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Ack kinase regulates CTP synthase filaments during Drosophila oogenesis

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

Editor: Esther Schnapp

1st Editorial Decision 02 April 2014

Thank you for the submission of your manuscript to EMBO reports. I am sorry for the delay in getting back to you; we have now received the full set of referee reports and referee cross-comments that are copied below.

As you will see, while the referees acknowledge that the findings are novel and potentially interesting, they raise several concerns that preclude publication of the manuscript at this point. The most important concerns are the weak functional link between DAck and CTPS and the specificity of the DAck antibody. Upon cross-commenting on each others reports, all referees agree that it should be investigated whether expression of a gain-of-function human CTPS allele rescues DAck86 mutant phenotypes, and whether DAck wt and/or DAck-K156A rescue the phospholipid and CTPS assembly defects. Referees 2 and 3 also indicate that it should be examined whether endoreplication defects contribute to the mutant phenotypes. Referee 2 further remarks that it should be distinguished whether elevated human CTPS activity or elevated CTP levels induce filament formation. The technical concerns raised by referee 3 also need to be addressed. While insight into filament formation in other cell types, overlap of DAck with clathrin and phosphorylation of CTPS would certainly be welcome additions to the manuscript, they are not strictly required for

publication of the study here.

Given these constructive comments, we would like to invite you to revise your manuscript with the understanding that the referee concerns (as mentioned above and in their reports) must be fully addressed and their suggestions taken on board. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision. Please contact us if a 3 months time frame is not sufficient for the revision. Also, the revised manuscript may not exceed 30,000 characters (including spaces, references and figure legends) and 5 main plus 5 supplementary figures, which should directly relate to their corresponding main figure. The materials and methods section cannot be shortened any further.

Regarding data quantification, can you please specify the number "n" for how many experiments were performed, the bars and error bars (e.g. mean +/- SEM, SD) and the test used to calculate p-values in the respective figure legends? This information is currently incomplete and must be provided in the figure legends.

I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have any questions or comments regarding the revision.

REFEREE REPORTS:

Referee #1:

Ack1, a tyrosine kinase capable of interacting with GTP-bound Cdc42, has been implicated in metastasis, thus a better understanding of Ack function under physiological conditions is relevant to cell biology and cancer research. This manuscript, submitted by Strochlic et al., describes the role of Drosophila Ack in regulating the CTPS filaments during oogenesis. Ack mutant females exhibit reduced fertility and produce oocytes with membrane defects. In oocytes, Ack co-localizes with CTPS in filamentous structures, and regulates the morphology of these CTPS-containing filaments. The authors argue that CTPS activity in Ack mutants is disrupted, resulting in reduction of PIP2 staining and RNA level. While the findings are novel, the functional link between Ack and CTPS is weak. Thus I feel the work is too preliminary for publication at EMBO Report at this point.

Summary
• 1. Does this manuscript report a single key finding? YES/NO

Yes, the key finding is that Ack regulates the formation of CTPS-containing filaments during Drosophila oogenesis.

• 2. Is the reported work of significance (YES), or does it describe a confirmatory finding or one that has already been documented using other methods or in other organisms etc (NO)? YES/NO

Yes, the work is original, as there has been no report describing the association of Ack family kinases with these filamentous structures.

• 3. Is it of general interest to the molecular biology community? YES/NO

I would say no. While the work is novel, it is unclear if these results are specific to this particular system or applicable to other cell types. In addition, I feel additional experiments are needed to bolster the key point.

• 4. Is the single major finding robustly documented using independent lines of experimental evidence (YES), or is it really just a preliminary report requiring significant further data to become convincing, and thus more suited to a longerformat article (NO)? YES/NO

No, I feel the work is too preliminary and requires additional data (see below).

Specific Comments
1. Ack is known to colocalize with clathrin in mammalian cells. Does fly Ack overlap spatially with clathin in oocytes? Can the images be shown at a higher resolution? In addition, does Ack overlap with CTPS filaments in other Drosophila cell types?
2. While the authors show that Ack kinase is required for rescuing the egg-laying defect, rescue of the CTPS-positive filaments by germ cell-specific expression of Ack wild type and kinase-dead mutant should be shown. In addition, while no Ack-dependent phosphorylation of CTPS was seen, can the filaments be stained for anti-phosphotyrosine antibody? I would expect these filaments to be phosphotyrosine-positive because Ack is autophosphorylated.
3. The functional link between Ack and CTPS is rather weak. Since the authors show that expression of a gain-of-function human CTPS allele rescues CTPS mutant, couldn't the same experiment be done for Ack86? It seems to me that this will be a good way of functionally linking these two genes.
4. If the expression of the gain-of-function human CTPS in cultured cells forms filaments, is mammalian Ack1 detected in these structures? Furthermore, does the expression of this allele in other Drosophila cell types form filament? If yes, do these structures also contain Ack?
5. The authors show that Ack and CTPS mutant oocytes exhibit defects in plasma membrane integrity and RNA synthesis. Are these defects independent contributing factors to the reduced female fertility? I understand that these are difficult issues but they should be discussed in greater depth.

Referee #2:

The manuscript " Ack kinase regulates CTP synthase filament morphology and activity during Drosophila oogenesis" by Strochlic et al analyzes the role of the Ack kinase in regulating CTP synthase filament formation and uses mutations in CTP synthase itself to argue that the filaments are comprised of the active form of the enzyme. In particular the authors find that mutations in Ack cause reduced fertility in female Drosophila. Furthermore, the these mutations cause alterations in egg chamber membranes that are phenocopied by loss of function mutations in CTPS, suggesting that the membrane defect might be due to the fact that CTP levels are perturbed in both mutations causing a defect in phospholipid biosynthesis. However, the most intriguing results in the paper are the findings that 1) Ack co-localizes with CTPS filaments, 2) mutations in ack cause a decrease/loss of CTPS filaments, and 3) mutations in CTPS that remove feedback inhibition promote filament formation. Together, these results are highly novel and would be of great interest to the general readership of EMBO reports, however, there are several technical concerns that I have the preclude recommending publication at this time. If the authors address these concerns, I would gladly support publication.

Specific Concerns
1) One major area of concern is the specificity of the Ack antibody. Many antibodies have been found that cross react with CTPS, including the Cup antibody that was originally used to identify them in Drosophila. Even some affinity purified antibodies that are specific by western recognize the CTPS filaments. Since the colocalization is a major finding of the paper a GFP-Ack transgenic line would be necessary to show colocalization unambiguously. This is of particular concern since the staining of the ack mutant egg chambers with the ack antibody is not well characterized/described in the paper.

2) Related to the specificity of the antibody, the staining of ack mutants that are also expressing a PH-GFP mutant with the ack antibody does not provide sufficient information to evaluate the antibody. I would suggest that the authors stain straight ack mutant egg chambers and those that

have the Ack rescue construct simultaneously for ack and CTPS (if possible, otherwise in simultaneous parallel experiments). Providing counts of the egg chambers with and without Ack/CTPS filaments would provide a firm basis for evaluating the reagent.

3) The experiments with the human E161K CTPS are very interesting since they suggest that the filament formation of CTPS may be differentially regulated between organisms since the same mutation in yeast causes both a loss of filaments and a massive increase in the formation of smaller structures. One concern, however, in the interpretation of the experiments in both this paper and the yeast work is that it is unclear if the effect is due to a change in the enzyme or an indirect effect of high levels of CTP. The authors should address this by analyzing the effects of a catalytically dead mutant alone and in combination with E161K. This would unambiguously connect elevated enzyme activity with polymer formation and separate it from the possible effects of elevated CTP shutting down the enzyme.

Referee #3:

Summary
In this manuscript, the authors report that DAck mutant females lay fewer eggs and bear egg chambers with discontinuities in germ line membranes, and that DAck co-localizes with CTPS filaments in the germ line. Since CTPS is involved in phospholipid production, they hypothesize that the membrane discontinuities observed in DAck mutant germ lines are a result of defective CTPS mediated phospholipid synthesis in the absence of DAck. In support of this hypothesis, they report decreased levels of a PIP2 reporter in DAck mutant egg chambers, as well as discontinuities in CTPS mutant egg chambers. They also found that a form of CTPS that is catalytically active can rescue the membrane discontinuities and female sterility associated with loss of CTPS.

Comments
1. The phospholipid membrane changes and CTPS filament disruption in DAck86 mutants are crucial phenotypes in their proposed model. Can they be rescued by the UAS-DACK WT construct? Their observation that this construct rescues the egg laying and membrane discontinuity phenotypes may suggest, but does not show, that these other phenotypes are also DAck-dependent.
2. The authors show that the DAck kinase activity domain is important for the stabilization of the plasma membrane and for egg production. The abstract further implies that this domain is also involved in stabilizing the CTPS filaments too, but I found no data supporting this statement. Rescue of the CTPS filament phenotype by the UAS-DACK-K156A construct would support this claim, or the abstract can be rephrased to clarify this point.
3. Since the authors hypothesize that phospholipid changes are related to the membrane discontinuity and egg laying phenotypes, and that DAck86 and CTPSe/f mutants have the same phenotype, it is important to test if the lack of CTPS filaments (and then the lack of CTPS enzymatic activity) in the CTPSe/f heteroallelic egg chambers causes the same phospholipid plasma membrane composition change in oogenesis that the authors report for DAck86 mutants. Their underlying rationale, that reduction of CTP levels (i.e. upon loss of CTPS filament organization and/or function in the absence of DAck) leads to phospholipid defects, would imply that reduction of CTP in the CTPS mutant should have the same effect.
4. The authors speculate that a role for CTPS in membrane phospholipid production might underlie the membrane discontinuities observed in DAck and CTPS mutants. But although they mention that the CTPS mediated production of CTP is essential to support endoreplication and that endoreplication is important for oogenesis, they do not consider the possibility that endoreplication may be affected in their mutants (indeed, the nurse cell nuclei in Fig. 3D appear somewhat smaller than wild type) or that a defect in endoreplication might contribute to reduced egg production.
5. Given that the active mutant CTPS1-E161K is known to assemble spontaneously into filaments and produce CTP, might one predict that the overexpression of this construct should rescue the DAck86 mutant phenotypes?
Technical concerns
6. Additional detail should be provided about the quantitation of CTPS filaments presented in Figure 3H. The reported increase in CTPS filament number in DAck86 mutant egg chambers (Fig. 3H),

compared to wild type, seems clear, but additional details should be provided about how quantitation was accomplished in various stages. In particular, although the authors provide data for stage 14 egg chambers, the staging criteria outlined by Spradling (1993) state that this stage is defined by the absence of nurse cell nuclei (which would make filament counting impossible). Were the authors using different staging criteria? Also, was visualizing the filaments straightforward in stage 12/13 egg chambers, where the nurse cells are beginning to disappear, and in very early stages, where the egg chambers are very small?
7. The data in Fig. 4D show reduced total RNA in DAck86 mutant egg chambers. However, it is not clear what this difference represents. For example, since mutants lay fewer eggs, it seems quite possible that their ovaries would have fewer later stage egg chambers (which, as the authors state, have more total RNA and DNA than younger stages). If so, the reduced total RNA could simply reflect a bias toward younger stages in the mutants vs. the wild type, even if the total "volume" of tissue analyzed was the same. On a related note, although the mutants lay fewer eggs but still apparently contain sufficient stages for staining and CTPS filament quantitation, it would be interesting to know where the "block" occurs. For example, are there fewer total egg chambers, do they arrest at some point during development, etc.?
8. The penetrance of the "membrane discontinuity" phenotype should be presented. The percentage of egg chambers per stage presenting the phenotype should be clearly specified for the mutant and recue conditions (Fig. 1C, 3A, 3D). Indeed, the mutant egg chamber in Fig 3A does not appear to exhibit this phenotype, suggesting that it is not completely penetrant. In this particular image it would be useful to stain the egg chamber with a different membrane marker.
9. More detail about the way the egg laying phenotype was scored should be provided (Fig. 1, 4). Since the normal egg laying number depends on the fly's age (as well as other factors), it should be documented that the differences observed are due to the genotype itself and not due to other variables (such as age, temperature, nutrition, time of day, etc.).
Minor points
10. The notation " **" indicates "not significant" in Figs. 3G and 4C, but indicates "significant" in Fig 4D.
11. Use of the word "significant" without supporting statistical analysis should be avoided.
12. The conclusions for each section should be clear and based only on the data presented. Any interpretation and speculation should be clearly separated from the conclusion.
13. The manuscript would be strengthened by clearer explanation of underlying rationale for the authors' hypotheses/interpretations. For example, in section 2 the authors conclude that "Overall, this suggests the possibility that the germ cell membrane integrity defects observed in DAck86 flies might result from loss of DAck mediated regulation of CTPS in filaments". However at this point in the manuscript they haven't mention that CTPS is involved in CTP and then phospholipid production, and consequently in membrane stability. A bit more background information would make the rationale for this statement more clear.

Cross-comments from referee 2:

Reviewer#1 Rescue:
1) Expression of a gain-of-function human CTPS allele to rescue Ack86. I think this is a good experiment, but I suspect it will likely fail since A) the E161K mutant is known to alter phospholipid profiles in yeast and B) Ack likely regulates multiple genes contributing to phospholipid metabolism.

It is a useful experiment for refining the proposed model by excluding a simple pathway from ack to CTPS to phosopholipids. I also suspect that if their observations are true and E161K forms filaments in an unregulated manner that they won't be disassembled at nurse cell dumping causing an oogenesis defect - this in itself would be very interesting since it would provide a basis for future work at taking apart how polymerization is regulated.

Reviewer #3 Rescue:
1) Does UAS-DACK WT and/or K156A rescue the phospholipid and CTPS assembly defects? I think this is a critical experiment since it ties the kinase activity to filament assembly. I would also add that my suggestion of examining a catalytically dead mutant of CTPS that polymerizes (already reported in e coli) for the ability to rescue dack or ctps mutant would allow further separation of

CTP levels, dack function, and polymer formation.

2) Expression of a gain-of-function human CTPS allele to rescue Ack86. (see reviewer #1)

3) Reviewer #3 provides an excellent alternative model that some of these defects are actually due to defects in endoreduplication. The Drosophila meeting just ended and Cbl seems to regulate both filament formation and endoreduplication although, like here, the question of cause and effect is unclear. This model should be investigated, perhaps by using other endoreduplication mutants to demonstrate that the polymer effects are not secondary to alterations in gene copy number.

Cross-comments from referee 3:

Yes, I think the rescue experiments are crucial. The current data only show that the Dack kinase activity is important for egg production and for membrane continuity in the germline. Without a rescue experiment in which they put back a wild type Dack transgene (e.g. as they do in Figure 1A for the egg laying phenotype) they cannot even conclude that Dack is required for CTPS filament architecture; it's entirely possible that the filament defects are due to another lesion on the Dack chromosome or even due to normal variation between genetic backgrounds (particularly since they see a reduction in filament size but not total filament volume). This experiment is therefore important for establishing even the "baseline" result that Dack affects CTPS filaments. Moreover, without testing for rescue by the kinase-dead Dack, the authors have NOT shown (contrary to what they say in the abstract) that Dack kinase activity is required for CTPS filament architecture.

If the authors were to demonstrate a functional link between Dack and CTPS, I think the manuscript would be potentially suitable for publication. But I'm not sure what that link would be. For example, they show that the filaments are shorter in the Dack mutant, but the total filament volume is not reduced so the significance of this phenotype is not clear to me. Also, their only read-out of whether CTPS function is altered in the absence of Dack is the reduction of a single phospholipid marker in the absence of Dack, and even then it's not clear that this reduction has anything to do with altered CTPS function (ie as opposed to being a result of a non-CTPS-mediated consequence of loss of Dack). Reviewer 1 and I mention testing for rescue with the activated human form of CTPS, which could support the notion that the relevant defect in Dack mutants is altered CTPS activity.

I agree with the majority of the other reviewers' comments and suggestions, but would like to add (if I may) that I found the suggestions about looking at clathrin and at CTPS phosphorylation very interesting but possibly outside the scope of the current work. Also, in his/her summary, Reviewer 3 refers to the finding that loss of ack leads to a decrease/loss of CTPS filaments, when really what the authors have shown is much more subtle -the filaments are still present (albeit shorter) and have the same total volume - and it's therefore less clear whether these changes would even be expected to alter CTPS activity.

Finally, I want to re-iterate my technical concerns about the interpretation of the reduction of total RNA shown in Fig 4D. Despite the convincing bar graph and error bars, the fundamental design of the experiment may well be flawed. As I mention in my review, the reduced total RNA may simply reflect the presence of fewer later-stage egg chambers (which have less total RNA) in the mutant, which is fairly likely given the fact that they produce fewer mature eggs. If the authors have considered this caveat, they should address this in the text.

1st Revision - authors' response 03 July 2014

While the reviewers noted that “the findings are novel” and “would be of great interest to the general readership of EMBO Reports”, they also raised several important issues and suggested specific experiments to address them. We thank the reviewers for their thoughtful comments and insight and we have incorporated their suggestions into a revised manuscript that we believe is much improved.

Primary issues raised by multiple reviewers

All three reviewers suggested that genetic rescue experiments should be performed to confirm aspects of our model. The first is to demonstrate that re-expression of wild type DAck rescues all of the phenotypes of the DAck-deficient flies. Second, is to determine whether DAck kinase activity is required for its function in CTPS filament morphology by testing transgenic rescue with a kinase-dead DAck (K156A). Finally, does expression of constitutively active CTPS1-E161K in the female germline rescue the phenotypes associated with loss of DAck?

We have completed all three of these genetic rescue experiments, resulting in three new figure panels. The results are consistent with our model and demonstrate that: a) all phenotypes associated with *DACK*-deficiency are rescued by DAck re-expression but not kinase-dead DAck (K156A): fertility (Fig. 1A), plasma membrane defects (Figs. 1C & 1D), and CTPS filament morphology (Fig. 3H). Finally, as predicted in the cross-comments by reviewer #2, we show that expression of CTPS1-E161K fails to rescue the reduced fertility of *DACK*-deficient flies (Fig. 4D) and offer an intriguing potential explanation for this result. We suggest, based on evidence from new catalytically inactive CTPS mutants (suggested by reviewer #2), that DAck controls CTP production via morphological organization of the filaments rather than by direct activation of CTPS enzymatic activity. Specifically, we found that while CTPS1-E161K forms normal filaments in the context of wild-type flies, in DAck^{86} mutants CTPS1-E161K filaments are morphologically disrupted (Fig. 4E) and therefore likely not fully active in this background. Taken together, these rescue experiments strongly bolster our mechanistic model linking DAck catalytic activity to filament assembly and highlight the importance of normal filament morphology for CTPS catalytic activity.

Two reviewers suggested examining if endoreplication is affected when DACK is deleted and filament morphology is abnormal since this could contribute to the reduced female fertility. In addition, in the cross-comments, Reviewer #2 suggests that alterations in CTPS filaments might be due to changes in CTPS gene copy number secondary to defects in endoreplication.

As discussed below in the response to Reviewer #3 (comment 4), we provide new evidence (Fig. 4A) that endoreplication defects may indeed be present in DAck^{86} flies, an unsurprising finding given the evidence for reduced CTP levels in this genetic background. Importantly for the reviewer's particular concern here, however, we find that CTPS protein levels are unchanged in DAck^{86} ovaries compared with wild-type (Fig. E3), demonstrating that filament defects are not a result of decreased CTPS expression.

Complete Comments from Reviewer #1 with responses

1) Given what is known about mammalian Ack, the reviewer suggested looking at the co-localization of clathrin and DAck in egg chambers and to examine whether Ack co-localizes with CTPS in other Drosophila cell types or tissues.

While these questions are certainly interesting, we believe that they are outside the scope of the work described here, particularly in a focused "short report" format. A similar sentiment was expressed by Reviewer #3 in the cross-comments.

2) While the authors show that Ack kinase is required for rescuing the egg-laying defect, rescue of the CTPS-positive filaments by germ cell-specific expression of wild-type and kinase-dead Ack should be shown.
AND
Can the filaments be stained with an anti-phosphotyrosine antibody?

The requested rescue experiments were suggested by multiple reviewers and are addressed above.

The reviewer asks if the filaments can be detected with an anti-phosphotyrosine antibody since activated Ack is known to be autophosphorylated. We are unable to detect anti-phosphotyrosine staining of CTPS filaments in *Drosophila* egg chambers but it is unclear whether this negative data

is due to inadequate detection sensitivity or poor antibody accessibility. However, we are able to detect endogenous filaments that contain both CTPS1 and Tyr284-phosphorylated Ack in cultured mammalian cells under conditions of nucleotide depletion. This experiment also addresses the concern of this reviewer that these results are specific to this particular system and are not applicable to other cell types or organisms. We have included this new data in revised Figure 2C.

3) Does the gain of function mutant CTPS-E161K rescue the DAck^{86} mutant?

The suggestion to test whether CTPS-E161K rescues the DAck^{86} mutant was raised by multiple reviewers and is addressed above.

4) If expression of the gain-of-function human CTPS in cultured cells forms filaments, is mammalian Ack detected in these structures? Furthermore, does the expression of this allele in other Drosophila cell types form filaments? If yes, do these structures also contain Ack?

We now demonstrate in revised Figure 2C that endogenous phospho-Y284-Ack1 and endogenous CTPS1 co-localize in filaments in mammalian cells, supporting their relevance beyond *Drosophila*. Similar to comment 1, we have not tested if expression of this allele forms filaments in other *Drosophila* cell types or if DAck is localized there since we feel it is outside the scope of this report.

5) The authors show that Ack and CTPS mutant oocytes exhibit defects in plasma membrane integrity and RNA synthesis. Are these defects independent contributing factors to reduced female fertility?

Our genetic evidence demonstrates directly that DAck regulates CTPS morphology and indirectly that DAck regulates CTPS catalytic activity. New data in the revised manuscript shows that constitutively active CTPS1-E161K fails to rescue the fertility defect of DAck^{86} mutants (Fig. 4E), demonstrating that DAck plays both CTPS-dependent and CTPS-independent roles in oogenesis. This important point is discussed in the text along with the results of this experiment.

Complete Comments from Reviewer #2 with responses

1) One major area of concern is the specificity of the Ack antibody as many antibodies have been found to cross-react with CTPS. Since the co-localization is a major finding of the paper, a GFP-Ack transgenic line would be necessary to show co-localization unambiguously.
ALSO
2) Related to the specificity of the antibody, the staining of Ack mutant egg chambers that are also expressing a GFP-PH-PLCδ reporter does not provide sufficient information to evaluate the antibody.

These comments question the specificity of the anti-DAck antibody, an important point. The revised manuscript now includes three separate pieces of evidence that the anti-DAck staining of CTPS filaments is specific (two are new to the revised manuscript and are presented in new Expanded View Figure 1).
1) In Fig. 3A we mixed wild-type and DAck^{86} egg chambers and show that these are easily distinguished by the loss of anti-DAck staining in DAck^{86} mutants.
2) In Fig. E1A we demonstrate antibody specificity by lack of staining in DAck^{86} egg chambers and restoration of filament detection in DAck^{86} egg chambers re-expressing wild-type DAck.
3) Finally, as suggested by the reviewer, we generated transgenic flies expressing UAS-FLAG-DAck for Gal4-mediated expression in the female germline. This epitope-tagged version localizes as expected to germ cell filaments (Fig. E1B) and demonstrates definitively that Ack is a *bona fide* component of these structures in *Drosophila* egg chambers.

3) The authors should analyze the effects of a catalytically dead mutant of CTPS alone and in combination with the E161K mutation [on filament formation].

This clever experiment was proposed to determine if filament formation by CTPS-E161K is due to "a change in the enzyme or is an indirect effect due to high levels of CTP." We examined filament formation by CTPS containing the E161K or catalytically inactive G148A mutants alone or in combination and the results have been incorporated into revised Fig. 4B. In short, we find that filaments form when the E161K mutation is present regardless of whether the enzyme is catalytically active, thus demonstrating that filament formation is not a consequence of high levels of CTP.

Complete Comments from Reviewer #3 with responses

1) The phospholipid membrane changes and CTPS filament disruption in DAck^{86} mutants are crucial phenotypes in their proposed model. Can they be rescued by the UAS-DACK WT construct? Their observation that this construct rescues the egg laying and membrane discontinuity phenotypes may suggest, but does not show, that these other phenotypes are also DAck-dependent.
2) The authors show that the DAck kinase activity domain is important for the stabilization of the plasma membrane and for egg production. The abstract further implies that this domain is involved in stabilizing the CTPS filaments too, but I found no data supporting this statement. Rescue of the CTPS filament phenotype by the UAS-DACK-K156A construct would support this claim, or the abstract can be rephrased to clarify this point.

These comments were echoed by other reviewers and were addressed above.

3) Since the authors hypothesize that phospholipid changes are related to the membrane discontinuity and egg laying phenotypes, and because DAck^{86} and CTPS$^{e/f}$ mutants have the same phenotype, it is important to test if the CTPS$^{e/f}$ egg chambers display the same phospholipid defects as DAck^{86} egg chambers.

We agree and have examined phosphoinositide membrane staining with the GFP-PH-PLCδ domain in CTPS$^{e/f}$ egg chambers (new Fig. E2). As expected, we observe reduced GFP staining at the plasma membrane of germ cells of this genotype, much like those of DAck^{86} mutants.

4) Although they mention that the CTPS-mediated production of CTP is essential to support endoreplication and that endoreplication is important for oogenesis, they do not consider the possibility that endoreplication may be defective in their mutants or that a defect in endoreplication might contribute to reduced egg production.

We thank the reviewer for this insightful suggestion and have now measured germ cell nuclear diameter as an indirect measure of endoreplication in wild-type and DAck^{86} egg chambers. The results demonstrate that, indeed, there is a statistically significant reduction in nuclear diameter in DAck^{86} compared to wild-type germ cell nuclei, consistent with reduced DNA content and a likely defect in endoreplication. This finding is entirely consistent with, and is possibly directly due, to decreased CTP levels in these genotypes. This data has been included in revised Fig. 4A.

5) Given that the active mutant CTPS1-E161K is known to assemble spontaneously into filaments and produce CTP, might one predict that the overexpression of this construct should rescue the DAck^{86} mutant phenotypes?

This suggestion was made by multiple reviewers and is addressed above.

This reviewer also raised a number of technical concerns/issues outlined below:

6) Additional information is requested regarding the staging method of egg chambers for filament quantitation in Figure 3H.

Filaments were counted regardless of whether nurse cell nuclei were present. Staging was based on egg chamber morphology when stained with fluorescently-conjugated phalloidin according to the characteristics described in Spradling, 1993. As a side note, the filaments are very easily to visualize and count in all stages in which they are present.

7) *The data in Fig. 4D (now Fig. 4F) show reduced total RNA in DAck86 mutant egg chambers. However, it is not clear what this difference represents. For example, since mutants lay fewer eggs, it seems quite possible that their ovaries would have fewer later stage egg chambers (which, as the authors state, have more total RNA and DNA than younger stages). If so, the reduced total RNA could simply reflect a bias toward younger stages in the mutants vs. the wild type, even if the total "volume" of tissue analyzed was the same. On a related note, although the mutants lay fewer eggs but still apparently contain sufficient stages for staining and CTPS filament quantitation, it would be interesting to know where the "block" occurs. For example, are there fewer total egg chambers, do they arrest at some point during development, etc.?*

We have not noticed a stage-specific block in oogenesis nor an increase in the total number of early stage egg chambers compared to late stages in DAck^{86} flies compared to wild-type flies. Nonetheless, fewer eggs are laid indicating that accumulating defects ultimately result in reduced fertility. We have added a sentence to the text to clarify this point and justify the normalization of tissue by volume.

8) *The reviewer states that the penetrance of the membrane discontinuity phenotype should be presented since it appears that it is not completely penetrant.*

The requested information has now been added by counting the number of stage 10 egg chambers that either do or do not display this phenotype, and indeed, the phenotype is largely, but not completely, penetrant. Additional panels showing phenotypic quantitation are now incorporated into Figures 1D, 3A, and 3D.

9) *The reviewer requests further details regarding how the egg laying assay (fertility analysis) was performed.*

This information has now been included in the Methods section.

Minor points:

*10) The notation "**" indicates "not significant" in Figs. 3G and 4C but indicates "significant" in Fig. 4D.*

This has been corrected and uniform notation is now used.

11) *Use of the word "significant" without statistical supporting analysis should be avoided.*

The word "significant" has been removed from the text in all instances in which there is not statistical information to support this claim.

12) *The conclusions for each section should be clear and based only on the data presented. Any interpretation and speculation should be clearly separated from the conclusion.*

Throughout the manuscript we have tried to clearly delineate the conclusions based on our data from interpretation and speculation.

13) *The manuscript would be strengthened by clearer explanation of the underlying rationale for the authors' hypotheses/interpretations.*

The reviewer specifically highlights a sentence at the end of section 2 that states that the germ cell membrane integrity defects in DAck^{86} flies might result from loss of DAck-mediated regulation of CTPS in filaments. However at this point in the manuscript, the role that CTP synthase plays in the biosynthesis of phospholipids has not yet been introduced. We agree that this statement seems out of place within the context of the manuscript. We have therefore removed it from the end of this section, as the rationale for this hypothesis is stated at the beginning of the following section. We hope that this and other minor textual changes have improved the clarity of the presentation.

2nd Editorial Decision 23 July 2014

Thank you for the submission of your revised manuscript to EMBO reports. We have now received the reports from the referees that were asked to assess it, as well as cross-comments. As you will see, both referees 1 and 2 still raise a few concerns that I would like you to address and incorporate in the manuscript before we can proceed with its official acceptance.

Referee 1 notes that the evidence that Ack regulates CTPS activity is weak, and I agree with this concern. The title and abstract (before last sentence) should therefore be modified accordingly. Referee 2 further mentions that the data should be interpreted and discussed more carefully and suggests that the G148A data could be removed from the manuscript, which I leave up to you to decide.

Regarding statistics, please specify the test used to calculate p-values in the respective figure legends, and please mention the number "n" for how many experiments were performed. This information is missing in some of the main figure legends.

Since January this year, EMBO press papers are accompanied online by 1) a short summary of the findings and their significance, 2) 2-3 bullet points highlighting key results and 3) a synopsis image. Can you please send us this information, and may be draw a model for the synopsis image, which needs to be exactly 211x157 pixels large? Otherwise, a key result or image could also be depicted in the synopsis image. Please note that the final size is rather small, and that not too much information can be included in the synopsis image. Text needs to be readable at the final size.

I look forward to seeing a final form of your manuscript as soon as possible. Please let me know if you have any questions or comments.

REFEREE REPORTS:

Referee #1:

This manuscript, submitted by Strochlic et al., describes the role of Drosophila Ack in regulating the CTPS filaments and activity during oogenesis. Ack mutant females exhibit reduced fertility and produce oocytes with membrane defects. In oocytes, Ack co-localizes with CTPS in filamentous structures, and regulates the morphology of these CTPS-containing filaments. The authors argue that CTPS activity in Ack mutants is disrupted, resulting in reduction of PIP2 staining and RNA level, which contributes to disrupted oogenesis.

This revised version includes additional molecular genetic experiments, demonstrating that re-expression of functional Ack rescues all the phenotypes associated with Ack86 in oogenesis, namely the reduced egg laying, the membrane defects, and the abnormal CTPS filaments. These results

show clearly that Ack kinase activity regulates the morphology of CTPS filaments. However, as the gain-of-function CTPS construct fails to rescue Ack86, the evidence supporting that Ack regulates CTPS activity remains weak. Although the authors suggest that this gain-of-function CTPS construct is not fully active in the absence of Ack, it seems likely that a mechanism other than regulating the CTPS activity could be involved. Nevertheless, as the paper has nicely demonstrated a role of Ack in regulating the formation of CTPS filaments, I will support its publication in EMBO Report.

Summary
• 1. Does this manuscript report a single key finding? YES/NO

Yes, the key finding is that Ack regulates the morphology and activity of CTPS-containing filaments during Drosophila oogenesis.

• 2. Is the reported work of significance (YES), or does it describe a confirmatory finding or one that has already been documented using other methods or in other organisms etc (NO)? YES/NO

Yes, the work is original, as there has been no report describing the association of Ack family kinases with these filamentous structures.

• 3. Is it of general interest to the molecular biology community? YES/NO

I would still say No, as it remains unclear if these results are specific to this particular system or applicable to other cell types.

• 4. Is the single major finding robustly documented using independent lines of experimental evidence (YES), or is it really just a preliminary report requiring significant further data to become convincing, and thus more suited to a longerformat article (NO)? YES/NO

The evidence for Ack regulates the morphology of CTPS filaments is nicely done. The evidence for Ack regulates the activity of CTPS is quite weak.

Specific Comments
1. Given that Ack localizes to these filaments and Ack kinase activity is required for the proper morphology of these filaments, the observation that these filaments cannot be stained by 4G10 is surprising. As Ack is heavily autophosphorylated, this should be investigated further.
2. In figure 4F, the difference in total ovarian RNA between Ack86 and E161K-rescued flies does not seem to be significant. Since Ack86 exhibits defects in oogenesis and E161K-rescued flies do not, wouldn't this suggest that the reduction in RNA synthesis is not the cause for the phenotypes?

Referee #2:

The manuscript " Ack kinase regulates CTP synthase filament morphology and activity during Drosophila oogenesis" by Strochlic et al analyzes the role of the Ack kinase in regulating CTP synthase filament formation and uses mutations in CTP synthase itself to argue that the filaments are comprised of the active form of the enzyme. These results are highly novel and would be of great interest to the general readership of EMBO reports. My major concern, the specificity of the Ack antibody, has been addressed. However, there are several technical concerns that I have that the authors should address within the manuscript and that might require additional experiments. Once those corrections are made, I wholeheartedly recommend publication.

Specific Concerns
1) I have to admit when I suggested the inactivating mutation experiment, I thought the authors would do this experiment in the fly not in tissue culture cells. Many of these metabolic polymers behave oddly in transient expression due to the high degree of variation in protein levels from cell to cell. I would caution the authors not to push these experiments too heavily, particularly without any

controls for protein levels in the individual cells they are analyzing.

2) Along similar lines, the authors use a G148A mutation to inactivate the CTPS. This mutation disrupts the UTP binding site as well as tetramerization. Thus, it is inactivating, but also disrupts other aspects of enzyme regulation. The C404G mutant is often used for a clean catalytically dead mutant.
The authors note that "These data corroborate the results observed in S. cerevisiae [28] with the notable exception that the yeast E161K mutant forms foci instead of filaments." Actually, their results are the opposite of those in the cited paper. The G148A mutation causes an increase in filament formation in [28] while in the submitted paper it seems to block filament formation. The C404G-E161K combination was used in [28] to argue that CTP levels were not the cause, but since G148A and E161K both affect tetramer formation the double mutant is difficult to interpret cleanly. One possibility is that E161K can suppress the tetramerization defects in the G148A mutant.

My suggestions:
I believe that the Dack localization alone is a very significant finding and I would not like for this paper to be held up over the mutant analysis. However, it seems like the mutant analysis is not particularly well controlled for protein expression and that the choice of mutations is problematic. I would suggest that the authors either:
A) address these concerns experimentally or B) be more cautious in their interpretation within the text (i.e note the effects of the mutants and other possible interpretations).
Alternatively, since I think the key finding is that there is a tyrosine kinase on a metabolic filament, C) I would also support the author's removing the G148A data for use in a more systematic study of the regulation of CTPS polymerization if they should desire to pursue that possibility.

Referee #3:

In the revised version of the manuscript by Strochlic et al. which describes a new role for the non-receptor tyrosine kinase DAck in regulating the assembly and activity of the CTP synthase macromolecular filaments, the authors have adequately addressed all the comments made in our previous review. All the crucial rescue experiments were performed and integrated to the text in the appropriate place, and the paper is better written and organized.

Cross-comments:

Referee #1:

The issues raised by Reviewer 2, who is clearly knowledgeable of CTPS, are good and should be addressed. I think the easiest solution will be to remove the G148A results.

Referee #2:

I think many of the issues raised by reviewer#1 and myself can be addressed within the text. One of the concerns raised by reviewer#1 is the lack of 4G10 staining on the filament. I did not comment on this in my review, but one possibility is that the CTPS filament could be regulating Ack signaling and that the focus on CTPS activity is misplaced. This would also be consistent with some of the rescue data. I think in general, the manuscript would be greatly strengthened by a broader exploration of what the relationship between the filament, enzyme activity, and Ack signaling might represent and the recognition that this is the start of the story rather than the final word on this.

I hesitate to rewrite their title, but I think a title more in line with 'Ack kinase is a component of CTP synthase filaments and regulates their morphology during Drosophila oogenesis' might be preferable and safer. Also, the authors don't really play up the fact that they have a completely new component of these structures - the first such discovery! It might be worthwhile to emphasize the importance of this finding in the text and title.

2nd Revision - authors' response 11 August 2014

Attached, please find a final revised version of our manuscript (#EMBOR-2014-38688V3) now entitled "**Ack kinase regulates CTP synthase filaments during *Drosophila* oogenesis**" for publication in EMBO Reports. Only files that required revision are attached. I have also attached separate files containing our summary of findings, a bullet point list of results, and a graphical synopsis, as you requested.

As recommended by yourself and the reviewers, we have revised our manuscript to address the three remaining concerns:

1) *Overemphasis on the link between Ack and CTPS catalytic activity*.
   To address this, we have removed the reference to CTPS catalytic activity in the title and altered the second to last sentence of the abstract to state that our results "suggest that DAck may regulate CTP synthase activity" rather than definitively demonstrate it.
2) *Difficulty in interpreting the G148A mutant CTPS data*
   As suggested by the reviewer, we removed the experiment with this mutant from the manuscript.
3) *Missing statistical information*
   Statistical tests of significance are listed in the Methods and all figure legends state "n" clearly.

We hope that you will now find this revised manuscript acceptable for publication.

3rd Editorial Decision 12 August 2014

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports.

Thank you for your contribution to EMBO reports and congratulations on a successful publication. Please consider us again in the future for your most exciting work.

