presented the RNAi knockdown for genetic interactions. We agree that genetic interactions could only be supportive results for their roles in molecular mechanism, and our genetic interaction results only suggest that CTPsyn activity is involved in trafficking pathways. Therefore, we removed the no genetic interaction result with DNRab7. We also agree that the effect of CTPsyn is on particular trafficking activities, since the location and expression of many cell polarity proteins, such E-cadherin, aPKC, and armadillo were not affected (Fig. 6 and S5).

10) In BC, Rab5 blocks Rac activation and Rab11 regulates Moesin activity, Rac activity and protrusion restriction, Cdc42 and Misshapen localization, among other things (Ramel et al., Colombié et al., Plutoni et al.). Ideally, the author should look at pMoe, RacFRET, Cdc42 and Msn distribution. At least, they should analyze protrusions numbers and distribution.
Response: We have now looked closely on actin for protrusion and found a clear defect on actin enrichment at leading edge (Fig. 3J and K). We agree that Rab5 and Rab11 downstream effectors are critical for border cell migration, but here we focused on the role of CTPsyn on border cell

migration through mutant clone analysis and initially identified the PIP2 alteration and the defects of asymmetric trafficking pathways. In the future, these are very interesting questions to be further analyzed.

11) Along these lines, most of the phenotypes described would be consistent with an overall loss of polarization (that may or may not be due to trafficking defaults). Indeed, a loss of overall polarization would lead to a change of distribution of actin, of endocytic compartments and possibly of the PIP2 probe. It may also lead to genetic interaction with other pathways involved in BC migration (such as trafficking). The data that aPKC is normal is based on a single image. Like many other data presented in the manuscript (not listed here), this should be quantified precisely and ideally, other markers should be studied (Dlg, Par3 or Par6).
Response: We agree that the overall cell polarity should be examined, therefore, we performed CTPsyn RNAi knockdown experiments to observe aPKC and Dlg expression with immunofluorescence staining in migration delayed border cell clusters. The results showed no obvious reduction or mis-localization of aPKC and Dlg compared to wild-type border cell clusters. Figure S5 showed different apical and side views of aPKC and Dlg distribution, and aPKC was found to localize apically and Dlg was concentrated at basolateral region. Moreover, the distribution of E-cad, and Armadillo were not affected in CTPsyn mutant clonal analysis or RNAi depletion (Fig.2, 6, and S2), we think it is unlikely the cell polarity was affected by the reduction of CTPsyn activity.

12) Why is the FYVE probe still on vesicles when CTPsyn is perturbed. As for the PIP2 probe, it should be soluble if PIPs synthesis is dramatically affected. It seems that the phenotype is a loss of endocytic polarization and not of PI3P polarization. Again, this question the pertinence of the loss of function background used here. (Also, Rab5 is not a FYVE domain protein, l.227)
Response: The PHPLCδ-GFP signals of wild-type border cell in Fig. 3 A-E showed the most concentrated signal at the leading edge of the cluster (Fig. 3 A-E marked with asterisks). This enrichment was not maintained in CTPsyn reduced border cell cluster (Fig. 3F', H and H'), and the differences of enrichment at leading edge to trailing edge were quantified in Fig. 3G in both genotypes (Fig. 3H). However, the overall levels of GFP signal was not dramatically altered. We proposed that high production of PIP2 at front leading edge which anchors the PHPLCδ-GFP depends on CTPsyn activity. The reduction of PIP2 polarity may only lead to reduction of asymmetrical trafficking, and not enough to cause drastic reduction of overall endocytic activity, therefor, the GFP signals of FYVE probe for early endosomes were not dramatically reduced either. Even though, the asymmetrical distribution of FYVE-GFP was altered which is quantified in Fig. 5C.

13) Several data are not relevant. For example, the DON inhibitor phenotype might be extremely indirect. The PI3K-CAAX experiment is meaningless as no phenotype does not mean no involvement (a validated loss-of-function would be more interesting).
Response: We thank Reviewer's suggestion to make the manuscript easier for readers to follow and we have removed these results as suggested above.

Minor points:
1) There are multiple problems of labelling in the figures. See above for Fig.5. Fig.7: labels are misplaced on the lower part. Fig.3: for dsRNA, overexpression is not the right working.
Response: We have remade all figures and changed labels to give correct information for readers to follow our thinking.

2) The author should choose between PI4,5P2 and PIP2.
Response: We have now made it consistent throughout the manuscript as PI4,5P2 or PIP2.

3) Several data need to be quantified, including the distribution of CTPsyn in figure 1.
Response: We have now quantified the enrichment of CTPsyn and F-actin at the cortex of border cell cluster in Fig. 1C.

4) In most of the figures, the morphology of control vs loss of function clusters is significantly different, with control clusters forming robust protrusions. The difference of morphology could explain some of the phenotypes, in particular the polarization of PIP2 probe.

Response: We agree it is better to compare border cell clusters with similar morphology and we have added more images of wt and reduced CTPsyn border cell cluster for the PI4,5P2 probe.
5) The PIP2 probe also distributes at the border cell – nurse cell interphase. This should be described in the text.
Response: We have added this description in result section on page 7, line 156.

6) Statistical analyses should be more precisely described (please, systematically indicate what is compared to what).
Response: We have clearly described the statistical analyses in details in the method section and figure legends.

7) The model in Fig.7 is useless, as it does not explain the role of CTPsyn at all.
Response: We have modified the model to show the polarized vesicle trafficking downstream of RTK and enrichment of CTPsyn and PIP2 in Fig. 6. Furthermore, reduction of CTPsyn led to reduction of PI4,5 P2 enrichment in the raft domain which may result in reduced endocytic trafficking that diminished the polarized trafficking events.

Reviewer 2 Advance Summary and Potential Significance to Field:

In this manuscript, the authors identify a novel role of cytidine triphosphate synthase (CTPsyn) in border cell migration during Drosophila oogenesis. The authors use genetic perturbations along with fixed sample imaging to show that border cell migration is impaired in the CTPsyn mutant background. This defect is further pronounced when several proteins involved in the endocytic process are perturbed genetically in the mutant background. The authors then provide evidence through imaging techniques for the role of CTPsyn in establishing polarized distributions of endocytic components and phosphorylated RTKs.

It has been shown that there is a polarized endocytic cycle that regulates border cell migration (Assaker et al., 2010). What was most interesting in this paper was that it appears the mechanism to localize PI(4,5)P2 synthesis involves not just localized PI kinase(s), but localization of an enzyme that is further upstream in the biosynthesis pathway. This will be of broad interest to the field, because we do not commonly think of this as a regulatory point in the direction of PIP synthesis. I found the manuscript to be well written and the conclusions to be largely supported by the data.

Reviewer 2 Comments for the Author:
Comments:

1) The authors describe border cell movement, but do not define what border cells are and their role in oogenesis. It could be informative to add a short sentence to give some context for the purpose of this process, such as consequence(s) of defective border cell migration, for readers unfamiliar with the system.
Response: We thank reviewer for the support of our work and we have now mentioned the function of border cells in introduction on page 3, line 65 as following “the border cells migrate to meet the dorsal-anterior follicle cells in the stage 10 egg chamber to participate in micropyle formation which is the path for sperm entry in fertilization”.

2) The authors show that PIP2 localization and activated RTKs are less polarized in the CTPsyn mutant background, yet surprisingly, the actin network appears polarized in these border cells (Fig. 2 and 6), even though PIP2/RTKs are a known regulator of actin. It would be insightful if the authors could discuss further whether leading edge actin levels and dynamics are affected in the CTPsyn mutant background.
Response: It’s been previously demonstrated that PIP2 regulates actin polymerization. Since CTPsyn depletion driven by C306 gal4 expressing strongly in border cells caused severe migration delay phonotype, we took this advantage to closely examined the F-actin at leading edge and found that the depletion of CTPsyn resulted in the reduction of actin enrichment at the leading edge in Fig. 3. Quantification results showed significant reduction of F-actin enrichment at leading edge (Fig. 3L).

3) The authors show that CTPsyn depletion affects the polarized distribution of PIP2. But, are PIP2 levels lower in CTPsyn mutant border cells? Can the authors assess whether the total levels of membrane-bound PHPLC are unchanged or use another method to assess PIP2 levels?
Response: From the images, we realized that the total levels of PIP2 were not dramatically affected in CTPsyn reduction border cells (Fig. S3A). We now quantified the levels of PIP2 as shown in Fig. S3A.

Minor comments:

1) If the formatting allows, it would be helpful for microscopy figures to be bigger. It's hard to read the text/labels on some figures, such as 4B and 6A.
Response: We have remade all figures to make images clearer, and corrected labels to match the text and figure legends.

2) It would be useful to note why the CTPsyn d06966/+ heterozygote is used. Is the homozygous mutant lethal?
Response: Yes, indeed homozygote mutants of CTPsyn d06966 die within 7 days of egg deposition, therefore, we now provide new data from mosaic mutant border cell clones (Fig. 2G-I).

3) Line 64-65: This sentence "During this process, the extracellular signals are ligands of RTK as guidance cues" is missing a word or two
Response: We have now changed the sentence as “of receptor tyrosine kinase (RTK) which act as guidance cues”.

4) Line 68: Should it be "migratory border cell cluster" instead of "migration border cell cluster?"
Response: Yes, we have now corrected this mistake.

5) Line 111: There isn’t a citation for how the asymmetric distribution of CTPsyn is needed for actin polymerization. Was this shown previously?
Response: This is the first study examining the effect of CTPsyn on actin polymerization, so we have carefully quantified the different F-actin enrichment at the front/back axis of border cell cluster in Fig. 3L.

- Adam Martin

Reviewer 3 Advance Summary and Potential Significance to Field:

Wang et al. report on the function of a biosynthetic enzyme, CTP synthase, that is required for phosphatidyl (PI) synthesis in the context of collective cell migration of border cells during fly oogenesis. They show that CTP synthase is required for the efficient migration of border cells, and that PIP2 asymmetries in the migrating border cells are decreased after CTPsynth function is compromised. They then go on to examine endosomal, and possibly recycling endosomal, function in permitting polarized RTK signaling required for border cell migration. The first part of the manuscript is largely done well, although the phenotypes are often on the weaker side, but addressing CTP function in 3 different ways is thorough. The second part of the manuscript becomes more scattershot, leaping between PIP2, PI3P, and PI4P as well as a variety of different compartments and rationales. There are also concerns about data quality, data omissions, and tests for statistical significance.

Reviewer 3 Comments for the Author:

1) This is a sloppy manuscript, and needs significant refinement. For example, in Line 111 the authors state, “Moreover, this asymmetrical distribution of CTPsyn is required for actin polymerization”, but two paragraphs later they state that F-actin is unaffected after disrupting CTP synthase function, “Interestingly, the pattern of actin did not show significant alteration” (Line 140). F-actin is not well-shown or quantified, and should be shown in detail and quantified in a revision.
Another example, remarkably both the abstract and the methods mention ER-enrichment with a

Sec61 marker, and yet this isn't presented in the text or figures (I don't think I could've missed this, but apologies if so…).
And another example, the main text says that a Rab5DN, a Rab7-GFP, and a Rab11 shRNA were used to address function – comparable functional analyses should be performed. The Rab field has moved away from using dominant negative approaches, so it should be explained why a Rab5 DN is used, while shRNAs are used for other Rabs. There is also little difference between the Rab7 and Rab11 results (compare Fig. 5C to 5D), so drawing strong conclusions from this data should be avoided. The authors are encouraged to submit a more carefully reviewed manuscript in the future – this took considerable time to figure out these inconsistencies.

Response: We apologize for some unclear descriptions in our previous version of the manuscript. We now have clear answers for these three questions. **1)** We have now closely examined the actin at leading edge at early stage 9 by phalloidin staining and found that the depletion of CTPsyn resulted in the reduction of actin enrichment at the leading edge in Fig.3. About more than 50% of egg chambers showed this defect in quantification results. **2)** To answer why the PIP2 is enriched at leading edge, we came up with one possibility is that the local enrichment was generated by more active CTPsyn activity regionally, for example CTPS associated ER might locate proximity to the leading edge. We have tried to examine the ER marker, sec61. Unfortunately, it is very difficult to clearly show a co-local enrichment of sec61 and CTPsyn at the front of border cell cluster by our current facility. Therefore, we only can discuss this possibility in discussion and have removed the statement from abstract. **3)** We agree that we should have only used the Rab RNAi for genetic interactions, and now we have only included the Rab7 and Rab 11 RNAi results.

2) More detail is needed on the image quantitation – are the shown images identically imaged and leveled? In some panels, it appears that overall levels could be affected rather than asymmetries. These data need to be carefully stated and controlled for. In Fig. 6A it particularly looks like there is less overall Rab11.
Response: We have now added quantification results of PIP2, FYVE-GFP, and Rab11-GFP results in Fig. 3 and 5. For the asymmetrical distribution we measured the GFP intensity at the leading edge to that at the tail. We have now also quantified the total level of PIP2 and Rab11 in Fig. S3. The total PIP2 levels were not significantly affected, however the level of total Rab- 11 was elevated a bit with CTPsyn reduction.

3) One major concern is if the authors have identified the relevant PIP species that is affecting border cell migration – PIP3, PIP2, PI3P, and PI4P are all examined and/or implicated. For example, PIP2 localization and function is examined thoroughly, while PIP3 localization is not (but function is addressed). PI4P and PI3P are also both implicated, but not rigorously examined. It would be helpful if similar criteria was used in the evaluation of each of these species (although it is understood there are varying reagents available for both).
Response: The function of PIs in membrane dynamics has been intensively studied, and the role of PIP2 in migration has been demonstrated in mammalian cells. PIP2 regulates migration through control of actin polymerization and symmetrical trafficking. Here, we are the first to report PIP2's role in collective cell migration and found that the enrichment of PIP2 in the leading edge is regulated by CTPsyn activity. PI3P on the endosome recognized by FYVE probe, is the downstream metabolic products of PIP2 which promotes endocytosis, and the asymmetrical distribution of early endosome in border cell cluster. Our work here cannot determine the role of PI4P in border cell migration as we only performed genetic interaction experiments to address whether PI biosynthesis enzyme could involve in the collective migration. To determine the role of PIP4, one will need to look at the mutant of PI4KIIIalpha and apply other reagents as well. To provide solid results for the role of CTPsyn in collective cell migration, here we only focus on the effect of CTPsyn depletion.

For example, is a PIP3 sensor (available from Bloomington) also asymmetrically enriched? Are the changes in PIP distributions actually changes in distribution or overall levels (above point #2) – this should be carefully examined. Given the emphasis on PIP2 function, it is odd that Sktl does not genetically interact with CTPsyn. This also goes to the writing of the manuscript – there is a strong emphasis on plasma membrane PIP2 in the first part of the manuscript, but mechanistically what this accomplishes for border cell migration is not completely clear – it seems most likely

(from the manuscript data) that the primary function of PIP2 enrichment is to establish a gradient of downstream PIPs (PI3P, PI4P).

Response: The genetic interaction results indicate that PIP3 is not involved, therefore, we did not examine the distribution of PIP3. One possible explanation for no genetic interaction between CTPsyn and Sktl is that CTPsyn functioning in the generation of PIP2 is 5 steps upstream and the reduction of CTPsyn might be compensated by enzymes functioning between these steps. Even though, Sktl is the closest one to PI(4,5)P2, and knockdown of itself actually has a stronger effect in border cell migration than other biosynthetic enzymes of this pathway. In this revised version, we discussed one hypothesis for role of compartmentalized CTPsyn is to build the raft domain where the RTK mediated polarized trafficking activities maintained the feedback loop of polarized RTK. Alternatively, PIP2 gradient may influence the gradient of downstream PIPs that control the endocytic pathways which is discussed in the discussion.

4) The Methods section is very brief and should be more detailed. For example, when statistical analysis is used the method should be stated in each figure legend (is missing in some). Also, the method of statistical analysis should be justified. The student's t-test must be applied to normally distributed data (so normality should be stated in Methods), and is (to my knowledge) not appropriate for applying to categorical data such as the bins on cell migration (non-parametric analyses such as Mann-Whitney should be applied).
Response: We have now described the statistical analyses clearly in the methods and all used student's test to obtain the p values. For the cell migration results, we used student's t-test (2 tail) for complete migration egg chamber only, and we did not compare other migration categories.

5) Lines 209-212 suggest the primary role of PIP2 in endocytosis is to recruit the PI phosphatases – this is incorrect, most people in the field would say PIP2 is deeply implicated in recruiting the correct coat and adaptor proteins for endocytosis, and then the PI phosphatases terminate this recruitment. This goes to a deeper issue in the presentation of the logic of the paper. There is endocytosis, which is the uptake of material from the cell surface, and then there is endosomal function (and potentially the recycling function the authors seem interested in). These are different, but linked processes, and this needs to be carefully delineated in the manuscript.
Response: We agree that the role of PIP2 in endocytosis should be addressed clearly because the asymmetrical distribution of PIP2 plays a central role in this study. According to reviewer's suggestion we have now modified the text on page 8, line 181 as follows: "PIP2 is involved in recruiting the correct coat and adaptor proteins during endocytosis, and PI phosphatases like Synaptojanin and src-homology 2 containing 5- phosphatase (SHIP2) terminate this recruitment and subsequently PIP2 is hydrolyzed to PI3P.".

6) Fig. S3 – please quantify PIP2 distribution.
Response: At this moment, we don't have enough data to confirm the effect of Pis knockdown on PIP2 distribution, and now we have removed this result.

7) Is there evidence for CTP synthase function outside of the ER? Or do the authors believe the CTP synthase localization is in the ER but juxtaposed near the PM? Their own schematic (Fig. 3A) shows synthesis in the ER, though they notably do not include CTP synthase in the schematic. Again, the abstract mentions ER enrichment, but I do not see this data in either the main text or figures.
Response: We suspect that CTPsyn localized in the ER somehow became proximity to the leading edge during the border cell migration, but the images to proof this notion were very difficult to be obtained with our current facility. Without clear evidence, we can only discuss this possibility in the discussion and have to remove the image of sec61 results.

Minor notes:

There are a variety of data presentation styles – in some cases scatter plots are used, in other cases bar graphs of different types. It would be better if the authors decided on one style (in general, box and whisker or other more informational plots are the better choice).
Response: We have remade all figures and labels to improve the quality of the figures.

It is understandable, but there are a number of typos and/or awkward grammar:
-Lines 51-53, not sure what the “despite” statement refers to
-Line 54-55, “investigated how molecules are deposited in a polarized manner”, this sounds like ECM deposition, but really it’s how asymmetric distributions of proteins are established.
-Line 91, “therefore” doesn’t make sense here
-Line 126, “protein segments fused with GFP” doesn’t make sense
-Line 181, don’t capitalize eukaryotic
Response: We have corrected the mistakes on page 3 (line 52), page 3 (line 55), page 4 (line 91) and page 5 (line 122).

Barth, J.M.I., E. Hafen, and K. Köhler. 2012. The lack of autophagy triggers precocious activation of Notch signaling during Drosophila oogenesis. *BMC Developmental Biology*. 12:35.
Varnai, P., X. Lin, S.B. Lee, G. Tuymetova, T. Bondeva, A. Spat, S.G. Rhee, G. Hajnoczky, and T. Balla. 2002. Inositol lipid binding and membrane localization of isolated pleckstrin homology (PH) domains. Studies on the PH domains of phospholipase C delta 1 and p130. *The Journal of biological chemistry*. 277:27412-27422.

Resubmission

First decision letter

MS ID#: DEVELOP/2021/200190

MS TITLE: Compartmentalization of Drosophila CTP synthase regulates collective cell migration by controlling the polarized endocytic cycle

AUTHORS: Pei-Yu Wang, Archan Chakraborty, Hsin-Ju Ma, Wei-Cheng Lin, Anna C.-C. Jang, Hai-Wei Pi, Chau-Ting Yeh, Mei-Ling Cheng, Jau-Song Yu, and Li-Mei Pai

I am really sorry fpr the long delay but it has been really hard to find available reviewers. In fact, I obtained a single report and have reached a decision. The referees' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

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

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

Reviewer 1

Advance summary and potential significance to field

The manuscript by Wang et al., describes the role of the Drosophila CTP synthase (CTPsyn) during border cell migration. The border cells are a group of 8 to 10 cells that are part of the anterior end of the follicular epithelium, and that collectively migrate toward the oocyte at stage 9 of oogenesis. Migration depends on known guidance cues that activate receptor tyrosine kinases, such as EGFR and PVR. The activity of these receptors is mainly detected at the leading edge of the

migratory border cell cluster. Maintenance of this activity in leading cells is dependent on endocytic recycling cycles. Deciphering the different mechanisms that strengthen RTK activities in the leading cells is therefore important to understand cell migration in animals. Phosphatidylinositol (PI) 4,5-bisphosphate (PIP2) is a membrane molecule that has been shown to be involved in endocytosis. To test whether PIP2 could participate in maintaining high levels of TRK activity in leading cells, the authors analysed the role of the CTP synthase, a key enzyme to produce PIP2. The authors monitored CTPsyn and PIP2 expression (with the use of UAS-PH-PLCd-GFP reporter) during border cell migration and observe an enhanced expression at the leading edge of the border cell cluster. They also prove that CTPsyn is required for border cell migration by quantifying migration in either follicles with CTPsyn mutant border cells (clonal analysis) or follicles expressing RNAi against CTPsyn. Depletion of CTPsyn also leads to a reduction of PIP2 expression and of its hydrolysed form PI3P in the leading cells. In parallel, the spatial expression of pTyr signal is disrupted. From these correlations, the authors propose that CTPsyn is important for restricting RTK activities at the leading edge of the border cells by acting on the PIP2-dependant endocytic cycles.

Comments for the author

Based on the results, I am convinced that CTPsyn is required for border cell migration and that its depletion leads to low expression of pTyr in the leading cells in parallel to PIP2 expression. However, it remains uncertain whether the observed decrease of RTK activities is specific or is due to abnormal general sorting of membrane proteins. Indeed, the quality of figure 6 is not sufficient to be convinced about the absence of phenotype in Ecad, Arm, aPKC and Dlg expression. Furthermore, a more conclusive experiment would be to analyse the difference in migration, in pTyr, Eacd, aPKC and Dlg expression in border cell clusters composed of WT and mutant cells. Based on the authors' hypothesis, one would expect that when the leading cells are WT, no phenotype should be observed. In parallel, live experiment would be useful to determine whether mutant cells are never "chosen" to be leading cells.

Minor comments :
- line 64 : typo "cell" should be "cells "
- line 82 : should be Fig. 4A and not 3A
- line 141 : "when dicer was used" is unclear
- line 204 : "didn't resulted" should be "did not result "
- line 665 : "group" should be "groups "
- line 821 : "nanos-Gal4" should be italicized

First revision

Author response to reviewers' comments

Reviewer 1 Advance Summary and Potential Significance to Field:

The manuscript by Wang et al., describes the role of the Drosophila CTP synthase (CTPsyn) during border cell migration. The border cells are a group of 8 to 10 cells that are part of the anterior end of the follicular epithelium, and that collectively migrate toward the oocyte at stage 9 of oogenesis. Migration depends on known guidance cues that activate receptor tyrosine kinases, such as EGFR and PVR. The activity of these receptors is mainly detected at the leading edge of the migratory border cell cluster. Maintenance of this activity in leading cells is dependent on endocytic recycling cycles. Deciphering the different mechanisms that strengthen RTK activities in the leading cells is therefore important to understand cell migration in animals. Phosphatidylinositol (PI) 4,5- bisphosphate (PIP2) is a membrane molecule that has been shown to be involved in endocytosis. To test whether PIP2 could participate in maintaining high levels of TRK activity in leading cells, the authors analysed the role of the CTP synthase, a key enzyme to produce PIP2. The authors monitored CTPsyn and PIP2 expression (with the use of UAS-PH-PLCd-GFP reporter) during border cell migration and observe an enhanced expression at the leading edge of the border cell cluster. They also prove that CTPsyn is required for border cell migration by quantifying migration in either follicles with CTPsyn mutant border cells (clonal analysis) or follicles expressing RNAi against CTPsyn. Depletion of CTPsyn also leads to a reduction of PIP2 expression

and of its hydrolysed form PI3P in the leading cells. In parallel, the spatial expression of pTyr signal is disrupted. From these correlations, the authors propose that CTPsyn is important for restricting RTK activities at the leading edge of the border cells by acting on the PIP2-dependant endocytic cycles.

Reviewer 1 Comments for the Author:
Based on the results, I am convinced that CTPsyn is required for border cell migration and that its depletion leads to low expression of pTyr in the leading cells in parallel to PIP2 expression. However, it remains uncertain whether the observed decrease of RTK activities is specific or is due to abnormal general sorting of membrane proteins. Indeed, the quality of figure 6 is not sufficient to be convinced about the absence of phenotype in Ecad, Arm, aPKC and Dlg expression. Furthermore, a more conclusive experiment would be to analyse the difference in migration, in pTyr, Eacd, aPKC and Dlg expression in border cell clusters composed of WT and mutant cells. Based on the authors' hypothesis, one would expect that when the leading cells are WT, no phenotype should be observed. In parallel, live experiment would be useful to determine whether mutant cells are never "chosen" to be leading cells.
Response: We thank the Reviewer for the support of our work. To determine whether cell polarity is generally disrupted in CTPsyn mutant border cells, we performed immunostaining of Ecad, aPKC, and Dlg in border cell clusters containing mutant clones of CTPsyn in supplementary Figure 5. The results showed no disruption in the location of these cell polarity proteins. For the live image of migration of border cell cluster containing CTPsyn mutant clones, we found that rotation of the border cells during migration was disrupted in mutant clones, and migration was reduced (please see movie 1 of control and 2 with mutant clones). These results suggested that the CTPsyn activity might be crucial for the rotation of border cells, however, the molecular mechanisms need further investigation. We also examined the anterior/ posterior position of mutant border cells by marking wild-type cells with GFP (Fig. S2D), and no preferences were detected for the position of mutant cells.

We have corrected all the typo mistakes mentioned below and thank the Reviewer for the careful reading.

Minor comments:
-line 64 : typo "cell" should be "cells "
Response: Edited as "Once they reach the junction, the border cells migrate to meet the dorsal-anterior follicle cells in the stage 10 egg chamber to participate in micropyle formation which is the path for sperm entry in fertilization."

-line 82 : should be Fig. 4A and not 3A
Response: Edited as "Cytidine triphosphate synthase (CTPsyn) is involved in PIP biosynthesis to produce CTP for forming CDP-diacylglycerol (CDP-DAG) with phosphatidic acid (PA) in *de novo* synthesis (Chang and Carman, 2008) (Fig. 4A)."

-line 141 : "when dicer was used" is unclear
Response: Edited as "Furthermore, when the efficacy of CTPsyn gene silencing was increased using Dicer, complete border cell cluster migration reduced to 32% (Fig. S1E, J and K)."

-line 204 : "didn't resulted" should be "did not result "
Response: Edited as "Although, under *CTPsyn*$^{d06966/+}$ mutant background, depletion of either Rab11 or Rab7 did not result in a significant migration delay (Fig. S4A),"

-line 665 : "group" should be "groups "
Response: Edited as "The three isoforms of Phosphoinositides bearing two phosphate groups are mentioned in green."

-line 821 : "nanos-Gal4" should be italicized
Response: Edited as "Western blot image shows reduction of CTPsyn protein in *Drosophila* ovary when CTPsyn RNAi was driven by *nanos-GAL4*."

Second decision letter

MS ID#: DEVELOP/2021/200190

MS TITLE: Compartmentalization of Drosophila CTP synthase regulates collective cell migration by controlling the polarized endocytic cycle

AUTHORS: Pei-Yu Wang, Archan Chakraborty, Hsin-Ju Ma, Jhen-Wei Wu, Anna C.-C. Jang, Wei-Cheng Lin, Hai-Wei Pi, Chau-Ting Yeh, Mei-Ling Cheng, Jau-Song Yu, and Li-Mei Pai

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

The overall evaluation is positive and we would like to publish a revised manuscript in Development, provided that the referees' comments can be satisfactorily addressed. Please address in particular points 1, 4 and 5 of referee 2. 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.

Reviewer 1

Advance summary and potential significance to field

The manuscript by Wang et al., describes the role of the Drosophila CTP synthase (CTPsyn) during border cell migration. The border cells are a group of 8 to 10 cells that are part of the anterior end of the follicular epithelium, and that collectively migrate toward the oocyte at stage 9 of oogenesis. Migration depends on known guidance cues that activate receptor tyrosine kinases, such as EGFR and PVR. The activity of these receptors is mainly detected at the leading edge of the migratory border cell cluster. Maintenance of this activity in leading cells is dependent on endocytic recycling cycles. Deciphering the different mechanisms that strengthen RTK activities in the leading cells is therefore important to understand cell migration in animals.
Phosphatidylinositol (PI) 4,5- bisphosphate (PIP2) is a membrane molecule that has been shown to be involved in endocytosis. To test whether PIP2 could participate in maintaining high levels of TRK activity in leading cells, the authors analysed the role of the CTP synthase, a key enzyme to produce PIP2. The authors monitored CTPsyn and PIP2 expression (with the use of UAS-PH-PLCd-GFP reporter) during border cell migration and observe an enhanced expression at the leading edge of the border cell cluster. They also prove that CTPsyn is required for border cell migration by quantifying migration in either follicles with CTPsyn mutant border cells (clonal analysis) or follicles expressing RNAi against CTPsyn. Depletion of CTPsyn also leads to a reduction of PIP2 expression and of its hydrolysed form PI3P in the leading cells. In parallel, the spatial expression of pTyr signal is disrupted. From these correlations, the authors propose that CTPsyn is important for restricting RTK activities at the leading edge of the border cells by acting on the PIP2-dependant endocytic cycles.

Comments for the author

Based on the new data presented in the manuscript and on the responses to suggestions, I favour publication of this work in Development.

Reviewer 2

Advance summary and potential significance to field

Wang et al. report on the function of CTP Synthase during border cell migration in the Drosophila ovary.
Although CTP synthase produces the CTP pyrimidine which is a building block for a number of cellular macromolecules (DNA, RNA, lipids), the authors concentrate on its role in producing the forerunners for PIPs. They observe that a GFP protein trap line for CTP synthase shows a polarized

enrichment at the migratory leading edge of border cells, and that disruption of CTP synthase delays (or blocks?) migration.
The authors then see that F-actin levels at the leading edge are reduced, and that asymmetries in PIP2 are also disrupted. Lastly, they observe that exocyst subunits, Rab11, and RTK asymmetries and/or function are disrupted when CTP synthase function is compromised. This is an improved (and greatly changed) manuscript; however, responses to each of the reviewer points were not provided, and some important points still need to be addressed. The provided data is clear, although needs more clarification, and the figure composition is nice with helpful schematics. The writing is appropriate, though it needs more detailing of the results. There are several major points (genetics, measurement of absolute levels) that need addressing which are further described below:

Comments for the author

1) A key point that needs addressing – the description of the genetic interaction data required clarification/improvement. At many points in the manuscript it is stated that Gene X (Sec15, Sec3, Cbl, PI kinases) is necessary for border cell migration, and then the authors say that disrupting gene X in a heterozygous CTP synthase causes a phenotype, but it is not stated how much the individual gene knockdowns are enhanced when combined together. Given the statement that Gene X is necessary for BC migration, it would seem obvious that there could be a border cell phenotype when it is knocked out, so the point of these experiments would seem to be to say that there is a genetic interaction. It is especially important that it is a synergistic, and not additive, effect when combined to be able to claim an interaction so this needs to be made much clearer. An appropriate reference point is often not provided for these data in the manuscript text.
2) It would be good to further comment/explore how CTP synthase localization to the leading edge could produce PIP2 asymmetries. Since CTP synthase is fairly distant from the final PIP2 end product (there are a number of enzymes that must further process CTP to produce PIP2) are these additional enzymes also localized at the leading edge? For example, Sktl is often associated with the Golgi in many tissues, so how the enrichment of an early metabolic enzyme at the PM leads to a final PIP2 enrichment seems unclear. Is there evidence for this in other systems? Can the authors provide a model for how this occurs?
3) Also, when measuring potential changes in asymmetric enrichments to the leading edge, please provide measurements of the absolute levels of the proteins of interest. It could be that decreases in overall levels would also make it harder to observe the subtle fold changes that occur in some of these backgrounds.
Given that the levels of a major metabolite like CTP are being affected, it would not be surprising that the levels of a variety of cell factors may also be decreased.
4) Along these lines, the authors note that PIP2 levels are not disrupted after CTP synthase disruption, but only asymmetries, how could this be? Are PIP2 levels reduced later in development or in other tissues? Is this a perdurance issue? Are there other pathways that can produce PIP2? It would be good to comment on this.
5) Does disruption of CTP synthase delay or block migration? What fraction of BCs eventually make it to the anterior?

Minor notes:
The use of “compartmentalized” seemed confusing – at times it seemed to reference filament formation and at other times perhaps it references plasma membrane or other localization? I don’t know that it’s use for cytoplasmic localization adjacent to the PM seems appropriate.
Line 164, typo, “interphase” should be “interface”.

Second revision

Author response to reviewers' comments

Response letter

Reviewer 2 Advance Summary and Potential Significance to Field:
Wang et al. report on the function of CTP Synthase during border cell migration in the Drosophila ovary. Although CTP synthase produces the CTP pyrimidine which is a building block for a number of cellular macromolecules (DNA, RNA, lipids), the authors concentrate on its role in producing the forerunners for PIPs. They observe that a GFP protein trap line for CTP synthase shows a polarized enrichment at the migratory leading edge of border cells, and that disruption of CTP synthase delays (or blocks?) migration. The authors then see that F-actin levels at the leading edge are reduced, and that asymmetries in PIP2 are also disrupted. Lastly, they observe that exocyst subunits, Rab11, and RTK asymmetries and/or function are disrupted when CTP synthase function is compromised. This is an improved (and greatly changed) manuscript; however, responses to each of the reviewer points were not provided, and some important points still need to be addressed. The provided data is clear, although needs more clarification, and the figure composition is nice with helpful schematics. The writing is appropriate, though it needs more detailing of the results. There are several major points (genetics, measurement of absolute levels) that need addressing which are further described below:

Reviewer 2 Comments for the Author:

1) A key point that needs addressing – the description of the genetic interaction data required clarification/improvement. At many points in the manuscript it is stated that Gene X (Sec15, Sec3, Cbl, PI kinases) is necessary for border cell migration, and then the authors say that disrupting gene X in a heterozygous CTP synthase causes a phenotype, but it is not stated how much the individual gene knockdowns are enhanced when combined together. Given the statement that Gene X is necessary for BC migration, it would seem obvious that there could be a border cell phenotype when it is knocked out, so the point of these experiments would seem to be to say that there is a genetic interaction. It is especially important that it is a synergistic, and not additive, effect when combined to be able to claim an interaction, so this needs to be made much clearer. An appropriate reference point is often not provided for these data in the manuscript text.
Response: We agree that it is important to address whether the genetic interactions are synergistic, and we have now changed the context in the result section to describe the knockdown effect of individual genes, the phenotype of heterozygote P element insertion mutant of CTPsyn, and their interactions (Line 177-184, 214-216 and 226). The RNAi knockdown of Sec15, Sec3, Cbl, PI kinases by itself did not show defects in border cell migration, as there was no statistic difference between them and the control RNAi. Similarly, CTPsyn^{d06966} heterozygote mutant was not significantly different from the control RNAi either. Therefore, the genetic interactions between CTPsyn^{d06966} heterozygote mutant and RNAi of Sec15, Sec3, Cbl, PI kinases were synergistic, please see the new figure 4B and 5G.

2) It would be good to further comment/explore how CTP synthase localization to the leading edge could produce PIP2 asymmetries. Since CTP synthase is fairly distant from the final PIP2 end product (there are a number of enzymes that must further process CTP to produce PIP2) are these additional enzymes also localized at the leading edge? For example, Sktl is often associated with the Golgi in many tissues, so how the enrichment of an early metabolic enzyme at the PM leads to a final PIP2 enrichment seems unclear. Is there evidence for this in other systems? Can the authors provide a model for how this occurs?
Response: How CTP synthase localization to the leading edge could produce PIP2 asymmetries is a very interesting question, and one possibility is that the PIP2 local enrichment was generated by higher CTPsyn activity regionally (such as by multimerization of CTPsyn), as CTPsyn associated ER might locate in proximity to the leading edge. It is thought that PIP2 biosynthesis occurs in ER, and enzymes downstream CTPsyn in the pathway appear in ER. But whether enrichments of these enzymes in particular subcellular regions need further investigation. Moreover, the more CTP produced by CTPsyn could generate more PIP2 with the unaltered amounts of downstream enzymes.

3) Also, when measuring potential changes in asymmetric enrichments to the leading edge, please provide measurements of the absolute levels of the proteins of interest. It could be that decreases in overall levels would also make it harder to observe the subtle fold changes that occur in some of these backgrounds. Given that the levels of a major metabolite like CTP are being affected, it would not be surprising that the levels of a variety of cell factors may also be decreased.
Response: For the asymmetric enrichment of CTPsynGFP (Fig. 1C), PH-PLC GFP (fig 3I), actin (Fig. 3L), FYVEGFP (Fig. 5C), and Rab11GFP (Fig. 5F), we presented the results by the comparison of the leading edge to the tail, but we also noticed the interior signals were not dramatically reduced in CTPsyn depletion compared to that in wt. Since every batch of experiments would have variations in signal intensities, therefore, we did not present the absolute levels of signals.

4) Along these lines, the authors note that PIP2 levels are not disrupted after CTP synthase disruption, but only asymmetries, how could this be? Are PIP2 levels reduced later in development or in other tissues? Is this a perdurance issue? Are there other pathways that can produce PIP2? It would be good to comment on this.
Response: The total levels of PIP2 did not show a significant difference in the combination of RNAi and heterozygote mutant (now quantified in Fig.S3A), but the asymmetric distribution was reduced (Fig. 3). One possible reason could be the reduced level of CTPsyn still could make enough product in the biosynthesis of PIP2 and not cause a detectable overall change by IF staining, however, the asymmetric enrichment was easy to show a difference when a reduction occurred. Alternatively, the dynamics of CTP production by CTPsyn was not discussed previously, and we do not know whether CTP production was regulated locally by other factors. It is not known whether in *Drosophila* alternative pathways contribute to the PIP2 production, as shown in Fig. 4A. We have mentioned this issue in the results section (Line 182-183).

5) Does disruption of CTP synthase delay or block migration? What fraction of BCs eventually make it to the anterior?
Response: We examined stage 10B egg chambers to determine the effects of CTPsyn on migration. In RNAi knockdown and heterozygote mutant, there was about 35% of border cell clusters reached the anterior border of the oocyte (Fig. 1B). In mutant clone analysis, there were only about 5% of border cell clusters reached the anterior. However, in both cases, some clusters migrated to reach 50% or 75% of the nurse cell area, indicating the reduction of migration is not completely blocked. We think the disruption of CTPsyn suppressed border cell migration, therefore, we replaced the word “delay” to suppress (lines 35, 207 and 263). Thank the reviewer for the critical suggestion.

Minor notes:

The use of “compartmentalized” seemed confusing – at times it seemed to reference filament formation, and at other times perhaps it references plasma membrane or other localization? I don’t know that it’s use for cytoplasmic localization adjacent to the PM seems appropriate.
Response: We agree that not to confuse enrichment on the plasma membrane with CTPsyn filament and thus we have now removed the word “compartmentalization” from the title of the manuscript and edited the text in line 54 as “Here, we investigated how CTPsyn affected collective cell migration”.

Line 164, typo, “interphase” should be “interface”.
Response: We now have changed the “interphase” to “interface” in line 157.

Third decision letter

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MS TITLE: Drosophila CTP synthase regulates collective cell migration by controlling the polarized endocytic cycle

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ARTICLE TYPE: Research Article

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