JCB

Tld1 is a regulator of triglyceride lipolysis that demarcates a lipid droplet subpopulation

Natalie Speer, R Jay Braun, Emma Reynolds, Alicja Brudnicka, Jessica Swanson, and Mike Henne

Corresponding Author(s): Mike Henne, The University of Texas Southwestern Medical Center and R Jay Braun, University of Utah

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April 12, 2023

Re: JCB manuscript #202303026

Dr. Mike Henne
The University of Texas Southwestern Medical Center
Cell Biology
6000 Harry Hines Blvd NL6.120D
Dallas, Texas 75390

Dear Dr. Henne,

Thank you for submitting your manuscript entitled "Bsc2 is a novel regulator of triglyceride lipolysis that demarcates a lipid droplet subpopulation". The manuscript was assessed by expert reviewers, whose comments are appended to this letter. We invite you to submit a revision if you can address the reviewers' key concerns, as outlined here.

As you will see, the reviewers all agree that your study examines an interesting question regarding the functional distinction of lipid droplet populations. They have provided constructive feedback which we hope you agree will further strengthen your study. In revising, the most important general issues to focus upon are to provide some experimental evidence for the suggested model of LD binding, and to address the comment of reviewer 2 regarding the experiments examining the function of Bsc2 in lipolysis. However, while you should address all issues aimed at strengthening the current manuscript, the exact elucidation of the mechanism by which Bsc2 affects lipolysis can be the subject of future work. Furthermore, we agree that the suggested experiments to further test potential interactions between Bsc2 and Tgl need to be conducted. In addition, we hope that you will be able to respond to all the remaining reviewer comments in your revised manuscript.

While you are revising your manuscript, please also attend to the following editorial points to help expedite the publication of your manuscript. Please direct any editorial questions to the journal office.

GENERAL GUIDELINES:

Text limits: Character count for an Article is < 40,000, not including spaces. Count includes title page, abstract, introduction, results, discussion, and acknowledgments. Count does not include materials and methods, figure legends, references, tables, or supplemental legends.

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IMPORTANT: It is JCB policy that if requested, original data images must be made available. Failure to provide original images upon request will result in unavoidable delays in publication. Please ensure that you have access to all original microscopy and blot data images before submitting your revision.

Supplemental information: There are strict limits on the allowable amount of supplemental data. Articles may have up to 5 supplemental figures. Up to 10 supplemental videos or flash animations are allowed. A summary of all supplemental material should appear at the end of the Materials and methods section.

Please note that JCB now requires authors to submit Source Data used to generate figures containing gels and Western blots with all revised manuscripts. This Source Data consists of fully uncropped and unprocessed images for each gel/blot displayed in the main and supplemental figures. Since your paper includes cropped gel and/or blot images, please be sure to provide one Source Data file for each figure that contains gels and/or blots along with your revised manuscript files. File names for Source Data figures should be alphanumeric without any spaces or special characters (i.e., SourceDataF#, where F# refers to the associated main figure number or SourceDataFS# for those associated with Supplementary figures). The lanes of the gels/blots should be labeled as they are in the associated figure, the place where cropping was applied should be marked (with a box), and molecular weight/size standards should be labeled wherever possible.
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The typical timeframe for revisions is three to four months. While most universities and institutes have reopened labs and allowed researchers to begin working at nearly pre-pandemic levels, we at JCB realize that the lingering effects of the COVID-19 pandemic may still be impacting some aspects of your work, including the acquisition of equipment and reagents. Therefore, if you anticipate any difficulties in meeting this aforementioned revision time limit, please contact us and we can work with you to find an appropriate time frame for resubmission. Please note that papers are generally considered through only one revision cycle, so any revised manuscript will likely be either accepted or rejected.

When submitting the revision, please include a cover letter addressing the reviewers' comments point by point. Please also highlight all changes in the text of the manuscript.

We hope that the comments below will prove constructive as your work progresses. We would be happy to discuss them further once you've had a chance to consider the points raised in this letter.

Thank you for this interesting contribution to Journal of Cell Biology. You can contact us at the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Sincerely,

Tobias Walther, PhD
Monitoring Editor

Andrea L. Marat, PhD
Senior Scientific Editor

Journal of Cell Biology

Reviewer #1 (Comments to the Authors (Required)):

Emerging data indicate that there are distinct lipid droplet subpopulations that diff in their composition and function. However, how lipid droplet heterogeneity arises and how it influences lipid droplet function and cellular physiology remain key open questions.

In the current manuscript, a focused imaging screen of known lipid droplet proteins tagged with GFP revealed that Bsc2 is only present on a subset of lipid droplets. Analyses of yeast strains that produce triacylglycerol (TAG)-rich or sterol ester (SE)-rich lipid droplets revealed a strong preference of Bsc2 for TAG-rich lipid droplets. Deletion analyses and detailed molecular dynamics simulations reveal regions of Bsc2 which mediate the lipid droplet targeting and TAG-preference, suggesting that this is due to differences in Bsc2 domain structure in TAG-rich vs SE-rich lipid droplets. Functionally, deletion and overexpression data establish Bsc2 as a negative regulator of TAG lipolysis. Bsc2 does not stably interact with the TAG lipases and does not affect their regulation. The mechanism by which Bsc2 influences TAG lipases remains unresolved and the authors suggest a model that Bsc2 may compete with TAG lipases for TAG binding.

Overall, this is an interesting paper with high quality data exploring an important question in lipid droplet cell biology. The major findings reveal Bsc2 as new TAG lipase regulator that localizes to a subset of lipid droplets due to its preference for TAG-rich lipid droplets. These findings advance our understanding of the mechanisms that govern lipid droplet heterogeneity (i.e., a protein that targets TAG-rich lipid droplets through structural differences and thus "senses" composition). The findings are significant, and I am supportive of eventual publication. The proposed model of Bsc2 regulation of lipolysis is rather unsatisfying and seems unlikely given that Bsc2 does not impact lipase association with lipid droplets. Although a full understanding of the mechanism is probably beyond the scope of the current manuscript, some additional experiments that support the claims regarding lipid droplet heterogeneity and Bsc2 targeting would strengthen the conclusions.

Comment 1: Analyses of the yeast strains that solely generate TAG or SE-rich lipid droplets indicate a preference of Bsc2 for TAG-rich lipid droplets. These results imply that in the WT-type yeast there are lipid droplets with distinct lipid compositions, some with sufficient TAG to promote Bsc2 recruitment. Lipidomic analyses of Bsc2 positive (high) vs Bsc2 negative (low) would be useful to support this model and would provide some of the strongest data for lipid droplet heterogeneity. It is also interesting that lipid differences could promote Bsc2 recruitment and that the presence of Bsc2 and inhibition of TAG lipolysis could further lead to increases in TAG levels (i.e., reinforcing / reciprocal mechanism). Similarly, in Fig 2C Bsc2 only localizes to a subset of lipid droplets in U2OS, implying differences in relative TAG composition? Confirming this in yeast or in human cells would be an important result and support the conclusions about lipid droplet heterogeneity.

Comment 2: The molecular dynamics simulation data suggest an interesting model to explain Bsc2 TAG-rich lipid droplet preference. Is it possible to validate and support data biochemically? For example, the simulations predict very different amounts of exposed HR1 which is testable.

Comment 3: The model that the presence of Bsc2 reduces substrate accessibility to the lipases seems fairly unlikely given that the lipases still localize normally to the lipid droplet. Presumably these lipases are still fully embedded into the lipid droplet and would be able to access TAG. If the authors are suggesting that they are no longer fully embedded, then perhaps they would exhibit reduced association (chaotrope / salt stripping)? The lack of stable interaction of Bsc2 with the lipases still does not rule out weaker, important interactions that are important. Solubilizing and retaining the integrity of membrane-embedded complexes, especially lipid droplet protein complexes, can be challenging. It may be worth try in a bimolecular fluorescence complementation approach or similar method to assess interaction? My feeling is that understanding this mechanism will be beyond the scope of this current manuscript and it would be sufficient for me if a clear limitations section is included in the discussion.

Minor comments
Comment 1: Is Bsc2 conserved and is there a mammalian ortholog?
Comment 2: Figure 2B. The mNeon green sometimes appears to be saturated? For example for the livedrop fusion construct?

Reviewer #2 (Comments to the Authors (Required)):

This paper reports that yeast Bsc2 is a protein that localizes to a subset of LD where it regulates lipolysis. There are two main aspects to the paper - a description of the targeting, in conjunction with MD simulations, and functional analysis reporting that Bsc2 on LD slows lipolysis. In general, the paper has novel findings and is interesting. The targeting data are interesting although could be improved. The findings that Bsc2 regulates lipolysis are generally convincing but unfortunately a mechanism is not elucidated.

Figs 1-3 concern the targeting of Bsc2 to LD. Fig 1 reports it targets to a subset of LDs. Fig 2 reports domain analysis and identification of hydrophobic regions (HR) involved in targeting. Fig 3 reports MD simulation analysis of HR regions and targeting to ER and LD with different composition.

1. Fig 1 would benefit from a western blot showing endogenous protein expression during yeast expression (log to stationary).
2. In Fig 1a, are total Bsc2 protein levels increased?
3. One might expect LD with Bsc2 to be larger? Is this the case? Please quantify.
4. Fig 1d, please show western blost of Bsc2 - are protein levels reduced in SE conditions? If one over expresses, does it localize to SE LD?
5. Most of the evidence in Fig 2b highlights that HR1 is important for LD localization. An important mutant would be to introduce a charged residue into the hydrophobic helix of HR1 to disrupt binding to LD. This would be helpful here and later in the paper for testing functional analysis.
6. It is unclear about the N-terminal region before HR1. Adding this to HR1 seems to significantly increase targeting. Please investigate this region and it's requirement.
7. Again, and relevant to the last point, western blots or some means to quantify protein expression would help to interpret 2b results.
8. 2c is unconvincing as it shows one cell. Please show more cells and more LD and/or quantify proportion of LD with Bsc2. Related, do mammalian cells have an ortholog?
9. Regarding Fig 3, how do the authors think this targeting works? They report an interesting protein with both a hydrophobic helix and a hydrophobic hairpin (most LD proteins have one or the other). Does the protein insert into the ER and then the helix can take the protein to the LD if there are TG? If this is the case, one could conditionally express Dga1 in a SE-only background and turn on TG synthesis, thereby moving the protein to LD. This could be tested. Also, why not draw Fig 3E with the targeting reaction to TG or SE LD, rather than the situation that does not exist (apparently) of HR1/2 on SE LD.

Fig 4-7 investigate the functional consequences of Bsc2 targeting. The data are interesting but could be improved.

10. It is not clear why the authors investigate TG synthesis with Dga1 on a Gal promoter, thereby taking it's regulation out of the equation. Wouldn't a better experiment be to examine TG synthesis in the TGL triple deletion +/- Bsc2?
11. The overexpression experiments in Fig 5 are interesting and test important predictions. A nice control would be HR1 that has a charged point mutant preventing targeting to LD. This seems quite important.
12. Fig 5, are the LD that are targeted by Bsc2 larger than those that are not. Please plot correlation of targeting signal and size. This also seems important.
13. In Fig 6c, the condition with Bsc2 overexpression looks like it reduces Tgl3 on LD. This needs to be more closely examined, as a simple hypothesis is that Bsc2 crowds Tgl3 off of LD. In addition to better quantifying the microscopy data, LD purification and western blots would be helpful.
14. Specific and well controlled IP pulldowns of Bsc2 and Tgls should be attempted to additionally look for an interaction.
15. Further attempts should be made to identify a mechanism for the reported decrease in lipolysis.

Reviewer #3 (Comments to the Authors (Required)):

Lipid droplet (LD) targeting is an intriguing scientific subject with many unresolved scientific questions. In particular, how the composition of neutral lipids of LD affects the composition of LD proteins is largely unknown. The authors used yeast experiments and all-atom molecular dynamics (MD) simulations to identify Bsc2 as a negative regulator of triacylglycerol (TG) lipolysis and propose a plausible explanation of Bsc2's preferential TG-abundant LD targeting compared to sterol ester (SE)-abundant LD and ER bilayer targeting. Overall, the manuscript is of scientific value and will be of interest to the lipid simulation community. I recommend that it be published in Journal of Cell Biology. However, the authors should first address the concerns written below.

MAJOR

The authors expressed seven mNG-tagged fragments of BSC2 (Fig. 2B) and concluded that HR1 is necessary (page 6, line 159) and may be sufficient (page 5, line 151) for LD targeting. However, by looking at Fig. 2B, both the N-terminal region and HR2 seem to contribute to LD targeting. In addition, the structural model in Fig. S3C seems to suggest that the N-terminus can contribute to LD and membrane binding. There seems to be no obvious changes in the overall and LD binding signal levels of BSC2-N-HR1+HR2 and Bsc2-HR1+HR2 compared to Bsc2-FL. Bsc2-N-HR1 (deletion of HR2) caused a significant reduction in LD binding signal level. Bsc2-HR1 (deletion of N-terminus) had no specificity in its LD targeting, and the authors write "Bsc2-HR1 failed to express well in yeast" (page 5, line 155).

- The authors should provide experimental data or analysis to support the statement that Bsc2-HR1 failed to express well in yeast.
- The authors should discuss why Bsc2-HR1+HR2, which does not have the N-terminal region like Bsc2-HR1, is expressed well.

The authors predicted a structure of Bsc2-N-HR1+HR2 (residue 1-100) using RoseTTAFold. However, I do not see the N-terminus region 1-24 in the snapshots of the MD frames (Fig. 3A). The authors should highlight the residues that they used for the MD simulations or clarify whether the N-terminus was simply not shown in Fig. 3A for visual clarity.

The authors showed one frame for each simulation. Given that the authors mainly discuss the conformational changes of the protein upon binding to TG-abundant LDs, SE-abundant LDs, and bilayers observed in the MD trajectories, the authors should provide more snapshots including the initial structures.

One of the conclusions from this manuscript is that the three polar residues, Gln72, Cys75, and Ser76, near the kink of HR2 play a critical role in driving the HR2 conformational changes. The authors also suggest that these polar residues can explain Bsc2's differential targeting preference. If these residues are mutated to hydrophobic residues, would the mutant construct bind to both the ER bilayer and TG-abundant LDs but not SE-abundant LDs?

The authors cited Campomanes et al., 2021, which proposed a new TG forcefield with significantly reduced partial charges of the glycerol moiety of TG. If the authors used this model, they should explicitly mention this in the main text. If the authors reduced the partial charges of the glycerol moiety of SE from their original paper (Braun and Swanson, 2022), this should be also clearly indicated.

The authors mentioned the association of the HR1 amphipathic helix and the surface packing defects multiple times and attributed it to different binding behaviors of HR2 toward TG-abundant LDs, SE-abundant LDs, and ER bilayers (line 215 of page 7, line 222 of page 8, legends of Fig. 3A, S3I, Fig.S3J, Fig. S3K). However, I do not see any snapshots that contain packing defects. Additional figure of the snapshots should be included.

The authors performed 4.5 us simulations of the bilayer and TG-only LD system and 1.0 us simulation of the SE-abundant LD system. The authors should provide a justification for the different simulation times as well as the approximate timescale of HR1 association with the TG-only surface and bilayer.

The authors proposed a model in which Bsc2 coated the LD surface, making other lipase enzymes less accessible to the LD surface and TG binding sites. The authors should discuss why the overexpression of Pln1, perilipin-like protein that binds to the LD surface and expected to have a comparable role with Bsc2, did not affect the TG level (Fig. 5E).

MINOR

It would be more informative if the authors used the simulation time (us) instead of the number of frames in Fig. S3A, Fig. S3I, Fig. S3J, Fig.S3K.

It would be more informative if the authors included a single-letter amino acid in front of the residue number in Fig. S3D, Fig. S3E, and Fig. S3F.

In the setup of the MD simulations, the overlapping phospholipids and neutral lipids were removed. The authors should report the final number of phospholipids for each leaflet and for each system.

The authors should describe how the angle of HR2's helix-kink-helix structure was calculated in the MD simulations (Fig. S3B).

Packing defects lie on a 2 dimensional plane without a meaningful z-value. The authors should justify why the contacts between packing defects and residues, which are not commonly used, are significant and describe this procedure in detail.

Please review the simulation parameters for Anton simulations. I believe Anton uses MTK pressure (see below), the M-SHAKE algorithm (instead of LINCS) and the Gaussian split Ewald method (instead of PME).
Martyna, G. J.; Tobias, D. J.; Klein, M. L. Constant pressure molecular dynamics algorithms. J. Chem. Phys. 1994, 101 (5), 4177– 4189.
Lippert, R. A.; Predescu, C.; Ierardi, D. J.; Mackenzie, K. M.; Eastwood, M. P.; Dror, R. O.; Shaw, D. E. Accurate and efficient integration for molecular dynamics simulations at constant temperature and pressure. J. Chem. Phys. 2013, 139 (16), 164106.

I believe the authors used C36m for a protein force field, not C36 (page 20, line 607). The protein force field was not cited.

W. Mike Henne, Ph.D.
W.W. Caruth, Jr. Endowed Scholar in Biomedical Research
Associate Professor, Depts. of Cell Biology & Biophysics

September 9, 2023

Dear Dr. Reviewers,

We are excited to submit our revised manuscript now entitled "**Tld1 is a novel regulator of triglyceride lipolysis that demarcates a lipid droplet subpopulation"** for consideration at *The Journal of Cell Biology*. We have addressed the reviewer concerns, as detailed in our point-by-point discussion below.

Please note an update: we are proposing to change the name of Bsc2 to Tld1 (for Triglyceride-associated lipid droplet protein 1). **This is because Bsc2 is very similar to Bscl2**, another name for the lipid droplet protein Seipin. Several members of our community have suggested we do this to avoid confusing Bsc2 with Bscl2. Thus, we have changed Bsc2 to Tld1 in our revised manuscript. We will also communicate with the Saccharomyces Genome Database to officially rename this ORF.

We also provide a brief summary of some of the new insights in our revised manuscript below. Specific changes in the manuscript text are in yellow in the manuscript:

1) **Additional evidence for Tld1 selective targeting to TG-rich LDs and not SE-rich LDs (new Fig 3)**: A major finding of our work is that Tld1 displays preference for binding triglyceride (TG)-rich LDs as opposed to sterol-ester (SE)-rich LDs. Reviewers requested that we further dissect this selective LD targeting. We have done this in two ways: First, we now show the Tld1-EGFP targets to TG-rich LDs in human cells, but not SE-rich liquid-crystalline LDs in human cells (seen in new Figure 3). Second, we also conducted mutagenesis dissection of Tld1's hydrophobic regions (HRs) HR1 and HR2. We now identify specific mutations in both HR1 (R26E, F44D) and HR2 (S72A+Q76A) that perturb LD binding in yeast and human cell expression systems (in new Figure 5). Importantly, these mutations are predicted by MD simulations to disrupt the Tld1 interaction with TG, supporting a model where Tld1 interacts with TG. Collectively, our work supports a model where HR1 LD targeting is necessary for Tld1 LD localization, but HR2 undergoes conformational changes on the LD surface promote more stable LD association. This is consistent with our MD simulations show that the Tld1 HR1 regions fails to bind SE-rich LDs, leading to its disengagement from the LD surface.

2) **Additional mutational analysis via MD simulations (Fig 4, new Supp Fig 2-4)**: We have conducted additional MD simulations which address the concerns of Reviewer 3. These new experiments also now show that mutation of key resides in the HR2 helix-turn-helix region and HR1 amphipathic helix cause defects in Tld1 LD targeting. The details are shown in Figure 4 and Supp Figures 2-4, and discussed in the results section. Collectively, this supports a model where Tld1 initially localizes to the ER and engages LDs via HR1. Following LD localization, HR2 adopts a more kinked confirmation that may provide additional LD affinity.

3) **New *in vivo* testing of Tld1 mutants (new Figure 5)**: The reviewers requested that we further test how HR1 and HR2 contribute to LD binding by conducting mutagenesis and structure-function analysis. We have now screened many mutations, and have identified mutations in HR1 (K26E, F44D) as well as HR2 (S76A and Q72A+S76A) that perturb LD targeting and Tld1 stability. These mutations provide additional

6000 Harry Hines Blvd., Dallas, Texas 75390 Phone: 214.648.6778 email: mike.henne@utsouthwestern.edu
www.utsouthwestern.edu/labs/henne/

experimental support for the MD simulations, and underscore how both HR1 and HR2 contribute to LD targeting in vivo.

4) **Western blotting confirming Tld1 stability requires TG, its abundance in Log and Stat-phases of yeast growth, and how different Tld1 fragments express**: Per the reviewer requests, we have conducted Western blotting examining Tld1 abundance in Log and Stat phases of yeast growth, as well as its abundance when yeast contain TG-only or SE-only LDs (added in Figure 1). We find that Tld1 protein levels are elevated in Stationary-phase (consistent with imaging data). We also find that Tld1 requires TG to maintain its protein abundance; SE-only yeast display a near complete loss of endogenous Tld1, which we speculate is due to its degradation. Finally, we now show a Western blot displaying the abundance of the different Tld1 fragments we express (Supp Figure 1).

5) **Additional mechanistic insights into Tld1-mediated TG accumulation from over-expression**: Per the reviewer requests, we have further investigated how Tld1 influences TG levels. We find that Tld1 over-expression in mammalian HeLa cells is sufficient to increase LD size, similar to Tld1 over-expression in yeast (data now in Figure 7). While the full mechanism is unclear and (as the reviewers state) beyond the scope of this manuscript, this collectively supports a model where Tld1 over-expression may promote Tld1 binding to TG and TG sequestration, which inhibits TG lipolysis and gradual TG accumulation. Future studies will dissect the molecular mechanism by which Tld1 influences TG levels.

6) **Additional investigation of Tld1-Tgl3 functional interactions**: while the full mechanism for how Tld1 influences TG lipases was considered not necessary for revision of the manuscript, we conducted new experiments dissecting whether Tld1 and Tgl3 form tight interactions with one another. We now show co-immunoprecipitation (co-IP) experiments indicating that we do not detect strong co-IP interactions between Tld1 and Tgl3 (Supplemental Figure 5). Combined with the Tld1 proteomics data sets in Figure 8, our work indicates that Tld1 and Tgl3 do not form a tight physical interaction, although we cannot rule out that Tld1 may form weak allosteric interactions with Tgl3 or other lipases that influence lipolysis. We have added text and a “limitations of study” section which discuss this.

We sincerely hope these revisions will satisfy the reviewer concerns. Please contact me if you have any further questions.

Thank you for your time,

W. Mike Henne, Ph.D.
Associate Professor
W.W. Caruth Jr., Endowed Scholar

Point-by-point responses:

Re: JCB manuscript #202303026

Dr. Mike Henne
The University of Texas Southwestern Medical Center
Cell Biology
6000 Harry Hines Blvd NL6.120D
Dallas, Texas 75390

Dear Dr. Henne,

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As you will see, the reviewers all agree that your study examines an interesting question regarding the functional distinction of lipid droplet populations. They have provided constructive feedback which we hope you agree will further strengthen your study. In revising, the most important general issues to focus upon are to provide some experimental evidence for the suggested model of LD binding, and to address the comment of reviewer 2 regarding the experiments examining the function of Tld1 in lipolysis.

Response: Thank you for this feedback. We have now provided extensive mutagenesis data for HR1 and HR2 that more fully dissects LD binding, as well as new MD simulations that further investigate this. These are in the new Figs 3-5 and Supp Fig 2-4. Additionally, we conducted two new experiments investigating lipolysis. We conducted new co-IP experiments investigating Tld1 and Tgl3 interactions. We also now show that Tld1 over-expression in human cells is sufficient to increase LD size (suggesting it can impact TG LD storage independent of other yeast proteins).

However, while you should address all issues aimed at strengthening the current manuscript, the exact elucidation of the mechanism by which Tld1 affects lipolysis can be the subject of future work. Furthermore, we agree that the suggested experiments to further test potential interactions between Tld1 and Tgl need to be conducted. In addition, we hope that you will be able to respond to all the remaining reviewer comments in your revised manuscript.

Response: Thank you. As stated above, we now provide new co-IP data further investigating the Tld1 and Tgl3 interaction. We also have added a “limitations of study” section delineating that we do not fully understand the mechanism by this Tld1 regulates lipolysis. Future studies will investigate this further. We also addressed other concerns from the reviewers.

While you are revising your manuscript, please also attend to the following editorial points to help expedite the publication of your manuscript. Please direct any editorial questions to the journal office.

Reviewer #1 (Comments to the Authors (Required)):

Emerging data indicate that there are distinct lipid droplet subpopulations that diff in their composition and function. However, how lipid droplet heterogeneity arises and how it influences lipid droplet function and cellular physiology remain key open questions.

In the current manuscript, a focused imaging screen of known lipid droplet proteins tagged with GFP revealed that Tld1 is only present on a subset of lipid droplets. Analyses of yeast strains that produce triacylglycerol (TAG)-rich or sterol ester (SE)-rich lipid droplets revealed a strong preference of Tld1 for TAG-rich lipid droplets. Deletion analyses and detailed molecular dynamics simulations reveal regions of Tld1 which mediate the lipid droplet targeting and TAG-preference, suggesting that this is due to differences in Tld1 domain structure in TAG-rich vs SE-rich lipid droplets. Functionally, deletion and overexpression data establish Tld1 as a negative regulator of TAG lipolysis. Tld1 does not stably interact with the TAG lipases and does not affect

their regulation. The mechanism by which Tld1 influences TAG lipases remains unresolved and the authors suggest a model that Tld1 may compete with TAG lipases for TAG binding.

Overall, this is an interesting paper with high quality data exploring an important question in lipid droplet cell biology. The major findings reveal Tld1 as new TAG lipase regulator that localizes to a subset of lipid droplets due to its preference for TAG-rich lipid droplets. These findings advance our understanding of the mechanisms that govern lipid droplet heterogeneity (i.e., a protein that targets TAG-rich lipid droplets through structural differences and thus "senses" composition). The findings are significant, and I am supportive of eventual publication.

Response: We thank the reviewer for their positive comments and feedback on our manuscript.

The proposed model of Tld1 regulation of lipolysis is rather unsatisfying and seems unlikely given that Tld1 does not impact lipase association with lipid droplets. Although a full understanding of the mechanism is probably beyond the scope of the current manuscript, some additional experiments that support the claims regarding lipid droplet heterogeneity and Tld1 targeting would strengthen the conclusions.

Response: Thank you for these suggestions. As discussed below, we have now performed several new experiments that provide new insights into how Tld1 targets to LDs, and how it may influence TG pools.

1. We have conducted mutagenesis analysis of the Tld1 HR1 and HR2 regions to identify key residues necessary for LD targeting. This was guided by our MD simulations. We now identify key residues in the HR1 amphipathic helix (K26E, F44D) and HR2 (S76A, Q72A+S76A) which when mutated reduce or eliminate LD targeting. These observations support the MD simulations of Tld1 structure, and indicate that HR1 likely acts as an amphipathic helix inserting into the LD surface. It also supports the model where HR2 adopts the helix-kink-helix motif on the LD surface. These mutants are examined in Figure 5 and the Supplemental Figures.

2. We have now expressed Tld1 in human HeLa cells which have been induced to generate either TG-rich or SE-rich LDs. Consistent with observations in yeast, we find that Tld1 decorates the surfaces of TG-rich LDs, but does not co-localize with the SE-rich LDs. This supports the model where Tld1 display preference for TG-rich LDs in multiple cell types (see Figure 3).

3. We have now over-expressed Tld1-EGFP in human cells, and find this is sufficient to increase LD size as observed in yeast. This supports a model where Tld1-EGFP over-expression can directly impact LD lipid accumulation independent of other yeast protein machinery (now in Figure 7).

   Comment 1: Analyses of the yeast strains that solely generate TAG or SE-rich lipid droplets indicate a preference of Tld1 for TAG-rich lipid droplets. These results imply that in the WT-type yeast there are lipid droplets with distinct lipid compositions, some with sufficient TAG to promote Tld1 recruitment. Lipidomic analyses of Tld1 positive (high) vs Tld1 negative (low) would be useful to support this model and would provide some of the strongest data for lipid droplet heterogeneity. It is also interesting that lipid differences could promote Tld1 recruitment and that the presence of Tld1 and inhibition of TAG lipolysis could further lead to increases in TAG levels (i.e., reinforcing / reciprocal mechanism). Similarly, in Fig 2C Tld1 only localizes to a subset of lipid droplets in U2OS, implying differences in relative TAG composition? Confirming this in yeast or in human cells would be an important result and support the conclusions about lipid droplet heterogeneity.

Response: Thank you for this comment and these suggestions. While we were unable to conduct lipidomic profiling of Tld1+ and Tld1- LDs, we did further investigate how lipid composition influenced Tld1 protein abundance in vivo, as well as LD targeting. First, we found that Tld1-GFP proteins levels are drastically reduced in SE-only yeast compared to wildtype and TG-only yeast (Fig 1). While the nature of this protein reduction is not completely clear, we speculate that Tld1 protein stability is impacted by loss of TG, leading to Tld1 protein degradation.

Second, as mentioned above, we have examined Tld1 LD protein targeting in more depth in human cells. We now find that: 1) Tld1-EGFP display preference for binding TG-rich LDs, and does not colocalize with SE-rich LDs in HeLa cells. 2) Similar to its behavior in yeast, HR1 is sufficient for LD targeting when expressed in human cells. 3) We find HR2 alone targets to the ER network, but HR1+HR2 together localize to LDs. Collectively, this supports a model where HR1 promotes LD targeting, and HR2 further reinforces this LD targeting via its helix-turn-helix conformation on the LD surface.

As an additional observation, we now show that Tld1-EGFP over-expression in human cells is sufficient to expand LD size (Fig 7). This also supports a model where Tld1 may directly engage TG on LDs and influence LD size.

Comment 2: The molecular dynamics simulation data suggest an interesting model to explain Tld1 TAG-rich lipid droplet preference. Is it possible to validate and support data biochemically? For example, the simulations predict very different amounts of exposed HR1 which is testable.

Thank you for this suggestion: We have tried to test the MD simulation data on Tld1 structure in two ways:

1) We have now made mutations in Tld1 at predicted residues critical to HR1 LD targeting (K26E, F44D) and HR2 targeting (S76A, Q72A+S76A). All these mutations reduce LD targeting to LDs, supporting the model. We also expressed Q72A+S76A in human cells and observed reduced LD targeting. Tld1 S76A mutation alone appears to target to LDs in yeast, but displays reduced LD targeting when expressed in human cells (Fig 5). Collectively, these mutations support the MD simulations and demonstrate that HR1 and HR2 help in LD targeting.

2) The MD simulations suggest that Tld1 loses affinity for SE-rich LDs, which may lead to its LD targeting loss and subsequent degradation as has been observed for other proteins like Tgl3. We monitored endogenous Tld1 protein levels by Western blotting, and indeed found that Tld1-GFP protein levels are diminished in SE-rich LDs (Fig 1). In line with this, Tld1-EGFP does not localize to SE-rich LDs in HeLa cells (Fig 3). This supports the MD simulation that in the absence of TG, Tld1 loses LD affinity.

Comment 3: The model that the presence of Tld1 reduces substrate accessibility to the lipases seems fairly unlikely given that the lipases still localize normally to the lipid droplet. Presumably these lipases are still fully embedded into the lipid droplet and would be able to access TAG. If the authors are suggesting that they are no longer fully embedded, then perhaps they would exhibit reduced association (chaotrope / salt stripping)? The lack of stable interaction of Tld1 with the lipases still does not rule out weaker, important interactions that are important. Solubilizing and retaining the integrity of membrane-embedded complexes, especially lipid droplet protein complexes, can be challenging. It may be worth try in a bimolecular fluorescence complementation approach or similar method to assess interaction? My feeling is that understanding this mechanism will be beyond the scope of this current manuscript and it would be sufficient for me if a clear limitations section is included in the discussion.

Response: We agree that we do not fully understand the mechanism of Tld1's influence on TG. We cannot rule out a model where Tld1 influences TG lipolysis via several potential mechanisms. For example, Tld1 may interact directly with a TG lipase like Tgl3 and allosterically regulate its activity. We have specifically added text to the manuscript to clearly state that there are multiple possible mechanisms for Tld1's influence of TG lipolysis. We also added a "limitations of study" section which discusses this. Experimentally, we also have now added in co-IP data where we pulled down Tld1 and blotted for Tgl3. While we do not detect a strong co-IP interaction, it does not rule out that Tgl3 and Tld1 may weakly interact (see Supplemental Figure 5). We agree with reviewers that a full mechanistic dissection is beyond the scope of the current manuscript, but look forward to further dissection in the future.

Minor comments
Comment 1: Is Tld1 conserved and is there a mammalian ortholog?

Response: Thank you for the question. There is no clear Tld1 homolog by sequence alignment. We speculate that other proteins in metazoans may have similar protein folds as Tld1, and are exploring this in a separate study.

Comment 2: Figure 2B. The mNeon green sometimes appears to be saturated? For example for the livedrop fusion construct?

Response: Thank you for this comment. We found a new ROI that was not saturated for this.

Reviewer #2 (Comments to the Authors (Required)):

This paper reports that yeast Tld1 is a protein that localizes to a subset of LD where it regulates lipolysis. There are two main aspects to the paper - a description of the targeting, in conjunction with MD simulations, and functional analysis reporting that Tld1 on LD slows lipolysis. In general, the paper has novel findings and is interesting. The targeting data are interesting although could be improved. The findings that Tld1 regulates lipolysis are generally convincing but unfortunately a mechanism is not elucidated.

Figs 1-3 concern the targeting of Tld1 to LD. Fig 1 reports it targets to a subset of LDs. Fig 2 reports domain analysis and identification of hydrophobic regions (HR) involved in targeting. Fig 3 reports MD simulation analysis of HR regions and targeting to ER and LD with different composition.

1. Fig 1 would benefit from a western blot showing endogenous protein expression during yeast expression (log to stationary).

Response: Thank you for this suggestion. We have now added this Western blot (Fig 1).

2. In Fig 1a, are total Tld1 protein levels increased?

Response: Indeed, Western blotting now shown in Fig 1 indicates that STAT phase yeast display an increase in Tld1 protein.

3. One might expect LD with Tld1 to be larger? Is this the case? Please quantify.

Response: LDs are indeed slightly larger in STAT phase versus Log phase, as has been observed in other studies and is consistent with the accumulation of TG in STAT phase yeast. To quantify this, we expressed Tld1-EGFP in human cells and monitored LD size with and without Tld1. Indeed, Tld1-EGFP localization to LDs correlates with increased LD size (Fig 7).

4. Fig 1d, please show western blost of Tld1 - are protein levels reduced in SE conditions? If one over expresses, does it localize to SE LD?

Response: Thank you for this question. Indeed, Western blots confirm that Tld1-GFP protein levels drop to almost undetectable levels in SE-only yeast (Fig 1). We speculate that this may be due to protein degradation in the absence of TG.

5. Most of the evidence in Fig 2b highlights that HR1 is important for LD localization. An important mutant would be to introduce a charged residue into the hydrophobic helix of HR1 to disrupt binding to LD. This would be helpful here and later in the paper for testing functional analysis.

Response: Thank you for this excellent idea. We have now done this—we introduced the F44D mutation into HR1 of Tld1-GFP, and expressed it in yeast. As expected, this reduced Tld1 LD targeting (Fig 5). We also tested several other mutants based on the MD simulations of Tld1, and they also impact LD targeting. Collectively, this supports the MD simulations for Tld1 structure.

6. It is unclear about the N-terminal region before HR1. Adding this to HR1 seems to significantly increase targeting. Please investigate this region and it's requirement.

Response: Thank you for the question. We addressed this in a few ways. First, we monitored whether HR1 alone (without the preceding N-terminal region) would be able to target to LDs by expressing this construct in mammalian cells. This construct, $Tld1^{HR1}$-EGFP was able to localize to LDs, indicating the N-terminal leader region is not important for LD targeting. Second, we expressed the N-terminal small region alone in yeast, and it does not localize to LDs (Fig 2). Notably, when examining the 'N-HR1 alone' construct, loss of the N-terminal lead region in yeast causes HR1 to become less stable on its own (Supp Fig 1). Collectively, we speculate that the N-terminal region is not required for LD targeting per se, but helps maintain HR1 stability in yeast.

7. Again, and relevant to the last point, western blots or some means to quantify protein expression would help to interpret 2b results.

Response: Thank you. We have now included a Western blot showing the protein abundances for the different Tld1-GFP fragments (Supp Fig 1).

8. 2c is unconvincing as it shows one cell. Please show more cells and more LD and/or quantify proportion of LD with Tld1. Related, do mammalian cells have an ortholog?

Response: Thank you. We have exchanged this image and added several other images of Tld1-EGFP expressed in human cells (Fig 3). As stated above, while we do not find a clear mammalian ortholog for Tld1, we think there may be structural analogs. Investigating this is a separate project in the lab.

9. Regarding Fig 3, how do the authors think this targeting works? They report an interesting protein with

both a hydrophobic helix and a hydrophobic hairpin (most LD proteins have one or the other). Does the protein insert into the ER and then the helix can take the protein to the LD if there are TG?

Response: Thank you for this question. We collectively find that through expression of Tld1 and its fragments in yeast and human cells, that: 1) HR1 is sufficient to target to LDs, 2) on its own, HR2 targets primarily to the ER network, 3) HR1 and HR2 combined target strongly to LDs, 4) in MD simulations HR2 forms a "splayed out" structure in the ER bilayer, but a "helix-turn-helix hairpin" on LDs. Collectively, our working model is that Tld1 initially targets to LDs by binding an LD via HR1. This may initiate LD localization, and once Tld1 is localized to LDs, HR2 adopts its hairpin confirmation that further supports LD localization. This may involve ER-to-LD translocation of Tld1 via a lipidic bridge connecting the ER and LD, but this is speculation.

If this is the case, one could conditionally express Dga1 in a SE-only background and turn on TG synthesis, thereby moving the protein to LD. This could be tested. Also, why not draw Fig 3E with the targeting reaction to TG or SE LD, rather than the situation that does not exist (apparently) of HR1/2 on SE LD.

Response: Our intent in Fig 3E cartoon was to provide a simple guide for LD targeting for Tld1. We have amended the panel and generated a better image to summarize the targeting preference in our model.

Fig 4-7 investigate the functional consequences of Tld1 targeting. The data are interesting but could be improved.

10. It is not clear why the authors investigate TG synthesis with Dga1 on a Gal promoter, thereby taking it's regulation out of the equation. Wouldn't a better experiment be to examine TG synthesis in the TGL triple deletion +/- Tld1?

Response: The purpose of this experiment was to monitor whether any step of TG synthesis was elevated or altered when Tld1 was deleted, and in a background where Dga1 levels were the same. The GAL-induction system provides a simple way to induce TG production, and monitor it by TLC. Furthermore, the GAL-promoter system also allows yeast to express a standard amount of Dga1 between yeast strains. Thus, the only major variable is the presence or absence of Tld1. We find that TG levels increase by the same amount in yeast with or without Tld1, suggesting Tld1 loss does not impact a step leading up to TG synthesis.

11. The overexpression experiments in Fig 5 are interesting and test important predictions. A nice control would be HR1 that has a charged point mutant preventing targeting to LD. This seems quite important.

Response: Thank you for this suggestion. We have further tested how Tld1 over-expression impacts LD accumulation by also quantifying how Tld1-EGFP over-expression in mammalian cells impacts LD accumulation. Indeed, over-expressing Tld1 in these cells also causes LD size increases like in yeast (new data in Fig 7). While we did not test this for the HR1 mutant per se, we did image the localization of Tld1 HR1 mutant K26E in yeast cells and found it had reduced LD localization (Fig 5).

12. Fig 5, are the LD that are targeted by Tld1 larger than those that are not. Please plot correlation of targeting signal and size. This also seems important.

Response: When we over-express Tld1 in yeast, it decorates all detectable LDs in yeast. TG also accumulates in these cells, and we reason that Tld1 is targeting TG-rich LDs. To try to quantify this, we

have now quantified LD size in human cells either expressing Tld1-EGFP or not. Indeed, we find Tld1-EGFP correlates with larger LDs (Fig 7).

13. In Fig 6c, the condition with Tld1 overexpression looks like it reduces Tgl3 on LD. This needs to be more closely examined, as a simple hypothesis is that Tld1 crowds Tgl3 off of LD. In addition to better quantifying the microscopy data, LD purification and western blots would be helpful.

Response: Thank you for bringing this issue up. Indeed, we examined this closely. We find that LDs are larger in these Tld1 over-expressed yeast, as also quantified and discussed in the original Fig 5 (now Fig 7). We also find that Tgl3 protein levels do not change in this Tld1 OE background (Fig 8). While Tgl3-GFP does not de-localize from LDs in this background, we reason that the same number of Tgl3-GFP molecules are spread across a larger LD surface area when Tld1 is over-expressed. Thus, Tgl3-GFP may appear dimmer by imaging, but this is a consequence of increased LD size and surface area.

14. Specific and well controlled IP pulldowns of Tld1 and Tgls should be attempted to additionally look for an interaction.

Response: Thank you for this suggestion. We designed and conducted controlled IPs for Tld1 and Tgl3, which we focused on since loss of Tgl3 blunted the enhanced lipolysis observed in Tld1-KO yeast (Fig 6). This IP has two negative controls including the mNeonGreen tag alone, as well as Pln1-mNeonGreen which has not been observed to interact with Tgl3. We also included this IP data in the Supplemental Data. The IP does not defect a strong interaction between Tgl3 and Tld1. Although we cannot rule out a weak interaction between Tld1 and Tgl3, this supports a model where Tld1 and Tgl3 do not form a tight complex-like interaction.

15. Further attempts should be made to identify a mechanism for the reported decrease in lipolysis.

Response: Thank you for this suggestion. We conducted an additional experiment to try to address this point: We determined whether Tld1 OE could cause LD size increase/accumulation in human cells that do not have other yeast LD machinery. Indeed, Tld1 OE was also sufficient to cause an increase in total LD area in cells (Fig 7). This supports a model where Tld1 influences TG pools at the LD surface. While we agree with the reviewers and editor that a full molecular mechanism is beyond the scope of the study, we have added a section in the results section on the “limitations of study” where we discuss the various models that could explain Tld1 and its influence on TG lipolysis. Our data supports a model where Tld1 can potentially bind TG, or that it can weakly interact with Tgl lipases and influence their activity.

Reviewer #3 (Comments to the Authors (Required)):

Lipid droplet (LD) targeting is an intriguing scientific subject with many unresolved scientific questions. In particular, how the composition of neutral lipids of LD affects the composition of LD proteins is largely unknown. The authors used yeast experiments and all-atom molecular dynamics (MD) simulations to identify Tld1 as a negative regulator of triacylglycerol (TG) lipolysis and propose a plausible explanation of Tld1's preferential TG-abundant LD targeting compared to sterol ester (SE)-abundant LD and ER bilayer targeting. Overall, the manuscript is of scientific value and will be of interest to the lipid simulation community. I recommend that it be published in Journal of Cell Biology. However, the authors should first address the concerns written below.

MAJOR
The authors expressed seven mNG-tagged fragments of TLD1 (Fig. 2B) and concluded that HR1 is necessary (page 6, line 159) and may be sufficient (page 5, line 151) for LD targeting. However, by looking at Fig. 2B, both the N-terminal region and HR2 seem to contribute to LD targeting. In addition, the structural model in Fig. S3C seems to suggest that the N-terminus can contribute to LD and membrane binding. There seems to be no obvious changes in the overall and LD binding signal levels of TLD1-N-HR1+HR2 and Tld1-HR1+HR2 compared to Tld1-FL. Tld1-N-HR1 (deletion of HR2) caused a significant reduction in LD binding signal level. Tld1-HR1 (deletion of N-terminus) had no specificity in its LD targeting, and the authors write "Tld1-HR1 failed to express well in yeast" (page 5, line 155).
- The authors should provide experimental data or analysis to support the statement that Tld1-HR1 failed to express well in yeast.
- The authors should discuss why Tld1-HR1+HR2, which does not have the N-terminal region like Tld1-HR1, is expressed well.

Response: Thank you for these helpful comments. First, we have now provided a Western blot showing that in yeast Tld1-HR1 alone does not express well (Supp Fig 1). Second, we now provide new experiments in human cells showing HR1 alone expresses well and targets to LDs (Fig 3). We speculate that in yeast the N-terminal region preceding HR1 is necessary for HR1 stability, but this matters less when HR1 is highly expressed in human cells. Third, we have added additional text in the manuscript discussing the model where HR1 and HR2 both provide LD targeting ability and support one another for LD localization.

The authors predicted a structure of Tld1-N-HR1+HR2 (residue 1-100) using RoseTTAFold. However, I do not see the N-terminus region 1-24 in the snapshots of the MD frames (Fig. 3A). The authors should highlight the residues that they used for the MD simulations or clarify whether the N-terminus was simply not shown in Fig. 3A for visual clarity.

Response: We have clarified that the full sequence (residues 1-100) was simulated. We also added a figure showing the N-terminus (Supp Figure 3B) and discussed how it has some interactions with the PLs in each system, but they do not add significantly to stabilization and they are similar in all systems (shown for TG-LD and ER in Supp Fig 3C).

The authors showed one frame for each simulation. Given that the authors mainly discuss the conformational changes of the protein upon binding to TG-abundant LDs, SE-abundant LDs, and bilayers observed in the MD trajectories, the authors should provide more snapshots including the initial structures.

Response: Thank you for this suggestion. We have added conformations capturing the beginning, middle, and end of the simulations for all three systems (Supp Fig 3D, E, F).

One of the conclusions from this manuscript is that the three polar residues, Gln72, Cys75, and Ser76, near the kink of HR2 play a critical role in driving the HR2 conformational changes. The authors also suggest that these polar residues can explain Tld1's differential targeting preference. If these residues are mutated to hydrophobic residues, would the mutant construct bind to both the ER bilayer and TG-abundant LDs but not SE-abundant LDs?

Response: We appreciate this suggestion. In the revised manuscript we have verified the importance of these residues with mutations. As predicted, Q72 and S76 play the dominant role in opening the kinked region in the

ER membrane. Converged free energy profiles show that removing one residue (S76A) decreases the driving force to open in the ER membrane, which could reduce the kinetic barrier to transition to a kinked conformation in the LD but may also stabilize HR2 in the ER, consistent with partial LD targeting experimentally. Removing both residues (Q72A+S76A) further stabilizes HR2 in the ER membrane in a deeper conformation, significantly decreasing LD targeting experimentally. In line with this, when the Q72A+S76A mutant is expressed in yeast and human cells it shows reduced LD targeting (Fig 5). Mutants of HR1 also block LD targeting (K36E and F44D) (Fig 5), verifying HR1 plays the dominant role in LD targeting and TG-rich preference.

The authors cited Campomanes et al., 2021, which proposed a new TG forcefield with significantly reduced partial charges of the glycerol moiety of TG. If the authors used this model, they should explicitly mention this in the main text. If the authors reduced the partial charges of the glycerol moiety of SE from their original paper (Braun and Swanson, 2022), this should be also clearly indicated.

Response: We have clarified that we did not reduce the partial charges as proposed by Campomanes et al. 2021. This forcefield has not been verified to retain experimentally measured LD packing defects or area per lipid headgroup (Kim et al., 2022). While we believe it does better capture the neutral-lipid water line tension (Camponmanes et al., 2021), we also believe the chosen force field better represents LD properties. We look forward to polarizable models that can properly handle both a neutral lipid- water interface and LD APL properties. Once those are available, these types of studies should be compared to the results with polarizable force fields. However, while the errors in the employed force field may shift quantitative numbers a bit, we are confident they will not change our comparative analysis or the conclusions in this study.

The authors mentioned the association of the HR1 amphipathic helix and the surface packing defects multiple times and attributed it to different binding behaviors of HR2 toward TG-abundant LDs, SE-abundant LDs, and ER bilayers (line 215 of page 7, line 222 of page 8, legends of Fig. 3A, S3I, Fig.S3J, Fig. S3K). However, I do not see any snapshots that contain packing defects. Additional figure of the snapshots should be included.

Response: We have added a figure clearly highlighting the packing defects in Supp Fig 3G.

The authors performed 4.5 us simulations of the bilayer and TG-only LD system and 1.0 us simulation of the SE-abundant LD system. The authors should provide a justification for the different simulation times as well as the approximate timescale of HR1 association with the TG-only surface and bilayer.

Response: We now explain that we were limited on Anton2 simulation time and had to run the SE-rich LD on other resources. We present RMSDs to show the SE-rich LD simulations are stable in this time frame (Supp Fig S3A).

The authors proposed a model in which Tld1 coated the LD surface, making other lipase enzymes less accessible to the LD surface and TG binding sites. The authors should discuss why the overexpression of Pln1, perilipin-like protein that binds to the LD surface and expected to have a comparable role with Tld1, did not affect the TG level (Fig. 5E).

Response: Thank you for this concern. We agree that while we have some evidence to support a model where Tld1 can interact with TG, this is not sufficient to propose that Tld1 TG binding may limit lipase access to TG. We have adjusted the text in several places to address this with our working model. We also now provide a "limitations of study" section to the updated manuscript that discusses this in more depth.

MINOR
It would be more informative if the authors used the simulation time (us) instead of the number of frames in Fig. S3A, Fig. S3I, Fig. S3J, Fig.S3K.

Response: We have changed the x-axes accordingly.

It would be more informative if the authors included a single-letter amino acid in front of the residue number in Fig. S3D, Fig. S3E, and Fig. S3F.

Response: We have included the amino acid letters.

In the setup of the MD simulations, the overlapping phospholipids and neutral lipids were removed. The authors should report the final number of phospholipids for each leaflet and for each system.

Response: We have reported these numbers.

The authors should describe how the angle of HR2's helix-kink-helix structure was calculated in the MD simulations (Fig. S3B).

Response: We have included how the angles were calculated. Theta of the kink region (Gly78) was determined with reference to Met66 and Arg100 (the surface-oriented anchor residues).

Packing defects lie on a 2-dimensional plane without a meaningful z-value. The authors should justify why the contacts between packing defects and residues, which are not commonly used, are significant and describe this procedure in detail.

Response: We have addressed why we used contact analysis. Contacts between defined hydrophobic defects and hydrophobic heavy atoms of the protein describe the quantity of interactions in a given defect and allow us to compare the three different simulation systems quantitatively.

Please review the simulation parameters for Anton simulations. I believe Anton uses MTK pressure (see below), the M-SHAKE algorithm (instead of LINCS) and the Gaussian split Ewald method (instead of PME).
Martyna, G. J.; Tobias, D. J.; Klein, M. L. Constant pressure molecular dynamics algorithms. J. Chem. Phys. 1994, 101 (5), 4177− 4189.
Lippert, R. A.; Predescu, C.; Ierardi, D. J.; Mackenzie, K. M.; Eastwood, M. P.; Dror, R. O.; Shaw, D. E. Accurate and efficient integration for molecular dynamics simulations at constant temperature and pressure. J. Chem. Phys. 2013, 139 (16), 164106.

Response: We have corrected the simulation parameters for Anton.

I believe the authors used C36m for a protein force field, not C36 (page 20, line 607). The protein force field was not cited.

Response: We have corrected this error and added the appropriate protein forcefield citation.

September 26, 2023

RE: JCB Manuscript #202303026R

Dr. Mike Henne
The University of Texas Southwestern Medical Center
Cell Biology
6000 Harry Hines Blvd NL6.120D
Dallas, Texas 75390

Dear Dr. Henne:

Thank you for submitting your revised manuscript entitled "Tld1 is a novel regulator of triglyceride lipolysis that demarcates a lipid droplet subpopulation". We would be happy to publish your paper in JCB pending final revisions necessary to meet our formatting guidelines (see details below).

To avoid unnecessary delays in the acceptance and publication of your paper, please read the following information carefully.

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Full guidelines are available on our Instructions for Authors page, https://jcb.rupress.org/submission-guidelines#revised. **Submission of a paper that does not conform to JCB guidelines will delay the acceptance of your manuscript.**

1) Text limits: Character count for Articles is < 40,000, not including spaces. Count includes abstract, introduction, results, discussion, and acknowledgments. Count does not include title page, figure legends, materials and methods, references, tables, or supplemental legends.

2) Figures limits: Articles may have up to 10 main text figures.

3) Figure formatting: Scale bars must be present on all microscopy images, including inset magnifications. Molecular weight or nucleic acid size markers must be included on all gel electrophoresis. Please avoid pairing red and green for images and graphs to ensure legibility for color-blind readers.

4) Statistical analysis: Error bars on graphic representations of numerical data must be clearly described in the figure legend. The number of independent data points (n) represented in a graph must be indicated in the legend. Statistical methods should be explained in full in the materials and methods. For figures presenting pooled data the statistical measure should be defined in the figure legends. Please also be sure to indicate the statistical tests used in each of your experiments (either in the figure legend itself or in a separate methods section) as well as the parameters of the test (for example, if you ran a t-test, please indicate if it was one- or two-sided, etc.). Also, if you used parametric tests, please indicate if the data distribution was tested for normality (and if so, how). If not, you must state something to the effect that "Data distribution was assumed to be normal but this was not formally tested."

5) Abstract and title: The abstract should be no longer than 160 words and should communicate the significance of the paper for a general audience. The title should be less than 100 characters including spaces. Make the title concise but accessible to a general readership.

* The term "novel" needs to be removed from the title as per JCB policy

6) Materials and methods: Should be comprehensive and not simply reference a previous publication for details on how an experiment was performed. Please provide full descriptions in the text for readers who may not have access to referenced manuscripts.

7) Please be sure to provide the sequences for all of your primers/oligos and RNAi constructs in the materials and methods. You must also indicate in the methods the source, species, and catalog numbers (where appropriate) for all of your antibodies. Please also indicate the acquisition and quantification methods for immunoblotting/western blots.

8) Microscope image acquisition: The following information must be provided about the acquisition and processing of images:
a. Make and model of microscope
b. Type, magnification, and numerical aperture of the objective lenses
c. Temperature
d. Imaging medium

e. Fluorochromes
f. Camera make and model
g. Acquisition software
h. Any software used for image processing subsequent to data acquisition. Please include details and types of operations involved (e.g., type of deconvolution, 3D reconstitutions, surface or volume rendering, gamma adjustments, etc.).

9) References: There is no limit to the number of references cited in a manuscript. References should be cited parenthetically in the text by author and year of publication. Abbreviate the names of journals according to PubMed.

10) Supplemental materials: There are strict limits on the allowable amount of supplemental data. Articles may have up to 5 supplemental figures. Please also note that tables, like figures, should be provided as individual, editable files. A summary of all supplemental material should appear at the end of the Materials and methods section.

11) eTOC summary: A ~40-50-word summary that describes the context and significance of the findings for a general readership should be included on the title page. The statement should be written in the present tense and refer to the work in the third person.

12) Conflict of interest statement: JCB requires inclusion of a statement in the acknowledgements regarding competing financial interests. If no competing financial interests exist, please include the following statement: "The authors declare no competing financial interests." If competing interests are declared, please follow your statement of these competing interests with the following statement: "The authors declare no further competing financial interests."

13) ORCID IDs: ORCID IDs are unique identifiers allowing researchers to create a record of their various scholarly contributions in a single place. Please note that ORCID IDs are now *required* for all authors. At resubmission of your final files, please be sure to provide your ORCID ID and those of all co-authors.

14) A separate author contribution section following the Acknowledgments. All authors should be mentioned and designated by their full names. We encourage use of the CRediT nomenclature.

Please note that JCB now requires authors to submit Source Data used to generate figures containing gels and Western blots with all revised manuscripts. This Source Data consists of fully uncropped and unprocessed images for each gel/blot displayed in the main and supplemental figures. Since your paper includes cropped gel and/or blot images, please be sure to provide one Source Data file for each figure that contains gels and/or blots along with your revised manuscript files. File names for Source Data figures should be alphanumeric without any spaces or special characters (i.e., SourceDataF#, where F# refers to the associated main figure number or SourceDataFS# for those associated with Supplementary figures). The lanes of the gels/blots should be labeled as they are in the associated figure, the place where cropping was applied should be marked (with a box), and molecular weight/size standards should be labeled wherever possible.
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B. FINAL FILES:

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Thank you for this interesting contribution, we look forward to publishing your paper in Journal of Cell Biology.

Sincerely,

Tobias Walther, PhD
Monitoring Editor

Andrea L. Marat, PhD
Senior Scientific Editor

Journal of Cell Biology

Reviewer #1 (Comments to the Authors (Required)):

The authors have thoughtfully and thoroughly responded to my previous comments. I congratulate them on a beautiful and exciting study. I strongly support publication.

Reviewer #3 (Comments to the Authors (Required)):

The authors have strengthened their study during revision and provided more compelling experimental and computational data about the function and targeting of Tld1.

First, the evidence of Tld1 selective targeting to TG-rich LDs over SE-rich LDs have become stronger in the revised manuscript: A) The authors have shown that their previous conclusion, limited to yeast cells, can be drawn for the human cell lines (U2OS and HeLa). Tld1 selectively targets to TG-rich LDs in both human cells, and the overexpression of Tld1 can control the LD size in HeLa. B) The authors calculated the potential of mean force (PMF) of the wild-type peptide and mutant peptides binding to the monolayer and bilayer. C) The authors identified the critical residues in LD targeting, which were confirmed experimentally and computationally.

Second, the additional MD simulation data and analysis are convincing, and their MD setup and parameters were adequately described.

Finally, the authors have discussed the limitation of the current study and the future work on identifying the mechanism of Tld1 in TG lipolysis regulation.

Therefore, the authors have thoroughly responded to my previous comments. I strongly support publication.