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Characterization of lipoprotein lipase storage vesicles in 3T3-L1 adipocytes

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MS TITLE: Characterization of Lipoprotein Lipase Storage Vesicles in 3T3-L1 Adipocytes

AUTHORS: Benjamin S Roberts, Chelsea Q Yang, and Saskia Neher

ARTICLE TYPE: Research Article

We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

As you will see, the reviewers raise a number of criticisms that prevent me from accepting the paper at this stage. They suggest, however, that a revised version might prove acceptable, if you can address their concerns. A concern that was echoed by both reviewers was that some of the data is overinterpreted and conclusions are overstated. My feeling is that addressing the reviewers' comments will strengthen the manuscript. If you think that you can deal satisfactorily with the criticisms on revision, I would be pleased to see a revised manuscript. We would then return it to the reviewers.

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

Please ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion.

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. Please attend to

all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

In this manuscript from Roberts et al, the authors describe their work characterizing the insulin-stimulated secretion of LPL from adipocytes. They find that LPL secretion requires trafficking steps that are different from that of GLUT4 and partially different from those taken by leptin. They find that trafficking is regulated by calcium, but Golgi protein kinase D, and ARF1. These findings provide new insight into LPL secretion and suggest the possibility of a novel secretion pathway.

Comments for the author

Overall, the studies in this manuscript are well done and the observations are interesting and provide new insight into LPL secretion. Nonetheless, there are several minor issues that should be addressed as outlined below:
- It is not clear what the authors regard as “colocalizing”. In Figure 1 they say that LPL does not colocalize with GLUT4, but does colocalize with LAMP1, RAB4, and RAB10 (line 22), even though these also have an overlap coefficient of less than 0.5. What cutoff are they using?

-How western blots were quantified or normalized is not described anywhere in the test. It looks like normalized such that all the control points were set to one (instead of just setting the average of the control points to one). If this is the case, a t-test comparing the control to other samples is not valid because the variation in the control has been eliminated.

-The authors state that they see an increase in LPL cleavage with Ionomycin treatment (Fig. 5A), but the western blot does not support that statement. If anything the ratio of cleaved to full-length LPL seems to go down with treatment.

-The authors state that BFA entirely blocks LPL secretion, but only partially blocks adiponectin secretion. It is hard to judge if this is really true. From the graphs it looks like BFA blocks LPL by 90% and adiponectin by 80%. Not sure you can say this is really different especially if these data are from different experiments. Do the authors have any other evidence that LPL is not secreted by the same pathway as adiponectin?

-The authors show that insulin regulates biosynthesis of a protein required for IS LPL secretion. Did they test any candidates? Does insulin increase biosynthesis of SDC-1?

- Insulin-stimulated secretion of LPL is quite slow. Can the authors discuss the physiologic purpose of slow IS LPL secretion would be?

- Many of the micrographs have white arrowhead in the zoomed in boxes. These arrowheads presumably show colocalization but are not mentioned in the figure legend.

-The evidence that leptin and LPL use different trafficking pathways is not all that strong. Although the authors cite a study where ionomycin does not induce leptin secretion, they do not use leptin as a control for their own ionomycin experiments.

-Figure 8 is more speculative that a summary of the results in the manuscript. The authors don’t actually show that insulin signaling recruits additional ARF1 and PRKD2to these sites and induce scission of LPL containing vesicles. It’s fine to show such a model but it should be more clear that this is a hypothetical model rather than a model of established findings.

Reviewer 2

Advance summary and potential significance to field

In this paper Roberts and colleagues set out to characterize the trafficking pathways followed by Lipoprotein Lipase (LPL) in adypocytes. The authors investigate insulin-dependent localization of LPL compared to the better studied leptin and GLUT4. Interestingly, they find out that LPL secretion is slowly responding to insulin but rapidly responded to the calcium ionophore Ionomicyn. The authors propose a model in which trafficking of LPL is dependent on the GTPase ARF1 and PKD localized to caveolar membrane domains and to have characterized a new trafficking pathway in adipocytes. While some of the data is intriguing and the topic is of great physiological interest, I find that the author overinterpret their results and do not provide sufficient experimental evidence to support their claims. In my opinion the manuscript cannot be published as it is and would require substantial re-writing and definitely some more experimental validation to support the proposed conclusions. Major re-writing of the manuscript is necessary for all chapters, titles and figure legends to tone down the conclusions and harmonize with their results.

Comments for the author

1) In figure 1 the authors test the localization of LPL with various organelles and a GLUT4 marker. The localization of LPL to caveolin-positive structures is interesting. Caveolin has been shown to label early endosomal structures (markers EEA1, Rab5) which have been shown to have a role in fast recycling to the PM however EE markers were not tested. Little co-localisation is observed with the Golgi marker Syntaxin 6 again shedding doubts on the validity of the model.

2) A major concern here is that the authors make claims about the trafficking routes of LPL that cannot simply be made by analyzing the steady state localization of LPL in cell. The authors should tone down their claims. If they want to extract information about trafficking the authors should perform live cell imaging experiments possibly with a controlled release system like the RUSH system (doi: 10.1038/nmeth.1928)

3) The authors should explain more clearly about transcriptional/translational regulation of LPL trafficking. Could they clearly explain what is known from the literature?

4) From the data in figure 7 the authors conclude trafficking of LPL is ARF1 dependent as secretion is blocked by BFA. BFA blocks secretion of every secretory cargoes and it is not very specific for ARF1 dependent cargoes.

5) The authors see that a dominant negative ARF1 mutant only blocks insulin stimulated secretion of LPL. However, a different interpretation is that ARF1 T31N is less efficient at completely abolishing secretion as there's endogenous WT ARF1 in the background. A cargo to show the differential effect on LPL should be used here.

6) Additionally, while the localization of ARF1 in structures positive for caveolin is intriguing there doesn't seem to be any changes in the localization of LPL in caveolin positive structures in the presence of ARF1 T31N.

7) The authors state "Together, these data illustrate a model wherein insulin recruits an ARF1 WT-PRKD2 complex to the Golgi membrane rapidly and dynamically.

In cells expressing ARF1 T31N, insulin is unable to catalyse GDP to GTP exchange in ARF1 T31N, causing ARF1 T31N to sequester PRKD2 and other trafficking machinery in the cytoplasm, inhibiting LPL secretion". However, no data is shown that shows

- Insulin-dependent ARF1 recruitment to the Golgi
- Insulin-dependent GDP-GTP exchange on ARF1
- Any interaction between the above components under IS.

8) LPL localizes to structures positive for caveolin but the authors propose a model in which LPL sorting occurs at the level of caveolin and ARF1 structures at the TGN. The authors provide no proof of localization of LPL to the TGN and should therefore revise their model based on the data they present. Or provide more experimental data in support.

First revision

Author response to reviewers' comments

Thank you for considering this manuscript for publication. We appreciate your reviews and comments and we have made appropriate modifications and additions to the manuscript. Please consider our comments and additions below. All changes have been highlighted in red throughout the manuscript.

Reviewer 1:

1. It is not clear what the authors regard as "colocalizing". In Figure 1 they say that LPL does not colocalize with GLUT4, but does colocalize with LAMP1, RAB4, and RAB10 (line 22), even though these also have an overlap coefficient of less than 0.5. What cutoff are they using?
   We have amended the description of our colocalization results to refer to the numerical Mander's overlap coefficients for each sample and we have removed unclear, overly- interpretive language. We have also included more detailed methods on our colocalization analysis methods. We have removed the 0.5 line to clarify our results in figure 1Q.

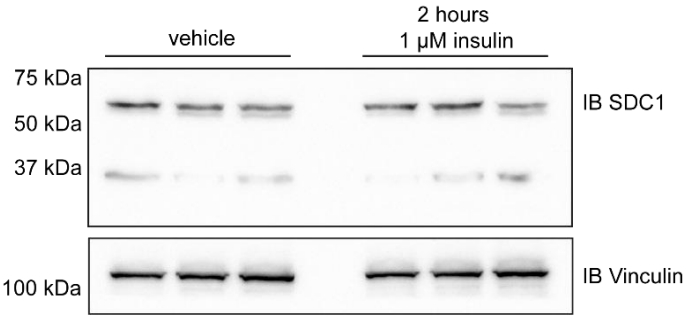
2. How western blots were quantified or normalized is not described anywhere in the test. It looks like normalized such that all the control points were set to one (instead of just setting the average of the control points to one). If this is the case, a t-test comparing the control to other samples is not valid because the variation in the control has been eliminated.
   We have clarified how we quantified Western blots in our methods section and in figure legends and we have re-analyzed all quantified Western blots to account for variations in background.
   We scan each blot at many different exposures to ensure that we are working in a linear range (as per journal standards) and therefore don't scan each blot at the same gain. We also do not include an absolute reference standard (of purified protein, for example). For these reasons, we cannot average the control between different blots. To the best of our knowledge, this is not a standard procedure. However, we believe that our general analysis method is sound. Our Western blots are thus quantified relative to a reference sample. Many other publications in the Journal of Cell Science report Western blot quantification in this way (PMID: 29661845, PMID: 29361522, PMID: 24105262).
   In addition, we have consulted with a biostatistician and we have provided updated statistics and additional methods on the tests we used throughout the manuscript.

3. The authors state that they see an increase in LPL cleavage with Ionomycin treatment (Fig. 5A), but the western blot does not support that statement. If anything the ratio of cleaved to full- length LPL seems to go down with treatment.
   We have amended the text to address this concern.

4. The authors state that BFA entirely blocks LPL secretion, but only partially blocks adiponectin secretion. It is hard to judge if this is really true. From the graphs it looks like BFA blocks LPL by 90% and adiponectin by 80%. Not sure you can say this is really different especially if these data are from different experiments. Do the authors have any other evidence that LPL is not secreted by the same pathway as adiponectin?
   We have provided statistical values (i.e. percent change in secretion) for these data in the respective figure legends (Figure 6A, Figure S5). There is a considerable difference in the secretion of adiponectin and LPL following BFA treatment in these datasets.

5. The authors show that insulin regulates biosynthesis of a protein required for IS LPL secretion. Did they test any candidates? Does insulin increase biosynthesis of SDC-1?
   We feel that searching for a partner protein where biosynthesis is regulated by insulin is outside of the scope of this study. However, we have analyzed the effect of insulin

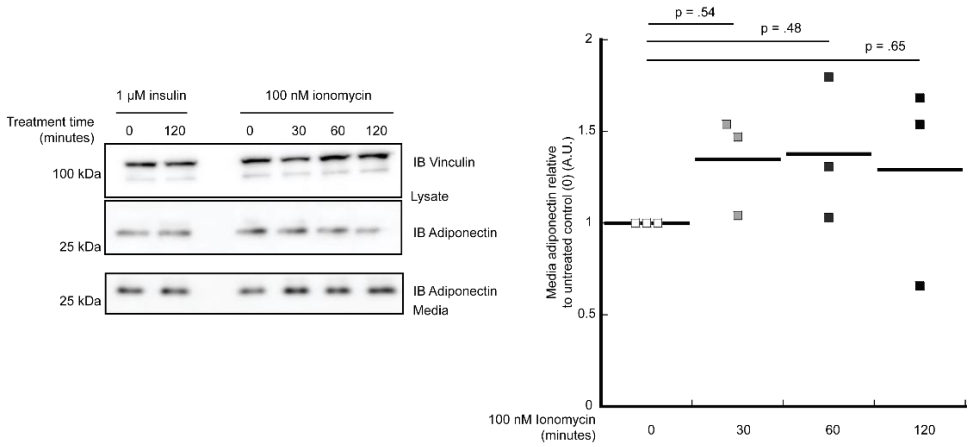
treatment on SDC1 protein levels by Western blot and we have presented these differences in a new supplemental figure (below). We did not find that SDC1 levels are significantly different in unstimulated (vehicle) and IS (2 hours) cells.



6. Insulin-stimulated secretion of LPL is quite slow. Can the authors discuss the physiologic purpose of slow IS LPL secretion would be?
We have included text and references in the discussion addressing this result.

7. Many of the micrographs have white arrowhead in the zoomed in boxes. These arrowheads presumably show colocalization but are not mentioned in the figure legend.
The arrowheads indicate colocalizing puncta in the zoom box. We have clarified these arrowheads in figure legends.

8. The evidence that leptin and LPL use different trafficking pathways is not all that strong. Although the authors cite a study where ionomycin does not induce leptin secretion, they do not use leptin as a control for their own ionomycin experiments.
Unfortunately, we were unable to detect leptin in media by Western blot. Instead, we have analyzed the secretion of adiponectin in ionomycin treated samples. We have included these results in a supplemental figure (below). We did not find that ionomycin significantly increased adiponectin secretion in these cells. Thus, LPL appears to be regulated in a highly specific trafficking pathway.



9. Figure 8 is more speculative that a summary of the results in the manuscript. The authors don't actually show that insulin signaling recruits additional ARF1 and PRKD2to these sites and induce scission of LPL containing vesicles. It's fine to show such a model but it should be more clear that this is a hypothetical model rather than a model of established findings.
We have modified this figure to focus on the experiments directly presented in this study, and we have adjusted the figure legend and heading to express that this figure shows a "hypothetical model for insulin stimulated LPL secretion." (See last figure below).

Reviewer 2

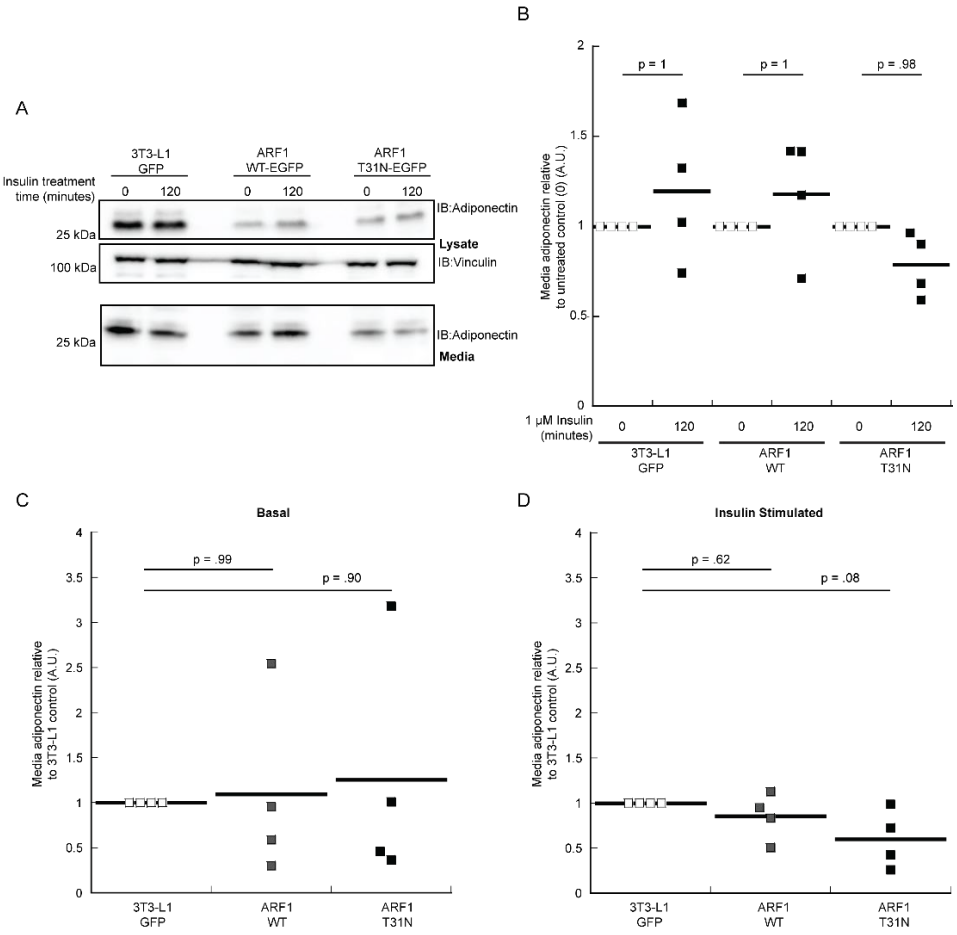
1. In figure 1 the authors test the localization of LPL with various organelles and a GLUT4 marker. The localization of LPL to caveolin-positive structures is interesting. Caveolin has been shown to label early endosomal structures (markers EEA1, Rab5) which have been shown to have a role in fast recycling to the PM however EE markers were not tested. Little co-localization is observed with the Golgi marker syntaxin 6 again shedding doubts on the validity of the model.
   We have probed fixed cells for LPL the early endosome marker EEA1 and alternative *trans*-Golgi markers: golgin 97 and GGA2, and measured their colocalization. We found that LPL colocalized with GGA2, golgin97 and EEA1. We have included these colocalization data and representative images in an updated figure 1.

2. A major concern here is that the authors make claims about the trafficking routes of LPL that cannot simply be made by analyzing the steady state localization of LPL in cell. The authors should tone down their claims. If they want to extract information about trafficking the authors should perform live cell imaging experiments possibly with a controlled release system like the RUSH system (doi: 10.1038/nmeth.1928)
   We have toned-down our descriptions and interpretations of our data to clarify that we are drawing conclusions off of steady-state data. Unfortunately, we did not believe that we have the ability to complete a live-cell imaging study at this time. We further believe that the RUSH system may interfere with the delicate packaging of mature LPL into secretory structures that have been described elsewhere (Gunn *et al.*, 2020 PMID: 32332168; Sundberg *et al.*, 2019 PMID: 31543446). We have suggested that future studies include live-cell imaging data to clarify the spatiotemporal trafficking of LPL. We hope that our re-writing clarifies the results and interpretation of our study.

3. The authors should explain more clearly about transcriptional/translational regulation of LPL trafficking. Could they clearly explain what is known from the literature?
   We have included additional background on the regulation of LPL biosynthesis in the introduction.

4. From the data in figure 7 the authors conclude trafficking of LPL is ARF1 dependent as secretion is blocked by BFA. BFA blocks secretion of every secretory cargoes and it is not very specific for ARF1 dependent cargoes.
   We have clarified our results in the text to indicate that BFA is an initial study, which we followed up with the figure 7B. BFA does affect multiple trafficking processes in cells, but there is evidence of an effect for BFA in the early secretory route and not the late secretory route in some cases which we have cited in this manuscript. We believe that we have clearly indicated that BFA is supportive of but not itself sufficient to describe our model.

5. The authors see that a dominant negative ARF1 mutant only blocks insulin stimulated secretion of LPL. However, a different interpretation is that ARF1 T31N is less efficient at completely abolishing secretion as there's endogenous WT ARF1 in the background. A cargo to show the differential effect on LPL should be used here.
   We have included a new supplemental figure (below) analyzing the secretion of adiponectin from 3T3-GFP, ARF1 WT, and ARF1 T31N expressing cells as a control. We found that ARF1 WT and ARF1 T31N did not significantly affect the secretion of adiponectin compared to 3T3-GFP cells, supporting the hypothesis that LPL and adiponectin follow different trafficking pathways which respond to exogenous ARF1 expression differently.

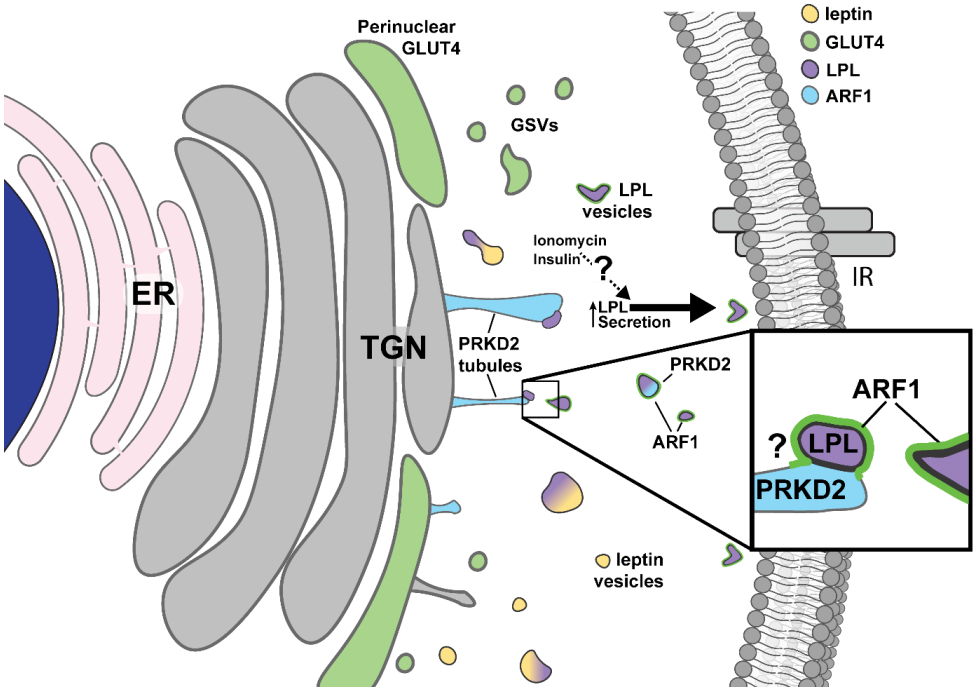


6. Additionally, while the localization of ARF1 in structures positive for caveolin is intriguing there doesn't seem to be any changes in the localization of LPL in caveolin positive structures in the presence of ARF1 T31N.
We believe LPL is already present in the caveolin-positive structures due to sorting by SDC1, independent of ARF1. We expect that ARF1 would act to traffic but not form, the vesicles. The adipocytes in the image were not insulin stimulated. We therefore do not expect to see any increase in the amount of LPL present in these structures based on the presence of ARF1 vs. ARF1 T31N, and the experiment was not designed to quantitate changes. Furthermore, based on prior studies we do not expect any change to the localization of the ARF1 T31N variant.
Specifically, previous studies have shown that the apparent distribution of ARF1 T31N in cells is not significantly different from ARF1 WT (PMID:22573888; PMID:15336557) despite a significant effect of ARF1 T31N expression on ARF1 client trafficking. Future studies should investigate the trafficking of ARF1 in insulin sensitive adipocytes using live cell imaging. We have clarified and commented on this result in the text.

7. The authors state "Together, these data illustrate a model wherein insulin recruits an ARF1 WT-PRKD2 complex to the Golgi membrane rapidly and dynamically.
In cells expressing ARF1 T31N, insulin is unable to catalyse GDP to GTP exchange in ARF1 T31N, causing ARF1 T31N to sequester PRKD2 and other trafficking machinery in the cytoplasm, inhibiting LPL secretion". However, no data is shown that shows
•Insulin-dependent ARF1 recruitment to the Golgi
•Insulin-dependent GDP-GTP exchange on ARF1
•Any interaction between the above components under IS.
We have amended our text to reflect only the observations presented in this manuscript including the text cited in this comment. We have removed language suggesting that PRKD2 and ARF1 interact in the model we are describing and we focus on the fluorescence microscopy data indicating that they localize to nearby structures.

It has been shown that ARF1 GTP binding is sensitive to insulin treatment (PMID: 9197239) and we feel that it is out of the scope of this study to describe insulin-dependent ARF1 GTP/GDP binding in 3T3 cells.
Future studies will be necessary to demonstrate the trafficking behavior of ARF1 itself in adipocytes.

8. LPL localizes to structures positive for caveolin but the authors propose a model in which LPL sorting occurs at the level of caveolin and ARF1 structures at the TGN. The authors provide no proof of localization of LPL to the TGN and should therefore revise their model based on the data they present. Or provide more experimental data in support.
We have modified figure 8 to reflect only the results presented in this manuscript. We have clarified the figure legend to indicate that this figure presents a hypothetical model of LPL trafficking based on our own results.



Second decision letter

MS ID#: JOCES/2021/258734

MS TITLE: Characterization of Lipoprotein Lipase Storage Vesicles in 3T3-L1 Adipocytes

AUTHORS: Benjamin S Roberts, Chelsea Q Yang, and Saskia Neher

ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Journal of Cell Science, pending standard ethics checks. Thank you for submitting this interesting manuscript to JCS and congratulations!