The LCLAT1/LYCAT acyltransferase is required for EGF-mediated phosphatidylinositol-3,4,5-trisphosphate generation and Akt signalling

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RE: Manuscript #E23-09-0361
TITLE: The LCLAT1/LYCAT acyltransferase is required for EGF-mediated phosphatidylinositol-3,4,5-trisphosphate and Akt signalling

Dear Dr. Botelho,

Thank you for submitting your manuscript "The LCLAT1/LYCAT acyltransferase is required for EGF-mediated phosphatidylinositol-3,4,5-trisphosphate and Akt signalling" for consideration for publication in the Molecular Biology of the Cell. I asked two colleagues who are experts in the field to review the paper and their verbatim comments are appended below. The referees find observations described in your manuscript to be novel and interesting. However, reviewer #2 raised a number of conceptual and technical concerns which undermine the strength of your conclusions. Although I cannot accept your manuscript for publication at this point, I believe that you will be able to address both referees' concerns, either by additional experimentation or revision of the text, and I look forward to receiving your revised manuscript. To expedite handling when you resubmit, please be sure to include a detailed response outlining how you have addressed each of the referees' concerns.

Sincerely,

Alexander Sorkin
Monitoring Editor
Molecular Biology of the Cell

Dear Dr. Botelho,

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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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

Reviewer #1 (Remarks to the Author):

This manuscript describes the role of the sn-1 acyltransferase LCLAT1 (a.k.a. LYCAT1) in PI 3-kinase signaling downstream of EGFRs. PI lipids are typically enriched in sn-1 stearate and sn-2 arachidonate and LCLAT1 is the enzyme that facilitates the former. After showing effective knockdown of the enzyme, the manuscript first demonstrates a lack of effect of this treatment on total or surface EGFR with and without stimulation in both ARPE-19 and MDA-MB-231 cells. Plasma membrane PI(4,5)P2 levels were shown to be decrease in the breast cancer cells, as already reported by this group for ARPE cells; moreover, EGF-stimulated PIP3 production is also markedly diminished in both cell lines. These observations are made both with genetically encoded lipid biosensors, as well as using mass spectrometry. Downstream of the lipid, Act activation along with effectors TSC2 ad GSK3beta are also inhibited when LCLAT1 is blocked. Altogether, the manuscript makes a compelling case for a requirement for stearoyl enrichment in sn-1 of PIs as being required for PI3K signaling.

Overall, the data are clear and compelling, and the manuscript is well presented and controlled. The reviewer saw no major technical flaws in the study. These are important findings that should be reported; given prior reports of effects on PI(4,5)P2, these data are novel but not necessarily that surprising. Indeed, in light of prior observations made by this group on requirements for LCLAT1, there are a number of points that I believe should be addressed in the manuscript regarding mechanism:

The discussion on p. 24 speculates on three mechanisms that may lead to diminished PI3K activity after LCLAT1 loss. The second posits that PI(4,5)P2 depletion may drive this. On that note, it is worth noting another recent article found that PIP3 synthesis in response to EGF in HEK293 cells scaled linearly with PI(4,5)P2 levels (doi: 10.1242/jcs.261494).

The third mechanism involves specific substrate pools, and perhaps local scaffolds. On this note, perhaps the enrichments of components of the PI3K signaling pathways in clathrin-coated structures in the plasma membrane, as described by the Antonescu group, could be a likely player? That would fit with the effects of depleted PI(4,5)P2, which also reduce the number of these structures.

Lastly, and related to the last point: in figure 1, rates of EGFR internalization are reported to be unaffected in these cells, but only after 1h of high dose (100 ng/ml) stimulation. This does not seem sufficient evidence to make the statement "EGFR was internalized at similar rates"; internalization with a much higher time resolution using more physiological ~ng/ml EGF concentrations would be required to confirm this statement. Therefore, either a more thorough determination of EGFR internalization is required, otherwise the authors should tone down the statement to sate that surface levels were not changed in unstimulated cells or cells exposed to a saturating stimulus for 1 hour. Given prior reports of effects of LCLAT1 on clathrin-mediated endocytosis, a complete lack of effect on EGFR internalization seems unlikely.

Minor points:

The first paragraph of the discussion states that the "enzyme is a target for drug targeting to treat cancer". I believe this statement should be toned down to a statement reflecting something more like "highlights a novel druggable pathway to modulate PI3K signaling". Pan-specific PI3K inhibitors have bombed in the clinic largely because of on-target adverse effects. Targeting a pathway that blocks all PI3Ks and several other PI-related pathways is not likely to be any better tolerated.

Figure 8e: the formatting of this panel appears screwed up in the exported pdf.

Reviewer #2 (Remarks to the Author):

In the manuscript, Chan and coworkers showed that LCLAT1 acyltransferase is responsible for the production of PI(3,4,5)P3 upon EGF stimulation. PI(3,4,5)P3 generation is increased upon EGF signaling, and the KD of LCLAT1 in HeLa and ARPE cells specifically affected this regulation. As a consequence, downstream AKT signaling is also impaired, as well as phospho-TSC2 and phospho-GSK3 signaling. However, the KD pf LCLAT1 seems to have pleiotropic effects, also independent on EGF, such as the impact on other PIP species and the effects on total MDM2 protein (both observed in basal conditions). The effects on proliferation and survival have not been linked to EGF signaling, and might well depend on these other functions.

The findings described here are potentially very interesting for the broad cell biology community, as they unveil a critical regulation of EGFR signaling by acyltransferase and lipid metabolism. However, some of the main conclusions are not fully supported by the data, and there are some conceptual and technical issues that need to be addressed.

Major Issues:

1) Fig. 1E. This assay is indirectly measuring cell surface levels of EGFR at a long time point (i.e., 1 h stimulation). This is not measuring EGFR internalization in any way. Authors should perform internalization assays with fluorescent EGF at early time points (i.e., 5-10 min), possibly comparing with internalization of Tf, which as this is a good positive control as they previously showed that it is affected by siLCLAT1.
2) Authors used different doses of EGF in different experiments (5 or 100 ng/ml). Critical experiments should be repeated at both doses (e.g., effects of siLCLAT1 on phospho-EGFR and downstream signaling)
3) Figure 3 is problematic as the IF pictures showed are of low quality, they lack some controls and do not allow to reach the conclusion. For instance:
- Fig 3A-B. How the authors recognize the membrane vs. cytosolic staining? They should perform a staining for a PM marker in parallel to PLCdelta, in order to quantify the signal at the membrane vs. the cytosolic one in the cytosol an automated way.
- Fig. 3C. Pictures are low quality. Authors should also analyze cells not stimulated with EGF, to check that, in this setting, EGF is indeed inducing an increase in the TIRF signal, as compared to basal condition.
- Fig. 3E. The siLCLAT1 cell shown is probably dying, as it is small and round, and the nucleus is not visible. If this is representative of the majority of the siLCLAT1 cells in the population, it would be very hard to draw any conclusion on the PM:cytosol ratio, as the cytosol is so constricted that the effects observed might be very indirect.
4) Figure 4. Authors should stress in the text and in the discussion that PI(3,4,5)P3 is the only one that is regulated by EGF in both cell lines. Thus, it would be important to show also the P-value of control/SS vs. +EGF condition and verify that this is significant.
5) Fig. 5. In parallel to AKT, authors should check whether the activation of other EGFR signaling branches like MAPK (phospho-Erk1/2) is affected by siCLAT1, in order to clarify whether the effects is specific for AKT or not.
6) Fig. 7 E-L. This figure raises a number of issues: i) MDM2 seems not be largely regulated by EGF; ii) on the contrary, the strong effects observed upon siCLAT1 are on the total protein level of MDM2, which is a strong regulator of p53 concentration in the cell. This data argues that siCLAT has a number of pleiotropic effects, well beyond the EGFR, as it impinges on a critical tumor suppressor pathway controlling proliferation and apoptosis (see also point 7). Have the authors confirmed this data with multiple oligos? Authors should also check p53 levels and its downstream target p21.
7) Fig. 8. The effects of siLCLAT1 on cell proliferation has not been directly linked to PI(3,4,5,)P3 metabolism nor to EGF signaling. It may well be, instead, that this phenotype is the consequences of MDM2 level downregulation. Experiments are not measuring effects on EGF-dependent proliferation. Authors should investigate cell proliferation stimulated by EGF, as compared to serum starved cells, in addition to serum/growing condition (note that in serum there are generally traces of EGF), to show if siLCLAT1 is also affecting the increase in proliferation specifically induced by EGF. Authors should also tone down their conclusions and discuss better the fact the effects on proliferation/survival might be due to multiple factors, beyond the EGFR-PIP-AKT axis. Please also change the Significance Statement accordngly, putting less emphasis on the impact of siLCLAT1 on cell proliferation, as being dependent on AKT and PIP metabolism.

Minor issues
1) Fig. 1 A, C. Since siCLAT1 affected cell growth authors should quantify total protein levels for WB. I don't see the point of just relying on the number of cells plated. In addition, authors should provide another loading control in addition to CHC, also considering the fact that CHC is also involved in cells division and its level may vary if proliferation is affected.
2) Quantitation of phospho-proteins in WBs in all figures need to be normalized first on the loading control on the same membrane and, then, on the corresponding total protein level.
3) Authors should provide details on EGF dose used and time of stimulations in all figure legends.
4) Fig. 2C Please provide lower exposures of the WB.
5) Please change the title adding the word "generation": The LCLAT1/LYCAT acyltransferase is required for EGF-1 mediated phosphatidylinositol-2 3,4,5-trisphosphate generation and Akt signalling

Response to Editor:

***Editor statement**: Thank you for submitting your manuscript "The LCLAT1/LYCAT acyltransferase is required for EGF-mediated phosphatidylinositol-3,4,5-trisphosphate and Akt signalling" for consideration for publication in the Molecular Biology of the Cell. I asked two colleagues who are experts in the field to review the paper and their verbatim comments are appended below. The referees find observations described in your manuscript to be novel and interesting. However, reviewer #2 raised a number of conceptual and technical concerns which undermine the strength of your conclusions. Although I cannot accept your manuscript for publication at this point, I believe that you will be able to address both referees' concerns, either by additional experimentation or revision of the text, and I look forward to receiving your revised manuscript. To expedite handling when you resubmit, please be sure to include a detailed response outlining how you have addressed each of the referees' concerns.*

> Response: Thank you for the positive outlook on our manuscript and for highlighting specific actions based on Reviewers' comments that would improve our work. Below, is an itemized response and changes that we implemented to improve our work. There have been some important changes to the manuscript as enumerated below and in the cover letter. We thank you and our peer-reviewers for their time.

Please note that to aid in review, we have highlighted in yellow changes to the text.

Responses to Reviewer 1

Reviewer 1 general statement: *"….Altogether, the manuscript makes a compelling case for a requirement for stearoyl enrichment in sn-1 of PIs as being required for PI3K signaling….. Overall, the data are clear and compelling, and the manuscript is well presented and controlled. The reviewer saw no major technical flaws in the study. These are important findings that should be reported; given prior reports of effects on PI(4,5)P2, these data are novel but not necessarily that surprising. Indeed, in light of prior observations made by this group on requirements for LCLAT1, there are a number of points that I believe should be addressed in the manuscript regarding mechanism: "*

> Response: We thank Reviewer 1 for the positive outlook on our work and for the important suggestions.

Reviewer 1 Specific Comments:

1. *The discussion on p. 24 speculates on three mechanisms that may lead to diminished PI3K activity after LCLAT1 loss. The second posits that PI(4,5)P2 depletion may drive this. On that note, it is worth noting another recent article found that PIP3 synthesis in response to EGF in HEK293 cells scaled linearly with PI(4,5)P2 levels (doi: 10.1242/jcs.261494).*

Response: Thank you for the suggestion. We have made this point, starting on line 496 and cited Wills et al. JCS. 2023.

2. *The third mechanism involves specific substrate pools, and perhaps local scaffolds. On this note, perhaps the enrichments of components of the PI3K signaling pathways in clathrin-coated structures in the plasma membrane, as described by the Antonescu group, could be a likely player? That would fit with the effects of depleted PI(4,5)P2, which also reduce the number of these structures.*

   Response: Again, thank you for the suggestion. Starting on line 500, we wrote that lower PtdIns(4,5)P2 may disturb clathrin scaffolds, which in turn promotes EGF-mediated PI3K signaling.

3. *Lastly, and related to the last point: in figure 1, rates of EGFR internalization are reported to be unaffected in these cells, but only after 1h of high dose (100 ng/ml) stimulation. This does not seem sufficient evidence to make the statement "EGFR was internalized at similar rates"; internalization with a much higher time resolution using more physiological ~ng/ml EGF concentrations would be required to confirm this statement. Therefore, either a more thorough determination of EGFR internalization is required, otherwise the authors should tone down the statement to state that surface levels were not changed in unstimulated cells or cells exposed to a saturating stimulus for 1 hour. Given prior reports of effects of LCLAT1 on clathrin-mediated endocytosis, a complete lack of effect on EGFR internalization seems unlikely.*

   Response: We agree with this criticism and edited our statement to tone down our conclusions; we now state that the surface EGFR levels after 1 h of EGF stimulation is similar between control and LCLAT1-silenced cells (line 325, Fig. 1E, 2E). We refrain from discussing rates of internalization, which would require time-resolved endocytic assays as the reviewer indicates.

Reviewer 1 minor points:

4. *The first paragraph of the discussion states that the "enzyme is a target for drug targeting to treat cancer". I believe this statement should be toned down to a statement reflecting something more like "highlights a novel druggable pathway to modulate PI3K signaling". Pan-specific PI3K inhibitors have bombed in the clinic largely because of on-target adverse effects. Targeting a pathway that blocks all PI3Ks and several other PI-related pathways is not likely to be any better tolerated.*

   Response: As written on line 469, we edited the sentence as suggested.

5. *Figure 8e: the formatting of this panel appears screwed up in the exported pdf.*

   Response: Thanks for catching this glitch. We fixed Fig. 8e.

Response to Reviewer 2

Reviewer 2 general statement: *"In the manuscript, Chan and coworkers showed that LCLAT1 acyltransferase is responsible for the production of PI(3,4,5)P3 upon EGF stimulation. PI(3,4,5)P3 generation is increased upon EGF signaling, and the KD of LCLAT1 in HeLa (sic; we used MDA-MB-231 cells, not HeLa) and ARPE cells specifically affected this regulation. As a consequence, downstream AKT signaling is also impaired, as well as phospho-TSC2 and phospho-GSK3 signaling. However, the KD pf LCLAT1 seems to have pleiotropic effects, also independent on EGF, such as the impact on other PIP species and the effects on total MDM2 protein (both observed in basal conditions). The effects on proliferation and survival have not been linked to EGF signaling, and might well depend on these other functions. The findings described here are potentially very interesting for the broad cell biology community, as they unveil a critical regulation of EGFR signaling by acyltransferase and lipid metabolism. However, some of the main conclusions are not fully supported by the data, and there are some conceptual and technical issues that need to be addressed.."*

Response: Thank you for both the positive comments and for raising issues related to our interpretation. We have addressed individual points below. We also acknowledge here that the effects on Mdm2 and cell proliferation that we observed initially with siLCLAT1-1 oligonucleotide was not recapitulated by other oligonucleotides against LCLAT1. This may be due to kinetic differences in silencing or a limited off-target effect by siLCLAT1-1. We have thus removed reference to cell proliferation (removed Figure 8) and explain the issue with Mdm2 in new Supplemental Figure S4.

Reviewer 2 Major Issues:

1. *Fig. 1E. This assay is indirectly measuring cell surface levels of EGFR at a long time point (i.e., 1 h stimulation). This is not measuring EGFR internalization in any way. Authors should perform internalization assays with fluorescent EGF at early time points (i.e., 5-10 min), possibly comparing with internalization of Tf, which as this is a good positive control as they previously showed that it is affected by siLCLAT1.*

   Response: This issue was also raised by Reviewer 1 and both reviewers are correct in that we did not measure rates of receptor internalization. We simply measured a steady state level of surface EGFR before and after 1 h of stimulation with 100 ng/mL EGF and compared this between control and LCLAT1-silenced cells. Starting in line 325, we modified our language to accurately reflect this.

2. *Authors used different doses of EGF in different experiments (5 or 100 ng/ml). Critical experiments should be repeated at both doses (e.g., effects of siLCLAT1 on phospho-EGFR and downstream signaling)*

   Response: We respectfully disagree that this is necessary. Most of our experiments were done at 5 ng/mL EGF, which is a physiological concentration. We also use 20 ng/mL during imaging experiments to facilitate changes during imaging. We used supra-physiological 100 ng/mL EGF because this was reported to induce strong

internalization and degradation of EGFR and to see if LCLAT1 silencing disturbed this phenomenon (Fig. 1E, 2E) – in other words, this served as a model for internalization. Given this, we do not believe that repeating other experiments with 100 ng/mL warrants the time and expense needed to do this.

3. Figure 3 is problematic as the IF pictures showed are of low quality, they lack some controls and do not allow to reach the conclusion. For instance:

3i: Fig 3A-B. How the authors recognize the membrane vs. cytosolic staining? They should perform a staining for a PM marker in parallel to PLCdelta, in order to quantify the signal at the membrane vs. the cytosolic one in the cytosol an automated way.

Response: Thank you for the suggestion. MDA-MB-231 cells stably expressing eGFP-PLCδ-PH to detect $PI(4,5)P_2$ were transfected with non-targeting siRNA or siLCLAT1 oligonucleotides. These cells were then incubated with FM4-64FX to label the plasma membrane and imaged for 10 min to minimize endocytosis of FM4-64FX. We then used FM4-64FX label to define the plasma membrane and cytosol, which was then used to measure eGFP-PLCδ-PH fluorescence on the plasma membrane and cytosol, followed a ratio calculation. This approach is described in Lines 236 and 266. The results as described in lines 337 and the new Figure 3A, B. As before, we observed a significant drop of eGFP-PLCδ-PH in the plasma membrane in LCLAT1-silenced cells relative to non-targeting cells, suggesting that LCLAT1 expression is required to maintain PI(4,5)P2 levels.

3ii: Fig. 3C. Pictures are low quality. Authors should also analyze cells not stimulated with EGF, to check that, in this setting, EGF is indeed inducing an increase in the TIRF signal, as compared to basal condition.

Response: We are not sure why the images in Figure 3C appeared low quality to the reviewer. They seemed fine on our end. Nevertheless, we repeated the experiments with ARPE-19 cells expressing Akt-PH-GFP to compare serum starved and EGF-stimulated cells using TIRF-M to epifluorescence microscopy ratio. This is described starting on line 350 and in the revised Fig. 3C, D. Compared to serum-starved cells, EGF causes an increase in Akt-PH-GFP on the TIRF field in control-silenced cells. However, this is abated in LCLAT1-silenced cells.

3iii: Fig. 3E. The siLCLAT1 cell shown is probably dying, as it is small and round, and the nucleus is not visible. If this is representative of the majority of the siLCLAT1 cells in the population, it would be very hard to draw any conclusion on the PM:cytosol ratio, as the cytosol is so constricted that the effects observed might be very indirect.

Response: As shown in other images (eg. Fig. 3A, C), LCLAT1-silenced cells do not typically undergo apoptosis within two days of LCLAT1 silencing. To avoid

confusion, we replaced the original image in Fig. 3E with an image that better reflects the morphology of MDA-MB-231 cells silenced for LCLAT1.

4. Figure 4. Authors should stress in the text and in the discussion that PI(3,4,5)P3 is the only one that is regulated by EGF in both cell lines. Thus, it would be important to show also the P-value of control/SS vs. +EGF condition and verify that this is significant.

Response: Our original statistical analysis was not set up as a repeated measures two-way ANOVA, which should have been since our data is based on independent experiments. We corrected the statistical analyses (along with other figures that used two-way ANOVA and we now show p values for all comparisons in all figures).

Our previous and current analysis had shown that LCLAT1-silenced ARPE-19 cells were different from control cells in their ratio of 38:4 to 36:x for PI and *bis*-PtdInsPs (Fig. 4A, 4C, Sup. Fig. S3B).

However, in our previous analysis we had indicated that MDA-MB-231 cells were resilient to acyl profile changes in LCLAT1-silenced cells and only PI(3,4,5)P3 shows a difference. We have now revised this. MDA-MB-231 cells silenced for LCLAT1 have altered 38:4-PI to 36:x-PI ratio (Fig. 4E), although other PIPs in most circumstances are not altered (with the exception of resting 38:4-mono-PIP to 36:x-mono-PIP (Sup. Fig. S3C). These comparisons are elaborated in results lines 384-387 and lines 395-404. We apologize for the error.

5. Fig. 5. In parallel to AKT, authors should check whether the activation of other EGFR signaling branches like MAPK (phospho-Erk1/2) is affected by siCLAT1, in order to clarify whether the effects is specific for AKT or not.

Response: We probed for phosphorylation of Erk1/2 in LCLAT1-silenced ARPE-19 and MDA-MB-231 cells finding a cell-type effect. For ARPE-19 cells, we do not readily observe a change in phosphorylated ERK1 using two oligonucleotides against LCLAT1 (Fig. 5G, 5H, lines 430-436). For MDA-MB-231 cells, however, we observed a drop in phospho-ERK by EGF after silencing LCLAT1 with two oligonucleotides (Fig. 6G, 6H, lines 430-436). We also asked if LCLAT1-silencing disrupted insulin-Akt signaling as indication of general applicability of our observations. As shown in Fig. 6G, 6H and indicated in lines 426-430, it seems that LCLAT1 silencing also inhibits insulin signaling.

6. Fig. 7 E-L. This figure raises a number of issues: i) MDM2 seems not be largely regulated by EGF; ii) on the contrary, the strong effects observed upon siCLAT1 are on the total protein level of MDM2, which is a strong regulator of p53 concentration in the cell. This data argues that siCLAT has a number of pleiotropic effects, well beyond the EGFR, as it impinges on a critical tumor suppressor pathway controlling proliferation and apoptosis (see also point 7). Have the authors confirmed this data with multiple oligos? Authors should also check p53 levels and its downstream target p21.

Response: The Reviewer raised an important point. When we tested if other siRNA sequences against LCLAT1 caused a drop in Mdm2 protein levels, we observed an inconsistency between these oligonucleotides and siLCLAT1-1 sequence, despite similar suppression of LCLAT1 and effects on phospho-Akt. There may be several explanations for this, including the kinetics of LCLAT1 silencing, a sharp threshold in silencing, or a limited off-target by siLCLAT1-1 sequence that affects Mdm2. This is shown in Sup. Fig. S4 for both ARPE-19 and MDA-MB-231 cells

We also tested the effects of these oligonucleotides on p53 and p21 protein levels. p53 protein levels remained similar across all treatments in both ARPE-19 and MDA-MB-231 cells. Similarly, p21 protein levels did not appreciably change in MDA-MB-231 cells treated with all three oligonucleotides. However, siLCLAT1-1 and siLCLAT1-5 oligonucleotides were inconsistent for their effect on p21 protein levels in ARPE-19 cells (Sup. Fig. S4C). This is all described starting in lines 450 and in the discussion, starting in line 559. We opted to show this in the manuscript for full-disclosure and advise not using siLCLAT1-1 in future work. However, we are confident that our observations on PI3K-Akt signaling hold because the effects on Akt were observed with up to four different oligonucleotides against LCLAT1 (Sup. Fig. S1, Sup. Fig. S2, Fig. 5, Fig. 6) and in two cell lines.

Nevertheless, given the inconsistency between silencing oligonucleotide sequences against LCLAT1 on Mdm2 and p21 protein levels, we opted to remove our observations on cell survival and proliferation altogether. We also opted to remove our data using inducible CRISPR-Cas9 deletion of LCLAT1 to be prudent.

7. *Fig. 8. The effects of siLCLAT1 on cell proliferation has not been directly linked to PI(3,4,5,)P3 metabolism nor to EGF signaling. It may well be, instead, that this phenotype is the consequences of MDM2 level downregulation. Experiments are not measuring effects on EGF-dependent proliferation. Authors should investigate cell proliferation stimulated by EGF, as compared to serum starved cells, in addition to serum/growing condition (note that in serum there are generally traces of EGF), to show if siLCLAT1 is also affecting the increase in proliferation specifically induced by EGF. Authors should also tone down their conclusions and discuss better the fact the effects on proliferation/survival might be due to multiple factors, beyond the EGFR-PIP-AKT axis. Please also change the Significance Statement accordngly, putting less emphasis on the impact of siLCLAT1 on cell proliferation, as being dependent on AKT and PIP metabolism.*

Response: We opted to remove these data given point 6 above.

8. The Statement of Significance is edited to reflect these changes.

Reviewer 2 minor issues:

9. Fig. 1 A, C. Since siCLAT1 affected cell growth authors should quantify total protein levels for WB. I don't see the point of just relying on the number of cells plated. In addition, authors should provide another loading control in addition to CHC, also considering the fact that CHC is also involved in cells division and its level may vary if proliferation is affected.

Response: We now routinely load equal proteins for these experiments instead of basing our measurements on the seeding cell number. We replaced Fig. 1A and Fig. 2A as examples. We also ran loading controls using CHC, cofilin, and vinculin. While we only show the quantification for CHC (Fig. 1B, 2B), we did not observe a difference with the other loading controls. Thus, we did not change 1C as suggested.

10. Quantitation of phospho-proteins in WBs in all figures need to be normalized first on the loading control on the same membrane and, then, on the corresponding total protein level.

Response: Our wording was not clear. This is exactly what we did. We have clarified this in the methods, lines 209-212 in the "Western blot analysis" section of the Methods.

11. Authors should provide details on EGF dose used and time of stimulations in all figure legends.

Response: We have now done this.

12. *Fig. 2C Please provide lower exposures of the WB.*

Response: These blots appear dark, but are not saturated.

13. Please change the title adding the word "generation": The LCLAT1/LYCAT acyltransferase is required for EGF-1 mediated phosphatidylinositol-2 3,4,5-trisphosphate generation and Akt signalling

Response: We added "generation" to the title.

RE: Manuscript #E23-09-0361R
TITLE: "The LCLAT1/LYCAT acyltransferase is required for EGF-mediated phosphatidylinositol-3,4,5-trisphosphate generation and Akt signalling"

Dear Dr. Botelho,

Thank you for submitting your revised manuscript, "The LCLAT1/LYCAT 1 Acyltransferase is Required for EGF-Mediated Phosphatidylinositol-3,4,5-Trisphosphate Generation and Akt Signalling." I appreciate that you addressed many of the reviewers' concerns by performing new experiments, providing additional clarifications and discussions, or omitting data that are difficult to interpret and peripheral to the main message of the manuscript. The use of multiple siRNAs and the acknowledgment of possible off-target effects as a caveat of RNA interference are commendable. I think the revised version of the manuscript is streamlined and better focused on key findings. I am willing to accept the manuscript after a minor revision. Specifically, the effect of LCLAT1 depletion on ERK activation in MDA-MA-231 cells but not in ARPE-19 cells might puzzle readers. It is important to discuss potential underlying mechanisms here. Could the difference be due to the expression of mutant K-Ras or other oncogenic mutations driving the ERK pathway in MDA-MA-231 cells, or due to more EGFRs in these cells compared to ARPE-19 cells? Alternatively, could it be due to different efficiencies of siRNA transfection and an off-target effect?

I look forward to seeing your revised manuscript in the near future.

Alexander Sorkin
Monitoring Editor
Molecular Biology of the Cell

Dear Dr. Botelho,

The review of your manuscript, referenced above, is now complete. The Monitoring Editor has decided that your manuscript requires minor revisions before it can be published in Molecular Biology of the Cell, as described in the Monitoring Editor's decision letter above and the reviewer comments (if any) 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 15 days to submit a revision. If this time period is inadequate, please contact us immediately at mboc@ascb.org.

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To submit the rebuttal letter, revised version, and figures, please use this link (please enable cookies, or cut and paste URL):
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Thank you for submitting your manuscript to MBoC. Please do not hesitate to contact this office if you have any questions.

Sincerely,

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

Response to Editor:

***Editor statement**: Thank you for submitting your revised manuscript, "The LCLAT1/LYCAT 1 Acyltransferase is Required for EGF-Mediated Phosphatidylinositol-3,4,5-Trisphosphate Generation and Akt Signalling." I appreciate that you addressed many of the reviewers' concerns by performing new experiments, providing additional clarifications and discussions, or omitting data that are difficult to interpret and peripheral to the main message of the manuscript. The use of multiple siRNAs and the acknowledgment of possible off-target effects as a caveat of RNA interference are commendable. I think the revised version of the manuscript is streamlined and better focused on key findings. I am willing to accept the manuscript after a minor revision. Specifically, the effect of LCLAT1 depletion on ERK activation in MDA-MA-231 cells but not in ARPE-19 cells might puzzle readers. It is important to discuss potential underlying mechanisms here. Could the difference be due to the expression of mutant K-Ras or other oncogenic mutations driving the ERK pathway in MDA-MA-231 cells, or due to more EGFRs in these cells compared to ARPE-19 cells? Alternatively, could it be due to different efficiencies of siRNA transfection and an off-target effect?*

Response: Thank you for the rapid and balanced assessment of the revised manuscript. We appreciate the feedback and suggestion to discuss possible reasons why LCLAT1-silencing might prevent ERK1/2 activation in ARPE-19 cells, but not in MDA-MB-231 cells.

Indeed, MDA-MB-231 cells carry mutations in KRAS and BRAF genes, which express K-Ras GTPase and B-Raf1 that regulate ERK1/2. So, it is thus quite possible that these mutations bypass the need for LCLAT1 in MDA-MB-231 cells. We discuss this starting in line 543 of the newly revised manuscript and cite Patra et al., 2017 and Wagner et al 2022, which speak to these mutations. We note other possibilities including differences in EGFR expression and silencing efficiency. We do not think that this is likely due to off-target effects given that these observations were seen using two oligonucleotides against LCLAT1.

We think this readily addresses the concern raised by you and should help readers understand how LCLAT1 silencing may impact the ERK1/2 pathway differently between cell types.

RE: Manuscript #E23-09-0361RR
TITLE: "The LCLAT1/LYCAT acyltransferase is required for EGF-mediated phosphatidylinositol-3,4,5-trisphosphate generation and Akt signalling"

Dear Dr. Botelho:

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

Sincerely,
Alexander Sorkin
Monitoring Editor
Molecular Biology of the Cell

Dear Dr. Botelho:

Congratulations on the acceptance of your manuscript! Thank you for publishing your work in Molecular Biology of the Cell (MBoC).

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