# LIPID- AND PHOSPHO-REGULATION OF CTP:PHOSPHOCHOLINE CYTIDYLYLTRANSFERASE α ASSOCIATION WITH NUCLEAR LIPID DROPLETS

Jason Foster, Michael McPhee, Lambert Yue, Graham Dellaire, Steven Pelech, and Neale Ridgway

*Corresponding author(s): Neale Ridgway, Dalhousie University*

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*Editor-in-Chief: Matthew Welch*

## Transaction Report:

(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.)

RE: Manuscript #E23-09-0354
TITLE: LIPID- AND PHOSPHO-REGULATION OF CTP:PHOSPHOCHOLINE CYTIDYLYLTRANSFERASE α ASSOCIATION WITH NUCLEAR LIPID DROPLETS

Dear Neale,

The review of your manuscript is now complete and the comments of two reviewers follow below.

As you will see the reviewers raised a number of concerns. Reviewer 1 was particularly concerned that some of the results do not appear to support the conclusions and may be partially redundant/contradictory in relation to your previous work. Both reviewers also raised a number of important technical issues. In view of these comments we cannot accept your manuscript in its present form but I would be happy to consider a revised manuscript. This would be returned to the original reviewers so it will be important to address their comments, both technical and regarding interpretation of the data with respect to previous work, in full.

We look forward to receiving a revised manuscript in due course.

Best regards,

Rob

Robert Parton
Monitoring Editor
Molecular Biology of the Cell

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Dear Dr. Ridgway,

The review of your manuscript, referenced above, is now complete. The Monitoring Editor has decided that your manuscript is not acceptable for publication at this time, but may be deemed acceptable after specific revisions are made, as described in the Monitoring Editor's decision letter above and the reviewer comments below.

A reminder: Please do not contact the Monitoring Editor directly regarding your manuscript. If you have any questions regarding the review process or the decision, please contact the MBoC Editorial Office (mboc@ascb.org).

When submitting your revision include a rebuttal letter that details, point-by-point, how the Monitoring Editor's and reviewers' comments have been addressed. (The file type for this letter must be "rebuttal letter"; do not include your response to the Monitoring Editor and reviewers in a "cover letter.") Please bear in mind that your rebuttal letter will be published with your paper if it is accepted, unless you have opted out of publishing the review history.

Authors are allowed 90 days to submit a revision. If this time period is inadequate, please contact us at mboc@ascb.org.

Revised manuscripts are assigned to the original Monitoring Editor whenever possible. However, special circumstances may preclude this. Also, revised manuscripts are often sent out for re-review, usually to the original reviewers when possible. The Monitoring Editor may solicit additional reviews if it is deemed necessary to render a completely informed decision.

In preparing your revised manuscript, please follow the instruction in the Information for Authors (www.molbiolcell.org/info-for-authors). In particular, to prepare for the possible acceptance of your revised manuscript, submit final, publication-quality figures with your revision as described.

If your manuscript contains a Significance Statement, please make sure that it consists of three separate bullet points totaling a maximum of 100 words and addressing each of the following points (see https://www.molbiolcell.org/curation-tools):
First bullet: What is the background context? What gap in knowledge does this study addres?
Second bullet: What are the key findings? What is unique or new about the approach?
Third bullet: Why is this paper significant? How might it influence future research?

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Thank you for submitting your manuscript to MBoC. We look forward to receiving your revised paper.

Sincerely,

Eric Baker
Journal Production Manager
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Reviewer #1 (Remarks to the Author):

CCTalpha is an enzyme that catalyzes the rate-limiting step of the PC synthetic Kennedy pathway. It was shown to be activated by binding to the nuclear envelope (NE) and nuclear lipid droplets (nLDs). Foster and the colleagues studied how the CCTalpha recruitment to nLDs is regulated and concluded that it is facilitated by lipin-1 and is suppressed by phosphorylation by CDK.

In the first half, the effect of two different fatty acids (oleate and palmitate) and lipin-1 overexpression on CCTalpha recruitment to nLDs is studied, but the result does not appear to support the conclusion. The second half is related to phosphorylation/dephosphorylation of CCTalpha, and the result is partially redundant and partially contradictory to the authors' previous paper in MBoC (Yue et al, 2020). The purpose of this study may be to study different cell types (Huh7 in this paper, HeLa and U2OS in the previous paper), in which nLDs form by different mechanisms, but the result is not discussed from that perspective sufficiently. Lastly, possible involvement of CDK is presented, but it was not studied in relation to the nLD recruitment of CCTalpha.

1. The authors concluded that oleate and lipin-1 facilitate binding of CCTalpha to nLD/LAPS, whereas palmitate suppressed it, based on the number of CCTalpha-positive nLD/LAPS per cell. In the conditions studied, however, the increase/decrease of CCTalpha-positive nLD/LAPS appears to simply reflect the increase/decrease of nLD/LAPS, which increases/decreases the surface monolayer for M-domain insertion (as discussed in p. 13). "Further enhancement" of the association of CCTalpha with nLD/LAPS (Abstract) cannot be concluded from this result.
2. In Yue et al, 2020, S319 in HeLa was shown to be dephosphorylated and distribute to the NE at 15-60 min of oleate treatment. In the present study, CCTalpha in Huh7 distributes to the NE at 15 min (less so at 30 min) (Fig. 4A), whereas S319 is not dephosphorylated at 3 hr (Fig. 4D) and whether S319 is dephosphorylated at 15 min not shown. If S319 in Huh7 is not dephosphorylated at 15 min, how is this result reconciled with that in HeLa? Conversely, if S319 is dephosphorylated at 15 min, how is the dephosphorylation (15 min)-rephosphorylation (3 hr) correlated with the PC synthesis?
3. In relation to the above point, at 12 hr of oleate treatment, CCTalpha is translocated to nLDs, but S319 phosphorylation does not change (Fig. 4D), or even increases (Fig. 5C). Please discuss this result. Is PC synthesis activated at this point, or only later, when S319 is dephosphorylated?
4. The previous paper (Yue et al, 2020) showed that S319 is dephosphorylated, whereas Y359/S362 remains to be phosphorylated when CCTalpha is translocated to nLD in U2OS, whereas in the present study, CCTalpha translocation to nLDs/LAPS in oleate-treated cells is accompanied by the dephosphorylation at S319 and S359/Y362 (Fig. 4D; p. 7, last line-). It is confusing that this seemingly contradictory result is presented without discussion. Does the difference have any meaning regarding activation and distributional change of CCTalpha in the two cell types?
5. Because CCTalpha exists as a dimer, presence of wild-type CCTalpha may affect the behavior of mutants by forming hetero-dimers. How do the authors exclude this possibility (especially in Fig. 9)?
6. To study whether CDK is involved in phosphorylation of CCTalpha, Huh7 cells were cultured with oleate for 30 min and then moved to an oleate-deficient medium containing CDK inhibitors. However, whether CCTalpha S319 is dephosphorylated at 30 min is not shown and its distribution in the NE appears to decrease compared to that at 15 min. 1) Please show whether S319 is dephosphorylated at 30 min. 2) The effect of CDK inhibitors on the CCTalpha distribution needs to be shown. 3) The effect of CDK inhibition needs to be studied in relation to nLD/LAPS, the main subject of this study.
7. LAPS, or lipid-associated PML structure, was defined as a subset of PML-NB, not as a subset of nLD. Throughout this paper, nLD and LAPS are treated in parallel, but this makes it difficult to understand sentences like "CCTa-positive LAPS and nLDs accounted for ~40% and 20% of total, respectively, with ~30% devoid of both PML and CCTa (Fig. 1B)" (p. 6). Classification of nLDs into PML-associated and -non-associated appears more reasonable to me.

Reviewer #2 (Remarks to the Author):

CTP:phosphocholine cytidylyltransferase (CCT) catalyzes the rate-limiting and regulated step in PC synthesis. Fatty acids trigger CCT recruitment to the inner nuclear membrane and, in some cell types, to nuclear lipid droplets. A portion of these nuclear lipid droplets are positive for promyelocytic leukemia (PML) protein.

In the current manuscript, the authors explore the mechanisms of CCT targeting to nuclear lipid droplets. As perhaps expected, oleate treatment and overexpression of lipin increased CCT recruitment to nuclear lipid droplets. Treatment with palmitate, which is less effective at inducing lipid droplets, had little effect on CCT localization to nuclear lipid droplets and also appeared to have an intriguing suppressive effect when added together with oleate. Through a series of mutagenesis studies it was discovered that phospho-memetic and phospho-null mutations of serine residues in the P-domain influenced CCT association with nuclear lipid droplets. The authors present additional evidence implicate cyclin-dependent kinase (CDK) 1 and 2 as two kinases involved in phosphorylating CCT .

While the findings are interesting and experiments mostly performed with high rigor, there are several areas where additional experiments and explanation would help to support the major conclusions.

Comment 1: The suppressive effect of palmitate on oleate-induced CCT targeting to nuclear lipid droplets is intriguing. However, the experiment is performed with less oleate in the palmitate co-treatment. If similar amounts of oleate are added does the additional of palmitate still have the suppressive effect? Is this due to the preferential incorporation of palmitate into phospholipids and a consequent reduction in packing defects on nuclear lipid droplets that are necessary for CCT recruitment?

Comment 2: An effect of the phospho-memetic and phospho-null mutations on CCT recruitment to nuclear lipid droplets is only observed when all 11 sites are mutated. This seems highly artificial. Would this ever happen in cells or is this an artificial situation that is not relevant to the actual regulation of the enzyme? Without evidence that this occurs in cells, the conclusions should be more conservative and speculative.

Comment 3: The primary evidence that CDK1 and CDK2 regulate the phosphorylation of CCT in cells comes from experiments that use inhibitors of these kinases. How specific are these inhibitors? Often kinase inhibitors hit multiple kinases. Are there substrates of these kinases that could be analyzed to support the specificity? Is it possible to use genetic approaches to disrupt these kinases to provide additional support?

Comment 4: Figure 4 would benefit from quantitation, particularly in panel C. It is mentioned that "palmitate-treated cells contained fewer cLDs, nLDs and LAPS, and LAPS and nLDs were devoid of CCTa." The reduction in CCTa is clear, but the changes in the amount of cLDs, nLDs and LAPS, and LAPS is not as obvious.

Comment 5: On page 9 it is mentioned that "In oleate-treated CHO-MT58 cells that do not form nLDs, the dephosphorylation of S319 was less pronounced (Fig. 6C)." Is the dephosphorylation of S319 less pronounced or just absent? It doesn't look like there is any dephosphorylation of S319 to me. In figure 5C, there seems to be a large increase in pS319 but this is not mentioned.

Comment 6: I found this sentence on page 6 incredibly difficult to decipher. Perphas it could be broken up into two sentences or additional clarification provided? "At 24 h, CCTa-positive LAPS and nLDs accounted for ~40% and 20% of total, respectively, with ~30% devoid of both PML and CCTa (Fig. 1B)."

Dear Dr. Parton,

We thank the reviewers for their thoughtful and constructive comments on our manuscript. Below is a point-by-point response to each comment, which includes the inclusion of new data and modification to the text. We hope that these revisions assuage the reviewers concerns and they now deem the manuscript suitable for publication in the *MBoC*.

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**Reviewer 1**

1. The authors concluded that oleate and lipin-1 facilitate binding of CCTalpha to nLD/LAPS, whereas palmitate suppressed it, based on the number of CCTalpha-positive nLD/LAPS per cell. In the conditions studied, however, the increase/decrease of CCTalpha-positive nLD/LAPS appears to simply reflect the increase/decrease of nLD/LAPS, which increases/decreases the surface monolayer for M-domain insertion (as discussed in p. 13). "Further enhancement" of the association of CCTalpha with nLD/LAPS (Abstract) cannot be concluded from this result.

*I agree and have modified that sentence and the concluding sentence in the Abstract to indicate that CCTα association reflects available nLD monolayer.*

2. In Yue et al, 2020, S319 in HeLa was shown to be dephosphorylated and distribute to the NE at 15-60 min of oleate treatment. In the present study, CCTalpha in Huh7 distributes to the NE at 15 min (less so at 30 min) (Fig. 4A), whereas S319 is not dephosphorylated at 3 hr (Fig. 4D) and whether S319 is dephosphorylated at 15 min not shown. If S319 in Huh7 is not dephosphorylated at 15 min, how is this result reconciled with that in HeLa? Conversely, if S319 is dephosphorylated at 15 min, how is the dephosphorylation (15 min)-rephosphorylation (3 hr) correlated with the PC synthesis?

*We now show in Figure 4B that CCTa-pS319 is dephosphorylated at 15 and 30 min but the Y359/S362 site is not, as is the case with outer cells we have tested. This suggests that in Huh7 cells acute treatment with oleate results in NE translocation and S319 dephosphorylation of CCTa but as the enzyme shifts to nLDs and LAPS during the 3-12 h period there is rephosphorylation.*

3. In relation to the above point, at 12 hr of oleate treatment, CCTalpha is translocated to nLDs, but S319 phosphorylation does not change (Fig. 4D), or even increases (Fig. 5C). Please discuss this result. Is PC synthesis activated at this point, or only later, when S319 is dephosphorylated?

*We have removed Figure 5C as it was redundant with Figure 4E and the sample load at 12 h time point is not consistent. As far as I'm aware, ours is the first report of CCTα phosphorylation status over the course of a 24 h oleate treatment. The consistent observation is that enzyme is extensively associated with nLDs and LAPS between 3-12 h while the phosphorylation status of S319 does not change. However, at no time do we see CCTa-pS319 on nLDs or LAPS. This suggests that between 3-12 h a pool of enzyme that is not phosphorylated on S319 associates with nLDs and LAPS. After treatment for >12 the pS319 pool starts to dephosphorylate and associate with nLDs and LAPS. This scenario is predicated on the assumption that there is heterogeneity in the phosphorylation of S319; it is not all or none. This is now discussed at the bottom page 14 and top page 15.*

*As in other cells, there is increased PC synthesis if the [$^3$H]choline and oleate are added together which reflects initial stimulation. The problem has been to get reliable shap-shots of PC synthesis over the 24 h time course to correlate with translocation and phosphorylation. Pulse-labelling with media changes gives an artificial stimulation PC synthesis while without media changes there is a variability, possibly linked to nutrient exhaustion (?). We are working on alternative approaches.*

4. The previous paper (Yue et al, 2020) showed that S319 is dephosphorylated, whereas Y359/S362 remains to be phosphorylated when CCTalpha is translocated to nLD in U2OS, whereas in the present study, CCTalpha translocation to nLDs/LAPS in oleate-treated cells is accompanied by the dephosphorylation at S319 and S359/Y362 (Fig. 4D; p. 7, last line-). It is confusing that this seemingly contradictory result is presented without discussion. Does the difference have any meaning regarding activation and distributional change of CCTalpha in the two cell types?

*To date, only in Huh7 cells do we observe dephosphorylation of Y359/S362 with long-term exposure to oleate (24 h). We are not sure of the cell-specific significance, but this point is now mentioned in the Discussion related to comment 3 (page 15).*

5. Because CCTalpha exists as a dimer, presence of wild-type CCTalpha may affect the behavior of mutants by forming hetero-dimers. How do the authors exclude this possibility (especially in Fig. 9)?

*In supplemental Figure 2A, we show immunoblots of U2OS cells that were mock transfected or expressing wild-type CCTa-V5 and probed with CCTa and V5 antibodies to illustrate the relative level of overexpression, which is about 6-8 fold. Thus, the location of transfected phospho-mutants would be minimally affected by dimerization with endogenous CCTa.*

6. To study whether CDK is involved in phosphorylation of CCTalpha, Huh7 cells were cultured with oleate for 30 min and then moved to an oleate-deficient medium containing CDK inhibitors. However, whether CCTalpha S319 is dephosphorylated at 30 min is not shown and its distribution in the NE appears to decrease compared to that at 15 min. 1) Please show whether S319 is dephosphorylated at 30 min. 2) The effect of CDK inhibitors on the CCTalpha distribution needs to be shown. 3) The effect of CDK inhibition needs to be studied in relation to nLD/LAPS, the main subject of this study.

*We now show in Figure 4B that CCTa is dephosphorylated at 15 and 30 min in Huh7 cells. After repeated attempts, we have been unable to show that CDK2 inhibitors effect CCTa localization to nLDs or LAPS. Part of the problem is that the inhibitors 1) start to have detrimental effects on cell growth with longer term 12-24 h incubations, and 2) global changes in phosphorylation status are required to observe an effect (based on the serine-to-alanine mutants in Figure 7). Because of this we have modified the final sentence in the Abstract to indicate that a role of CDKs in CCTa localization to nLDs is unresolved.*

7. LAPS, or lipid-associated PML structure, was defined as a subset of PML-NB, not as a subset of nLD. Throughout this paper, nLD and LAPS are treated in parallel, but this makes it difficult to understand sentences like "CCTa-positive LAPS and nLDs accounted for ~40% and 20% of

total, respectively, with ~30% devoid of both PML and CCTa (Fig. 1B)" (p. 6). Classification of nLDs into PML-associated and -non-associated appears more reasonable to me.

*Yes, LAPS are a subset of PML structures, but they also have an associated LD so are by definition a subset of nLDs that have a unique protein marker (ie. PML). There is a spectrum of nLDs that have PML, CCTa, Lipins etc. so it's difficult to come up with a term that defines the 'non-associated' pool. Reviewer 2 also had a issue with the sentence in question, which is now modified for clarity. We have included a sentence at the start of the Results section (bottom page 5) to define these terms; nLDs refers to the entire BODIPY-positive pool in the nucleus while LAPS are a subfraction of nLDs that have associated PML.*

**Reviewer #2:**

While the findings are interesting and experiments mostly performed with high rigor, there are several areas where additional experiments and explanation would help to support the major conclusions.

Comment 1: The suppressive effect of palmitate on oleate-induced CCTα targeting to nuclear lipid droplets is intriguing. However, the experiment is performed with less oleate in the palmitate co-treatment. If similar amounts of oleate are added does the additional of palmitate still have the suppressive effect? Is this due to the preferential incorporation of palmitate into phospholipids and a consequent reduction in packing defects on nuclear lipid droplets that are necessary for CCT recruitment?

*We initially wanted to maintain the total fatty acid concentration constant so reduced oleate to 0.3 mM, but I agree that for consistency should have used 0.4 mM. Experiments are now repeated at 0.4 mM oleate and 0.1 mM palmitate and the results quantified in Fig. 4E, F and G showing that addition of palmitate does not affect total nLDs but inhibited CCTα association by >50%.*

*Yes, would speculate that increased saturate-rich phospholipids in the LD monolayer prevents CCTα translocation because the total nLDs are unaffected (ie. not due to monolayer reduction).*

Comment 2: An effect of the phospho-memetic and phospho-null mutations on CCTα recruitment to nuclear lipid droplets is only observed when all 11 sites are mutated. This seems highly artificial. Would this ever happen in cells or is this an artificial situation that is not relevant to the actual regulation of the enzyme? Without evidence that this occurs in cells, the conclusions should be more conservative and speculative.

*Serine-to-alanine or -aspartate mutations are our best tool to sort out the functionality of sites. However, I would agree that a totally phosphorylated or dephosphorylated enzyme may not exist and the enzyme has a highly heterogenous phosphorylation state (as one might expect with 16 sites). Mutagenesis shows that large bulk changes in negative charge (phosphorylation) on CCTα are required to alter nLD localization.*

Comment 3: The primary evidence that CDK1 and CDK2 regulate the phosphorylation of CCTα in cells comes from experiments that use inhibitors of these kinases. How specific are these inhibitors? Often kinase inhibitors hit multiple kinases. Are there substrates of these

kinases that could be analyzed to support the specificity? Is it possible to use genetic approaches to disrupt these kinases to provide additional support*?*

*The CDK2 inhibitor binds the inactive state of the enzyme and prevents cyclin binding. We used it at concentrations (5-10 μM) reported in other studies. Thus, it is specific compared to active site CDK inhibitors (roscovitin) that are usually directed at the ATP binding site. The CDK1 inhibitor RO-3306 is an ATP active site inhibitor that is reportedly selective with a 10-fold-higher Ki for CDK2. Again, we used the inhibitor at concentration on cells reported in the literature (5-10 μm). Both of the inhibitors are reported to selectively prevent phosphorylation of downstream targets. In the Materials and Methods we have now included two references on the CDK1 and 2 inhibitors (top page 18). We have started to make shRNA knockdown cell lines for both kinases but there is issue with growth inhibition due to the role of CDKs in cell division.*

Comment 4: Figure 4 would benefit from quantitation, particularly in panel C. It is mentioned that "palmitate-treated cells contained fewer cLDs, nLDs and LAPS, and LAPS and nLDs were devoid of CCTa." The reduction in CCTa is clear, but the changes in the amount of cLDs, nLDs and LAPS, and LAPS is not as obvious.

*Data is now included from 3 separate experiments (Figure 4G) showing that palmitate does not elevate nLD much above the level of untreated cells (NA). The IF images in panel C purposely included some palmitate-treated cells with nLDs and LAPS to show the absence of CCTa.*

Comment 5: On page 9 it is mentioned that "In oleate-treated CHO-MT58 cells that do not form nLDs, the dephosphorylation of S319 was less pronounced (Fig. 6C)." Is the dephosphorylation of S319 less pronounced or just absent? It doesn't look like there is any dephosphorylation of S319 to me. In figure 5C, there seems to be a large increase in pS319 but this is not mentioned.

*Related to Figure 6C; yes, we should be more specific and note that in MT58 cell there is no dephosphorylation of S319 after 24 h in oleate (page 9). This contrasts Huh7 cells, where we show in Figure 5C and Figure 4D that the site is dephosphorylated at 24h. As mentioned in response to reviewer 1, we have removed Fig. 5C as it is redundant with the Fig. 4D. The key point in both those blots is the dephosphorylation of S319 and Y395/S362 that occurs by 24 h.*

Comment 6: I found this sentence on page 6 incredibly difficult to decipher. Perhaps it could be broken up into two sentences or additional clarification provided? "At 24 h, CCTa-positive LAPS and nLDs accounted for ~40% and 20% of total, respectively, with ~30% devoid of both PML and CCTa (Fig. 1B)."

*I agree and this was pointed out by reviewer 1. The sentence is now rewritten for clarity and includes a sentence to indicate how we define nLDs versus LAPS for quantification (page 5).*

Sincerely,
Neale D. Ridgway, PhD

RE: Manuscript #E23-09-0354R
TITLE: LIPID- AND PHOSPHO-REGULATION OF CTP:PHOSPHOCHOLINE CYTIDYLYLTRANSFERASE α ASSOCIATION WITH NUCLEAR LIPID DROPLETS

Dear Neale,

The review of your revised manuscript is now complete. As you will see the two reviewers differed in their opinion of the revised manuscript with one recommending acceptance and the other concerned that their concerns were not addressed. Having read the comments and considering your previous work in this area I am concerned that the new insights are fairly limited.

In view of these comments, and the differing opinions of the reviewers, I believe that the fairest way forward is to give you another chance to address these comments. I hope that this can be done by emphasising the new findings in the manuscript with respect to previous work rather than new experimentation. But I will leave that up to you. I hope that the manuscript can be revised accordingly and that re-review is not necessary.

I am sorry that I can't accept your manuscript for publication at this time but hope that the comments are useful. I will endeavour to provide a rapid final decision once you have revised the manuscript but I am also happy to discuss any revisions before the final submission.

Best regards,

Rob

Robert Parton
Monitoring Editor
Molecular Biology of the Cell

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Dear Dr. Ridgway,

The review of your manuscript, referenced above, is now complete. The Monitoring Editor has decided that your manuscript is not acceptable for publication at this time, but may be deemed acceptable after specific revisions are made, as described in the Monitoring Editor's decision letter above and the reviewer comments below.

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In preparing your revised manuscript, please follow the instruction in the Information for Authors (www.molbiolcell.org/info-for-authors). In particular, to prepare for the possible acceptance of your revised manuscript, submit final, publication-quality figures with your revision as described.

To submit the rebuttal letter, revised manuscript, and figures, use this link: Link Not Available

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Thank you for submitting your manuscript to MBoC. We look forward to receiving your revised paper.

Sincerely,

Eric Baker
Journal Production Manager
MBoC Editorial Office
mbc@ascb.org

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Reviewer #1 (Remarks to the Author):

The authors addressed some of my earlier comments by making corrections to the manuscript. However, they chose not to explore two crucial issues further: the potentiation of CCTα recruitment to nLDs and the impact of CDK inhibition on nLD distribution. While the remaining sections include some new findings, they are relatively minor. I am uncertain whether these additions meet the standards set by MBoC.

• What is shown in the first section is essentially an increase of nLD by oleate and lipin-1 overexpression, but this was already reported (Soltysik et al, 2019, 2021). The title "Lipin1 potentiates oleate-dependent CCTα recruitment to nLDs and LAPS" needs to be changed in accordance with the revision.

• The suppressive effect of palmitate shown in the second section, "nLDs and LAPS induced by palmitate do not recruit CCTα", is interesting, but it lacks further expansion and is only briefly discussed.

• The absence of the global phosphorylation-mimetic CCTα mutant in nLDs, shown in the third section "Global phosphorylation of the CCTα P-domain inhibits translocation to LAPS and nLDs", was reported in the authors' paper (Lee et al, 2020). The difference between U2OS/Caco2 and Huh7 on Y359/S362 phosphorylation/dephophorylation is potentially interesting, possibly reflecting different nLD formation mechanisms, but it was simply discussed as "cell-specific responses to long-term fatty acid exposure" (p. 15) and not explored further.

• The fourth section remains preliminary. It is unclear why the effect of CDK inhibitors on CCTα distribution cannot be tested. If detrimental effects of the inhibitor start to appear only after 12-24 h incubations (according to the rebuttal letter), the effect on the CCTα distribution should be able to be evaluated well before that time frame. Considering the different dephosphorylation/phosphorylation status of S319 for the NE and nLDs distribution in Huh7 cells (Fig. 4), it will be also interesting how CDK inhibition affects distribution of CCTα in the two locations. In its current form, what can be said about CDK is not much different from the paper in 1995 (ref. 31), and it is not clear whether CDK is related to recruitment of CCTα to nLD/LAPS, the main subject of this study.

Reviewer #2 (Remarks to the Author):

The authors have sufficiently responded to my concerns / comments.

Dear Dr. Parton,

Thank you for providing the reviewer comments on our resubmission and the opportunity to respond. I have provided a response to each comment, mostly to clarify how our study is an advancement in understanding the factors that regulate CCT$\alpha$ recruitment to nLDS and LAPS.

**• What is shown in the first section is essentially an increase of nLD by oleate and lipin-1 overexpression, but this was already reported (Soltysik et al, 2019, 2021). The title "Lipin1 potentiates oleate-dependent CCTα recruitment to nLDs and LAPS" needs to be changed in accordance with the revision.**

*The data in Figures 1-3 in this section is the first to show the temporal recruitment of CCT$\alpha$, PML and Lipin1 to nLDs in Huh7 cells, and that CCT$\alpha$ translocation to nLDs is coupled to Lipin1 activity that expands the nLD pool. We have renamed the section heading to reflect the finding that Lipin1 increases nLDs with associated CCTα.*

**• The suppressive effect of palmitate shown in the second section, "nLDs and LAPS induced by palmitate do not recruit CCTα", is interesting, but it lacks further expansion and is only briefly discussed.**

*The data in this section is the first to show that a saturated fatty acid does not promote nLD or LAPS formation and suppress oleate-dependent CCT$\alpha$ translocation without reducing the number of nLDs. Potential mechanisms are discussed on page 14.*

**• The absence of the global phosphorylation-mimetic CCTα mutant in nLDs, shown in the third section "Global phosphorylation of the CCTα P-domain inhibits translocation to LAPS and nLDs", was reported in the authors' paper (Lee et al, 2020). The difference between U2OS/Caco2 and Huh7 on Y359/S362 phosphorylation/dephophorylation is potentially interesting, possibly reflecting different nLD formation mechanisms, but it was simply discussed as "cell-specific responses to long-term fatty acid exposure" (p. 15) and not explored further.**

*Yes, we previously showed that mutating 16 serine residues to mimic the phosphorylated state blocked CCT$\alpha$ translocation to nLDs. However, this result did not exclude the possibility that individual serine phosphorylation sites were important. The only way to address this was to additively mutate sets of serine residues until we observed a response. The results indicate a mechanism involving a net charge threshold that might occur by sequential phosphorylation along the P-domain (at least in the context of S319 and immediately adjacent serine residues). In retrospect, 'global' phosphorylation is not the best term to describe the results presented here. Rather, a phosphorylation threshold of 11/16 serine residues (70%) must be achieved before enzyme localization is affected. This has been emphasized in the abstract and results section.*

**• The fourth section remains preliminary. It is unclear why the effect of CDK inhibitors on CCTα distribution cannot be tested. If detrimental effects of the inhibitor start to appear only after 12-24 h incubations (according to the rebuttal letter), the effect on the CCTα distribution should be able to be evaluated well before that time frame. Considering the different dephosphorylation/phosphorylation status of S319 for the NE and nLDs distribution in Huh7 cells (Fig. 4), it will be also interesting how CDK inhibition affects distribution of CCTα in the two locations. In its current form, what can be said about CDK is not much different from the paper in 1995 (ref. 31), and it is not clear whether CDK is related to recruitment of CCTα to nLD/LAPS, the main subject of this study.**

*Our results are an advancement by showing that inhibiting CDK1 or CDK2 prevents re-phosphorylation at S319 after oleate removal. The 1995 paper investigated in vitro phosphorylation of recombinant CCT$\alpha$ by cdc2 but made no attempt to validate the findings in cell models or identify the relevant phosphorylation sites, both of which we did.*

*As outlined in the previous response, we have been unsuccessful thus far in enhancing CCT$\alpha$ association with nLDs or the nuclear envelope by inhibiting CDK1 and/or CDK2. Besides a toxicity problem, inhibitors may not cause the level of global P domain dephosphorylation required to enhance CCT$\alpha$ association with nLDs (based on mutagenesis, effects are observed only after 11 serine residues are dephosphorylated). This could be related to redundancy within the large CDK family or involvement of other kinases. Our data here and a recent paper identifying Chk1 strongly implicates CDKs in CCT$\alpha$ phosphorylation. However, identifying their precise involvement may be difficult due to CDK redundancy and the subtle effects of phosphorylation on cell cycle and lipid regulation of CCT$\alpha$.*

We thank the reviewer for the effort taken to review our manuscript and hope these clarifications assuage any remaining concerns.

Sincerely,
Neale Ridgway, PhD

RE: Manuscript #E23-09-0354RR
TITLE: "LIPID- AND PHOSPHO-REGULATION OF CTP:PHOSPHOCHOLINE CYTIDYLYLTRANSFERASE α ASSOCIATION WITH NUCLEAR LIPID DROPLETS"

Dear Dr. Ridgway:

I am pleased to accept your manuscript for publication in Molecular Biology of the Cell.

Sincerely,
Robert Parton
Monitoring Editor
Molecular Biology of the Cell

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Dear Dr. Ridgway:

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