JCB

Zc3h10 regulates adipogenesis by controlling translation and F-actin/mitochondria interaction

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Re: JCB manuscript #202003173

Dr. Nico Mitro
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Dear Dr. Mitro,

Thank you for submitting your manuscript entitled "Zc3h10 regulates adipogenesis by controlling translation and F-actin/mitochondria interaction". The manuscript has been evaluated by expert reviewers, whose reports are appended below. Unfortunately, after an assessment of the reviewer feedback, our editorial decision is against publication in JCB.

You will see that while the reviewers find the potential description of a role of Zc3h10 in adipogenesis interesting, and appreciate that your study provides a great deal of phenotypic characterizations, they have brought up significant conceptual as well as technical concerns. Therefore, although your manuscript is intriguing, I feel that the points raised by the reviewers are more substantial than can be addressed in a typical revision period. If you wish to expedite publication of the current data, it may be best to pursue publication at another journal. Our journal office will transfer the reviewer comments to another journal at your request.

Given interest in the topic, I would be open to resubmission to JCB of a significantly revised and extended manuscript that fully addresses the reviewers' concerns and is subject to further peer-review. In particular, this would require (1) a reorganization and trimming of current data, (2) that you provide more mechanistic insights including (but not limited to) an examination of the cell cycle as well as metabolic studies (3) that you include studies at earlier time points. Please keep in mind a successful revision would require fully addressing the significant conceptual concerns, notably those from reviewers 1 and 2, as well as the concerns regarding statistics, methodology, and organization from all reviewers.

If you would like to resubmit this work to JCB, you must contact the journal office to discuss an appeal of this decision or you may submit an appeal directly through our manuscript submission system. In particular, you should prepare a detailed revision plan outlining how you will restructure and refocus your study, which we may discuss with the reviewers. Please note that priority and novelty would be reassessed at resubmission.

Regardless of how you choose to proceed, we hope that the comments below will prove constructive as your work progresses. We would be happy to discuss the reviewer comments further once you've had a chance to consider the points raised in this letter. You can contact the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Thank you for thinking of JCB as an appropriate place to publish your work.

Sincerely,

Johan Auwerx, MD, PhD
Monitoring Editor

Andrea L. Marat, PhD
Scientific Editor

Journal of Cell Biology

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

The aim of this manuscript is to prove the participation of the transcription factor Zc3h10 in adipogenesis. Zc3h10 was previously shown by the same lab to regulate mitochondrial activity and differentiation of myoblasts. They now claim that Zc3h10 controls the early phases of adipogenesis, notably the mitotic clonal expansion. The manuscript contains a substantial amount of data to characterize distinct events involved in adipocyte differentiation in Zc3h10 depleted or overexpressing cells. The studies include actin reorganization, mitochondrial dynamics and function, regulation of cell shape, lipid accumulation, protein translation, and signaling pathways such as Rho or mTOR targets. They show that Zc3h10 controls the expression of genes belonging to translation and actin cytoskeleton organization pathways, and modulates the expression of genes involved in mitochondrial function and cell cycle. They also show that the effects of Zc3h10 are mediated, at least partially, through the regulation of the Rho-GTPases activities. A lot of pathways are analyzed here, making the manuscript too heavy with too many figures and some data that are not necessarily relevant. Moreover, the authors do not provide mechanisms and the question of how specifically Zc3h10 regulates the different pathways that are shown, remains unanswered. The manuscript could be more focused on specific pathways or targets to demonstrate how Zc3h10 participates in lipogenesis. I also have the following concerns and suggestions.

1. It's confusing the precise timing of the effects of Zc3h10 in adipogenesis. From the data presented here, the effects of Zc3h10 in preadipocytes and in mature adipocytes can't be distinguished. Indeed, the expression of Zc3h10 is higher in fully differentiated adipocytes (Sup. Figure 1A), suggesting a role of the protein at this stage. Differential inhibition of Zc3h10 at the different phases of adipogenesis (at least in preadipocytes and mature adipocytes) would address this question.

2. The authors show that Zc3h10 silencing decreases mitochondrial activity, TCA cycle, and ETC in preadipocytes. They claim that these cells compensate the defects in glycolysis with an increase in fatty acid oxidation ("partial loss of Zc3h10 in pre-adipocytes led to TCA cycle defects, which blunted metabolic pathways sustained by glucose and glutamine utilization and imposed fatty acid oxidation to maintain energy homeostasis (Fig. 5F)"). How fatty acid oxidation could provide the energy if the TCA, and ETC are not properly functioning? Seahorse analysis (or other tests) specifically testing fatty acid oxidation and glycolysis could help to reveal more precisely how changes the energy metabolism.

3. In the figure 5L, a more precise representation of the flux metabolomics analysis would rather help to understand the results (see for instance a paper from the same authors as an example:

https://www.embopress.org/doi/full/10.15252/embr.201745531)

4. Cell cycle effects are paradoxical. Indeed, the authors say that MCE starts 4 days after induction of differentiation. This is far too late from a typical differentiation essay in which MCE starts after some hours of the addition of the differentiation cocktail. Moreover there is a discrepancy between the data in figure 6D-E, showing an increase in the cell number on day 4 after the beginning of differentiation, and the data presented in figure 6F-G illustrating the cell cycle distribution. Indeed, this last figure suggests that starting at day 3 the cells are quiescent in the control group (90% in G0/G1), which is not consistent with the high proliferation rate showed in 6D. Moreover, in the figure 6F, from days 1 to 3 a progression into the cell cycle can be observed. The authors could test the markers of the cell cycle, such as cyclins A, B, D, and E by western blot or RNA to better define the different cell cycle phases.

5. Another remark concerning the cell cycle experiment is the observation that post-confluent cells increase 4- to 5-fold their number. How is this possible assuming that the cell culture surface did not change? Also, at day 9 the authors assume that these cells are adipocytes, but as indicated in the panel D the cells are still proliferating.

6. Inhibition of Zc3h10 results in the arrest of the cells in the G2/M phase. In this phase, CDK1 phosphorylates and activates Drp1 to promote mitochondrial fission. Could the authors correlate Drp1 phosphorylation with the G2/M observed arrest and increased mitochondrial number?

7. Another paradox is that both inhibition and overexpression of Zc3h10 results in cell cycle arrest, although at different phases. How Zc3h10 control cell cycle progression during adipogenesis? Moreover, the authors showed in a previous publication that Zc3h10 has no effect on the proliferation of myoblasts.

8. From the data shown, Zc3h10-depleted cells become adipocytes but have deficient de novo lipogenesis. There is decreased lipid biosynthesis precursors and enzymes in Zc3h10 inhibited cells. Functional studies in differentiated cells +/- Zc3h10, such as lipogenesis, lipolysis, or glucose and lipid uptake, which are typical functions of adipocytes, should be tested.

9. The authors also show that both depletion and overexpression of Zc3h10 results in decreased cell size in preadipocytes and increased cell size in adipocytes. These results are paradoxical. What are the mechanisms?

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

Review of "Zc3h10 regulates adipogenesis by controlling translation and F-actin/mitochondria interaction"

Introduction:

In this manuscript, the authors characterize the role of zinc finger CCCH-type containing 10 (Zc3h10) in the adipogenic program, specifically adipose differentiation. There are two main RNA sequencing datasets in Zc3h10 knockdown cells that drive subsequent experiments. In pre-adipocytes, the authors find that Zc3h10 depletion causes altered expression of genes involved in cytosolic protein synthesis and actin cytoskeleton organization pathways. In mature adipocytes,

they find that Zc3h10 depletion causes dysregulation of cell cycle and mitochondrial genes. Throughout this manuscript extensive analysis of mitochondrial morphology, actin filaments, lipid droplets, and cell size is performed. In early adipocyte development, the model is that Zc3h10 promotes F-actin cytoskeleton reorganization to support mitochondrial dynamics and energetics important for differentiation. A general conclusion from these studies is that temporal expression of Zc3h10 is very important to proper adipocyte development and mitotic clonal expansion, and Zc3h10 depletion or overexpression has negative consequences.

This manuscript is extensive in analyses, which is in general positive, but it falls short firmly establishing links between the actions attributed to Zc3h10. It reads as a list of phenotypes without particular focus on an aspect of differentiation (whether mitochondrial or actin dynamic phenotypes, etc.), and development of a more complete narrative. This is not to be overly negative, as there are solid pieces of data in this manuscript especially concerning the transcriptional program of Zc3h10 and downstream effectors that the authors correctly investigate. Furthermore, it is not surprising that loss of a major regulator of adipocyte differentiation that function early in the differentiation program is going to lead to detrimental phenotypes. Focusing on earlier time points (and using the later time point data to emphasize dysregulated differentiation) would make the story cleaner, since attributing phenotypic changes to Zc3h10 at later time points - where you are essentially comparing terminally differentiated adipocytes to non-differentiated adipocytes, supported by adipocyte marker expression - is not entirely correct. Overall, this study establishes that deletion of Zc3h10 disrupts white adipocyte differentiation and prevents proper dynamics of mitochondria, actin, and lipid droplets throughout the differentiation process in these cells. Below are some additional comments:

Comments:
• Since Zc3h10 regulating protein synthesis is a major finding from paper, please bolster this data with other methodologies aside from p-4EBP1 western blots. Along these lines, are the pre-mRNAs detected in sequencing 5' TOP motif mRNAs, i.e. genes whose translation is sensitive to 4EBP1 phosphorylation status? If true, this would support your focus on 4EBP1 as a readout of translation rates.
• Along the lines, is hyperphosphorylation/deletion, i.e. loss-of-function, of 4E-BP1 sufficient to mimic the early phenotypes associated with Zc3h10 depletion on mitochondrial and F-actin networks.
• How direct are the effects of Zc3h10 on mitochondrial or metabolic phenotypes? It is difficult to discern effects of differentiation versus Zc3h10. Is there a biasing of data depending on cells quantified? For example, if you only analyze cells in the shZc3h10 population that contain lipid droplets, are mitochondrial/f-actin phenotypes still different than controls?
• What is the functional consequence of all the phenotypes discussed? For example, the initial colocalization of mitochondria with F-actin and then dissociation over time and how Zc3h10 negatively regulates this association. When mitochondria are bound to F-actin during differentiation is there more or less fusion dynamics occurring? Is a fusion phenotype important for glutamine mediated lipogenesis in order to form lipid droplets? Any effort to link the phenotypes towards a more functional outcome important for adipocyte differentiation would improve this manuscript.
• Genetic experiments could be used to bolster your data. For example, does promoting mitochondrial fusion genetically or pharmacologically bypass defects in F-actin remodeling in shZc3h10 cells and promote lipid droplet formation?
• The PKA epistasis experiments are not convincing. Removing PKA activator from the differentiation cocktail, does not preclude PKA modulation by Zc3h10. The RNA finding that Zc3h10 regulates a PKA subunit doesn't support your point either. Please target PKA genetically or pharmacologically or consider probing for PKA activity in your different conditions to demonstrate that PKA activity is lower without IBMX and is not positively modulated by Zc3h10 expression.

Minor comments:
• It is unclear how quantification is performed in this paper. For example, in Figure 1C, lipid droplet measurements only include 6 measurements per group. In the methods it states, "For lipid droplet size, images were first binarized and particles were then quantified." Considering each cell contains a dozen or more lipid droplets, how are we to interpret this data. Is this average lipid droplet size per cell? If so, it should be indicated on the axis. If not, then you need many more data points >30. This comment also holds true for quantification of F-actin networks. Please include more information regarding quantification methods for this paper.
• Please use blots with lower exposure for 4EBP1. Blots are overexposed hiding the normal ~4 band ladder pattern observed with phospho or total 4EBP1, which complicates quantitation. Also, in Fig 2B , Zc3h10 FLAG p-4EBP1 signal is quantified as 0.5 fold control despite roughly equivalent signal on the blot. I would double check this quantification. If it is a ratiometric quantification p-4ebp1/total 4ebp1 please indicate on axis or figure legends and methods.
• The goal in Figures 8 and 9 is interesting as you are asking questions regarding F-actin remodeling and its sufficiency in regulating mitochondrial and lipid droplet phenotypes. However, the data doesn't necessarily fit tightly and doesn't always repeat from previously in the manuscript. For example in Fig 9A, shZc3h10 cells are differentiating equivalent to controls (according to adipored staining) making it difficult to address a negative result from Y27632. Cell size data in Fig 9 is also not repeating from Fig 7 making data interpretation difficult.
• A linear model from f-actin>mitochondria>lipid droplets is complicated by the above issues with controls and the Ml141 data where mitochondrial phenotypes are rescued from Zc3h10 overexpression, but lipid droplet size and adipored cells are unchanged. Perhaps expand upon this latter discrepancy in the discussion.
• In discussion, "Functional validation experiments demonstrate that Zc3h10 depletion in pre-adipocytes is associated to translation inhibition as evidenced by the hyperphosphorylated status of 4E-BP1." This is backwards, it should read "translation activation". Hyperphosphorylated 4E-BP1 releases from eIF4E allowing for cap-dependent mRNA translation.
• Considering incorporating a model figure for this paper. This will help the reader and perhaps aid in the organization/emphasis of certain data in this manuscript.

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

In this study entitled: Zc3h10 regulates adipogenesis by controlling translation and F-actin/mitochondria interaction, Audano and colleagues propose a physiological role for the zinc finger CCCH-type containing 10 (Zc3h10) TF in the context of white adipogenesis. Zc3h10 was previously identified by the same group as a mitochondrial regulator that is required during myogenesis (Audano et al., 2018), as well as a factor regulating the thermogenic program in brown adipose tissue (Yi et al., 2019). It was also found to be one of the most enriched genes in mammalian adipose tissue (albeit based on sheep breed transcript data) (Li et al., 2018). To study the molecular processes that are regulated by Zc3h10 during white adipogenesis, a wide range of assays was used, revealing that Zc3h10, via its transcriptional activities, represses protein translation and promotes cytoskeleton remodelling. This in turn determines mitochondrial function, metabolic activation and cell shape, enabling mitotic clonal expansion (MCE) and, ultimately, lipid loading. The authors thereby suggest that Zc3h10 is involved in the early stages of adipogenesis, given that several of the modulated processes are most active early during this differentiation process.

The employed experimental approaches are based on classical methods of cell and molecular

biology including shRNA-mediated Zc3h10 depletion, its adenoviral overexpression, transcriptomics, metabolomics, oxygen consumption measurements, mitochondrial dynamics measurements, cell cycle analysis and others. The novelty of the study lies in the elucidation of the physiological role of Zc3h10 during white adipogenesis.

A strength of the study is certainly that multiple aspects of Zc3h10's involvement in adipogenesis are dissected by a myriad of approaches and assays. Overall, the manuscript and the science underneath it appear of reasonable to good quality. However, the multitude of experiments often leaves the reader lost with respect to the importance and relevance of the addressed features. Indeed, this data overload substantially clouds clarity on the findings, which is why a thorough structural and grammatical revision of the manuscript is highly recommended. The goal here is to simplify the paper's structure, to provide a better rationale of why certain analyses / data are presented, and to interpret the data in a more biologically meaningful way.

Comments:
1. The authors show the effects of both knockdown and overexpression of Zc3h10 in almost all the assays. It is however unclear why the consequences of these perturbations are not always consistent. For example, why does both knockdown and overexpression of Zc3h10 reduce the number of adipocytes (Figure 1B and E)? This question applies also to other assays, whose data is shown in Figures 2D/F, 3B/D, 5A, 6D/E. The authors should thus interpret the available data in a clearer way or at least provide sound explanations for these puzzling phenomena.

2. An important weakness of the current study is that overarching statements are made based on overall rather subtle differences. While often reported as "significant", the absence of details on which type of statistical test is exactly used (e.g. the authors often mention "Student's t test", but which one?) and on whether these analyses are corrected for multiple testing makes the interpretation of what "significant" precisely means unclear. One of the reasons why only subtle differences are detected in a knockdown setting could be the sizeable Zc3h10 protein amount that remains in cells after the knockdown. To support their findings, the authors would do well to generate a full Zc3h10 knockout cell line (e.g. by CRISPR) and to repeat some of the principal analyses to test whether this makes the overall impact of the perturbation more meaningful.

3. A point in case illustrating the subtle differences is the nascent mRNA analysis, which overall revealed few striking transcriptional differences, i.e. almost all differentially regulated pre-mRNAs were less than two-fold different. Downstream protein work validated some of the observations, yet it is surprising that the authors chose not to better integrate these data with other downstream analyses involving transcriptomic profiling of wildtype and knockdown mature adipocytes. This is especially relevant since, as one example, in earlier stages of adipogenesis, Zc3h10 knockdown appears to downregulate pre-mRNAs involved in actin filament-based processes, yet the opposite is true for mature mRNAs later in adipogenesis, even though even more Zc3h10 is being expressed then. A more profound data integration may allow the authors to reconcile or refute these contrasting observations…

4. It is laudable that the authors provide both the negative and positive data. But instead of simply showing all the data, it would be even more valuable if the authors could guide the reader in better understanding the biological meaning of their observations. For example, in Figures 2D and 2E, which property of F-actin does each of the parameters stand for? The same for Figures 3B and D.

5. Data shown in Figure 5A demonstrates that there is more oxygen consumption in a Zc3h10 overexpression context when oligomycin is added. How do the authors explain this proton leakage?

Are there more mitochondria that are damaged in these cells?

6. The authors claim that Zc3h10 controls the early stages of white adipogenesis. However, this conclusion seems to be only supported by an observation of Zc3h10-depleted adipocytes showing mitochondrial dysfunction in their mature state. How can the authors confidently conclude that Zc3h10 is exclusively relevant in the early stages of differentiation without targeting this TF at later stages of adipogenesis?

7. The observation that Zc3h10 may directly impact the cell cycle obviously has important consequences in terms of how this factor may influence adipogenesis, since the cell cycle and early differentiation are heavily intertwined. Can this factor be directly found on the mitotic spindle? And do the authors think that all other effects stem from the factor's role in the cell cycle? (chicken / egg question)

8. The methodology that is used to quantify in vitro adipogenesis, one of the main readouts employed in the study, is not at all clear.
The authors claim that the "Lipid accumulation and cell shape analyses" are conducted using flow cytometry:
The cytofluorometer settings for both side scatters (SSC) and forward scatters (FSC) to analyse cell shape were dependent on the analytic sensitivity of the machine. The voltages and compensation between scatters were set to the degree so that most control cells at day 0 were located above the scale of 105 for both the SSC and the FSC. Cell shape analysis was performed by measuring the median FSC- A of the whole range of the cells.
The way these results are represented and annotated in the manuscript leaves the reader to believe that the number of differentiated cells is inferred from the microscopic images, presented alongside (Fig. 1A and B, D and E, G and I, J and L, etc). Using flow cytometry measurements for in vitro adipogenesis quantification is not referenced and no supporting data is presented to demonstrate how this quantification is performed.

Concerning the measurements of lipid droplet sizes, this time based on the microscopy images, the authors included a sparse comment in the methods section:
Confocal microscopy image processing and analysis
For lipid droplet size, images were first binarized and particles were then quantified.
These two parameters, 1) the relative number of differentiated cells (adipored+) and 2) the lipid droplet size, are widely used throughout the study and serve as a principal basis for the main conclusions that are presented in the manuscript. A thorough documentation of how these parameters were obtained is absolutely necessary to support the proposed observations.

This is especially relevant since accurate quantification of in vitro adipogenesis is highly challenging and numerous methods have been described, starting from the classical Oil Red O staining of fixed cells and subsequent imaging of cells, inferring a ratio of lipid and nucleic acid-specific dye intensities over colorimetric measurements of adipocyte lysates to imaging live differentiating cells using specific/high throughput imaging platforms and employing mathematical models to analyse the degree of adipogenic lipid accumulation or/and size distribution of lipid-containing intracellular vesicles as a read-out of assayed conditions (Eom et al., 2018; Kraus et al., 2016; Varinli et al., 2015; Yuan et al., 2019).

9. A similar comment applies to assays addressing F-actin morphology and mitochondrial dynamics. Zc3h10 was shown to affect actin filament-based processes and actin cytoskeleton organisation, which in turn affects mitochondrial dynamics. The morphology of F-actin, including the shape and

length of the filaments, the number of punctuate/rounded filaments as well as the number and morphology of junctions and branches, were heavily used as a readout of the morphological changes that are affected by Zc3h10.
However, the methodology describing the image acquisition and analysis of the F-actin and mitochondrial networks is yet again inadequate. Even though the image treatment algorithm developed for this type of data is this time referenced (Valente et al., 2017), there is a complete lack of description of how the method needed to be adapted to the presented study. Intermediate images of the pipeline leading to image skeletonisation, presumably yielding the numerical values, are also missing (except representative binary images (i.e. Fig. 2C, E, G, H, Fig. 3A, C)).

10. In general, the methods section thus lacks a detailed description of many of the utilized, experimental procedures.

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JOURNAL OF CELL BIOLOGY MANUSCRIPT #202003173 Audano M. et. al.

Response to Reviewers

We thank the reviewers for their insightful comments. We have carefully received and addressed their suggestions to enhance our manuscript. Luckily, we were allowed to perform some key experiments during the COVID-19 pandemic that enabled us to revise the manuscript extensively and address each comment from the reviewers. We have paid particular attention to the experiments suggested to further strengthen the metabolic data and to elucidate the details of the molecular mechanisms governed by Zc3h10. In addition, with the aim of selecting data that would contribute to building a logical and convincing manuscript, as agreed by the editor, we have removed from the current manuscript all data related to the cell cycle (Fig. 6D-G of the original version of the manuscript) and cell size (Fig. 7A-B, 9D-F of the original version of the manuscript); therefore, the suggested experiments on these topics that were requested by the reviewers have not been performed. We have also thoroughly revised the text to enhance the flow of our manuscript as well as the clarity and presentation of our findings. As a result of these additions and changes, the paper is considerably improved, and we are indebted to the reviewers for their aid. We believe that we have addressed all concerns, as detailed below:

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

The aim of this manuscript is to prove the participation of the transcription factor Zc3h10 in adipogenesis. Zc3h10 was previously shown by the same lab to regulate mitochondrial activity and differentiation of myoblasts. They now claim that Zc3h10 controls the early phases of adipogenesis, notably the mitotic clonal expansion. The manuscript contains a substantial amount of data to characterize distinct events involved in adipocyte differentiation in Zc3h10 depleted or overexpressing cells. The studies include actin reorganization, mitochondrial dynamics and function, regulation of cell shape, lipid accumulation, protein translation, and signaling pathways such as Rho or mTOR targets. They show that Zc3h10 controls the expression of genes belonging to translation and actin cytoskeleton organization pathways, and modulates the expression of genes involved in mitochondrial function and cell cycle. They also show that the effects of Zc3h10 are mediated, at least partially, through the regulation of the Rho-GTPases activities. A lot of pathways are analyzed here, making the manuscript too heavy with too many figures and some data that are not necessarily relevant. Moreover, the authors do not provide mechanisms and the question of how specifically Zc3h10 regulates the different pathways that are shown, remains unanswered. The manuscript could be more focused on specific pathways or targets to demonstrate how Zc3h10 participates in lipogenesis. I also have the following concerns and suggestions.

1. It's confusing the precise timing of the effects of Zc3h10 in adipogenesis. From the data presented here, the effects of Zc3h10 in preadipocytes and in mature adipocytes can't be distinguished. Indeed, the expression of Zc3h10 is higher in fully differentiated adipocytes (Sup. Figure 1A), suggesting a role of the protein at this stage. Differential inhibition of Zc3h10 at the different phases of adipogenesis (at least in preadipocytes and mature adipocytes) would address this question.

Response: In response to the reviewer's concerns, we have added functional data related to Zc3h10 depletion in mature adipocytes (following the protocol in Supplementary Fig. 1J) that shows unchanged lipid accumulation (Oil Red O staining), basal respiration, and mtDNA content (Supplementary Fig. 1K-N). These results indicate that the effects of Zc3h10 on lipid accumulation

and mitochondrial function depend on the stage of adipocyte differentiation; more specifically, Zc3h10 impacts the beginning of the differentiation program.

2. *The authors show that Zc3h10 silencing decreases mitochondrial activity, TCA cycle, and ETC in preadipocytes. They claim that these cells compensate the defects in glycolysis with an increase in fatty acid oxidation ("partial loss of Zc3h10 in pre-adipocytes led to TCA cycle defects, which blunted metabolic pathways sustained by glucose and glutamine utilization and imposed fatty acid oxidation to maintain energy homeostasis (Fig. 5F)"). How fatty acid oxidation could provide the energy if the TCA, and ETC are not properly functioning? Seahorse analysis (or other tests) specifically testing fatty acid oxidation and glycolysis could help to reveal more precisely how changes the energy metabolism.*

Response: We thank the reviewer for bringing up this important point that prompted us to better describe the metabolic phenotype of Zc3h10-depleted cells. In this regard, *Zc3h10*-silenced pre-adipocytes do not experience obvious modifications of basal mitochondrial function; indeed, no differences in absolute levels of ATP or basal respiration were detected (Fig 5A and Supplementary Fig. 4A). However, we disclosed a significant rewiring of the metabolic pathways fueling the TCA cycle (Fig. 5B-G and Supplementary Fig. 4A-E), suggesting the presence of subtle metabolic defects. Notably, only metabolic stress-based experiments in Zc3h10-depleted cells unmasked defects in mitochondrial function. In the previous version of the manuscript, we reported that Zc3h10-depleted pre-adipocytes, when stressed, failed to cope with CCCP-mediated uncoupled maximal respiration (Fig. 5A of the new version of the manuscript). Along these lines, we performed metabolic stress-based experiments using Seahorse technology to investigate the contribution of different metabolic fuels to ATP generation. Energy (ATP) produced by cells can be derived from mitochondrial oxidation of glucose (pyruvate), glutamine (glutamate), and fatty acids; therefore, to examine the role of Zc3h10 in ATP synthesis, we exposed *Zc3h10*-silenced pre-adipocytes to Etomoxir (an inhibitor of long chain fatty acid oxidation), UK5099 (an inhibitor of the glucose oxidation pathway), and BPTES (an inhibitor of glutaminase I). Etomoxir inhibits carnitine palmitoyl-transferase 1 (Cpt1), which is critical for translocating long chain fatty acids from the cytosol into the mitochondria for beta oxidation (Fig. 5H of the new version of the manuscript). UK5099 blocks mitochondrial pyruvate carrier (Mpc), which is responsible for transporting pyruvate (generated through glycolysis) into the mitochondria so that it can be oxidized by the TCA cycle (Fig. 5J of the new version of the manuscript). BPTES inhibits the conversion of glutamine to glutamate, which is then converted to alpha-ketoglutarate and oxidized by the TCA cycle (Fig. 5L of the new version of the manuscript). Consistently, Seahorse XF real time ATP rate assays confirmed no differences in total ATP levels in untreated cells (Fig. 5I, K, and M, see total ATP production rate in the absence of metabolic inhibitors, right panels of the new version of the manuscript). In contrast, Zc3h10-depleted pre-adipocytes showed significant decreases in mitochondrial ATP production rates when challenged with inhibitors (Fig. 5I, K and M, see mitochondrial ATP production rate, middle panels of the new version of the manuscript), but these effects were maintained for total ATP levels only with Etomoxir and UK5099 treatments (Fig. 5I, K and M, see total ATP production rate in the presence of metabolic inhibitors, right panels of the new version of the manuscript). These data demonstrate the inability of *Zc3h10*-silenced cells to adapt to metabolic stress that is mainly due to glucose and fatty acid oxidation inhibition. Moreover, we also observed the ability of Zc3h10-depleted cells to cope with mitochondrial ATP impairment by glycolysis when glutaminolysis was inhibited (Fig. 5M, see glycolysis ATP production rate, left panel of the new version of the manuscript).

3. In the figure 5L, a more precise representation of the flux metabolomics analysis would rather help to understand the results (see for instance a paper from the same authors as an example: https://www.embopress.org/doi/full/10.15252/embr.201745531).

Response: Based on the reviewer's suggestion, we have now incorporated the key metabolites from the three different metabolic flux analyses into a TCA cycle diagram, as in our previous work (Fig. 5G). Single histograms of the three different metabolic flux analyses are also present for pre-adipocytes in Supplementary Figure 5B, D and E. To avoid redundant data, we decided to remove the metabolic flux analyses in mature adipocytes, as they described very similar effects compared to the ones depicted in pre-adipocytes.

4. *Cell cycle effects are paradoxical. Indeed, the authors say that MCE starts 4 days after induction of differentiation. This is far too late from a typical differentiation essay in which MCE starts after some hours of the addition of the differentiation cocktail. Moreover there is a discrepancy between the data in figure 6D-E, showing an increase in the cell number on day 4 after the beginning of differentiation, and the data presented in figure 6F-G illustrating the cell cycle distribution. Indeed, this last figure suggests that starting at day 3 the cells are quiescent in the control group (90% in G0/G1), which is not consistent with the high proliferation rate showed in 6D. Moreover, in the figure 6F, from days 1 to 3 a progression into the cell cycle can be observed. The authors could test the markers of the cell cycle, such as cyclins A, B, D, and E by western blot or RNA to better define the different cell cycle phases.*

5. Another remark concerning the cell cycle experiment is the observation that post-confluent cells increase 4- to 5-fold their number. How is this possible assuming that the cell culture surface did not change? Also, at day 9 the authors assume that these cells are adipocytes, but as indicated in the panel D the cells are still proliferating.

6. Inhibition of Zc3h10 results in the arrest of the cells in the G2/M phase. In this phase, CDK1 phosphorylates and activates Drp1 to promote mitochondrial fission. Could the authors correlate Drp1 phosphorylation with the G2/M observed arrest and increased mitochondrial number?

7. Another paradox is that both inhibition and overexpression of Zc3h10 results in cell cycle arrest, although at different phases. How Zc3h10 control cell cycle progression during adipogenesis? Moreover, the authors showed in a previous publication that Zc3h10 has no effect on the proliferation of myoblasts.

Response to point 4 to 7: To better focus and improve the flow of our manuscript, with the agreement of the editor, we have removed all data related to MCE, cell cycle, and cell shape. Indeed, the role of Zc3h10 in the cell cycle in general, and specifically, during adipogenesis, deserves an in-depth analysis. The reviewer's comments on this issue will help us in our future work in this direction.

8. From the data shown, Zc3h10-depleted cells become adipocytes but have deficient de novo lipogenesis. There is decreased lipid biosynthesis precursors and enzymes in Zc3h10 inhibited cells. Functional studies in differentiated cells +/- Zc3h10, such as lipogenesis, lipolysis, or glucose and lipid uptake, which are typical functions of adipocytes, should be tested.

Response: To address the reviewer's point, we have performed the following experiments in Scramble and Zc3h10-depleted pre-adipocytes and mature adipocytes:

1. Lipogenesis: This pathway is mainly sustained by glucose and glutamine metabolism. We have generated data showing that M3 Malonyl-CoA (the precursor of fatty acids) derived from either [U-$^{13}C_6$]glucose or [U-$^{13}C_5$]glutamine is decreased in Zc3h10-depleted adipocytes (Supplementary Fig. 4D and Supplementary Fig. 5F). These results suggest decreased glucose and glutamine-derived lipogenesis in *Zc3h10*-silenced cells.

2. Lipolysis: To assess effects on lipolysis, we quantified glycerol release (as a marker of triglycerides mobilization) in culture media from control and *Zc3h10*-silenced pre- (Fig. 5D) and mature adipocytes (Supplementary Fig. 5C). As expected, glycerol levels in Scramble control cells decreased during adipogenesis (from pre- to mature adipocytes). In contrast, *Zc3h10*-depleted pre-adipocytes showed increased levels of glycerol release compared to Scramble cells and failed to exhibit any loss of glycerol during differentiation (similar levels were observed between pre- (Fig. 5D) and mature adipocytes (Supplementary Fig. 5C)).

Together these results show that blunted lipogenesis and increased lipolysis occur when Zc3h10 is knocked down in adipocytes.

9. The authors also show that both depletion and overexpression of Zc3h10 results in decreased cell size in preadipocytes and increased cell size in adipocytes. These results are paradoxical. What are the mechanisms?

Response: To better focus and improve the flow of our manuscript, in agreement with the editor, we have removed all data related to MCE, cell cycle and cell shape. Indeed, the role of Zc3h10 in the cell cycle in general, and specifically, during adipogenesis, deserves an in-depth analysis. The reviewer's comments on this issue will help us in our future work in this direction.

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

Review of "Zc3h10 regulates adipogenesis by controlling translation and F-actin/mitochondria interaction"

Introduction:
In this manuscript, the authors characterize the role of zinc finger CCCH-type containing 10 (Zc3h10) in the adipogenic program, specifically adipose differentiation. There are two main RNA sequencing datasets in Zc3h10 knockdown cells that drive subsequent experiments. In pre-adipocytes, the authors find that Zc3h10 depletion causes altered expression of genes involved in cytosolic protein synthesis and actin cytoskeleton organization pathways. In mature adipocytes, they find that Zc3h10 depletion causes dysregulation of cell cycle and mitochondrial genes. Throughout this manuscript extensive analysis of mitochondrial morphology, actin filaments, lipid droplets, and cell size is performed. In early adipocyte development, the model is that Zc3h10 promotes F-actin cytoskeleton reorganization to support mitochondrial dynamics and energetics important for differentiation. A general conclusion from these studies is that temporal expression of Zc3h10 is very important to proper adipocyte development and mitotic clonal expansion, and Zc3h10 depletion or overexpression has negative consequences.

This manuscript is extensive in analyses, which is in general positive, but it falls short firmly establishing links between the actions attributed to Zc3h10. It reads as a list of phenotypes without particular focus on an aspect of differentiation (whether mitochondrial or actin dynamic phenotypes, etc.), and development of a more complete narrative. This is not to be overly negative, as there are solid pieces of data in this manuscript especially concerning the transcriptional program of Zc3h10 and downstream effectors that the authors correctly investigate. Furthermore, it is not surprising that loss of a major regulator of adipocyte differentiation that function early in the differentiation program is going to lead to detrimental phenotypes. Focusing on earlier time points (and using the later time point data to emphasize dysregulated differentiation) would make the story cleaner, since attributing phenotypic changes to Zc3h10 at later time points - where you are essentially comparing terminally differentiated adipocytes to non-differentiated adipocytes, supported by adipocyte marker expression - is not entirely correct. Overall, this study establishes

that deletion of Zc3h10 disrupts white adipocyte differentiation and prevents proper dynamics of mitochondria, actin, and lipid droplets throughout the differentiation process in these cells. Below are some additional comments:

Comments:
• Since Zc3h10 regulating protein synthesis is a major finding from paper, please bolster this data with other methodologies aside from p-4EBP1 western blots. Along these lines, are the pre-mRNAs detected in sequencing 5' TOP motif mRNAs, i.e. genes whose translation is sensitive to 4EBP1 phosphorylation status? If true, this would support your focus on 4EBP1 as a readout of translation rates.

Response: We want to thank the reviewer for bringing up this important point. All small and large ribosomal subunit (Rps/Rpl) mRNAs in metazoa contain the 5'TOP motif, a pyrimidine-rich stretch that plays a critical co-regulatory role in the expression of ribosomal protein genes (Hamilton, T.L., et al. 2006, Biochem Soc Trans 34, 12–16; Meyuhas, O., and Kahan, T. 2015, Biochim Biophys Acta 1849, 801–811; Perina, D., et al. 2011, Genomics 98, 56–63). The definition of a genome-wide TOP motif consensus is still a matter of study in the field, nevertheless, recent technological advances, including Cap Analysis Gene Expression (CAGE)-sequencing and cap-Cross-Linking Immunoprecipitation (capCLIP) (see Kirk B. Jensen 2020 bioRxiv preprint doi: https://doi.org/10.1101/2020.04.18.047571), have allowed for the refinement of a 7-nucleotide TOP motif (5'-CUYUYYC-3'). To gather information regarding whether up-regulated genes in Zc3h10-depleted cells are also significantly enriched in transcripts harboring a 5' UTR TOP motif, we annotated all the mm10 RefSeq 5' UTRs. We annotated both transcripts with a perfect match to the consensus "CUYUYYC" and transcripts with at most one mismatch in the same motif. Overall, 3699 out of 24483 genes (15.1%) had a perfect match and 12414 (50.7%) had at most one mismatch in the putative TOP motif in their 5' UTR. When analyzing the up-regulated genes in *Zc3h10*-silenced cells, we found that 191 out of 820 (23.3%) had a perfect TOP motif in their 5' UTR overlap. This number increased to 547 (66.7%) when considering RNAs with TOP motifs with at most one mismatch in the TOP consensus sequence. To assess whether these proportions of transcripts with TOP motifs were significant, indicating the specific enrichment of this motif in genes upregulated in Zc3h10-depleted cells, we performed a hypergeometric test (Fisher's exact test). Indeed, for both sets of transcripts (i.e., those harboring a perfect match to the TOP motif or those with at most one mismatch) the overlap was significant (p value = 1.27e-10 and p value = 1.6e-21 respectively, Fig. 8A). Furthermore, 5'TOP-positive transcripts were associated with Zc3h10 binding, with more than 50% of these genes being within 2kb of a Zc3h10 Chip-seq peak (Fig. 8B and C). The information regarding this analysis has been included in Supplementary Table 1 by adding the TOP motif annotation for both perfect match (0 mm) and at most one mismatch (1 mm). Taken together, these results support our focus on 4E-BP1 phosphorylation status as a readout of translation rates.

• Along the lines, is hyperphosphorylation/deletion, i.e. loss-of-function, of 4E-BP1 sufficient to mimic the early phenotypes associated with Zc3h10 depletion on mitochondrial and F-actin networks.

Response: To address this point, we silenced *4E-BP1* following the experimental protocol used for the silencing of *Zc3h10* (Supplementary Fig. 1D and Fig. 8F) and found no differences in F-actin/mitochondria interactions (Fig. 8G) or F-actin morphology (data not shown). In addition, the numbers of mitochondrial networks and rod-shaped mitochondria, along with mitochondrial length and branch length were increased, while the number of punctate/rounded mitochondria were decreased, indicating a slight difference only in the morphology of single mitochondria. Notably,

mitochondrial branches, junctions, and area (indexes of fusion) were unaffected, meaning that 4E-BP1 knockdown was unable to mimic the effects of Zc3h10 depletion on F-actin/mitochondria dynamics (Fig. 8H). Finally, the number of mature adipocytes was unchanged, while lipid droplet size was significantly reduced (Fig. 8I and J) with 4E-BP1 depletion. These results indicate that Zc3h10 negatively controls the expression of specific genes involved in protein translation pathways that are associated with a functional reduction in translational activity, a key step towards adipogenesis. In addition, the regulation of translation by Zc3h10 affects neither F-actin/mitochondria interactions nor mitochondrial fusion/biogenesis, which means that Zc3h10 controls protein synthesis and F-actin/mitochondria interactions in parallel during adipogenesis.

• *How direct are the effects of Zc3h10 on mitochondrial or metabolic phenotypes? It is difficult to discern effects of differentiation versus Zc3h10. Is there a biasing of data depending on cells quantified? For example, if you only analyze cells in the shZc3h10 population that contain lipid droplets, are mitochondrial/f-actin phenotypes still different than controls?*

Response: This is a very interesting point. The experiments performed at 36 hours after differentiation induction (pre-adipocytes) were pursued with the goal of avoiding ambiguity between Zc3h10- and differentiation-dependent phenotypes. However, it should be considered that at 36 hours after differentiation, only a few cells show lipid droplets (Supplementary Fig. 3A). Nevertheless, we analyzed the mitochondrial population of lipid positive and negative cells in both Scramble and *Zc3h10*-silenced pre-adipocytes (see figure below). First, we focused on Scramble control cells and we did not detect any differences in mitochondrial morphology between lipid droplet-positive and negative cells. On the other hand, Zc3h10-depleted cells showed impaired mitochondrial morphology in lipid-negative compared to lipid-positive pre-adipocytes. In addition, the mitochondria of lipid-positive *Zc3h10*-silenced cells did not show significant morphological alterations compared to Scramble cells. These data suggest that Zc3h10-depleted cells, whose mitochondria are compromised, are less efficient at accumulating lipids.

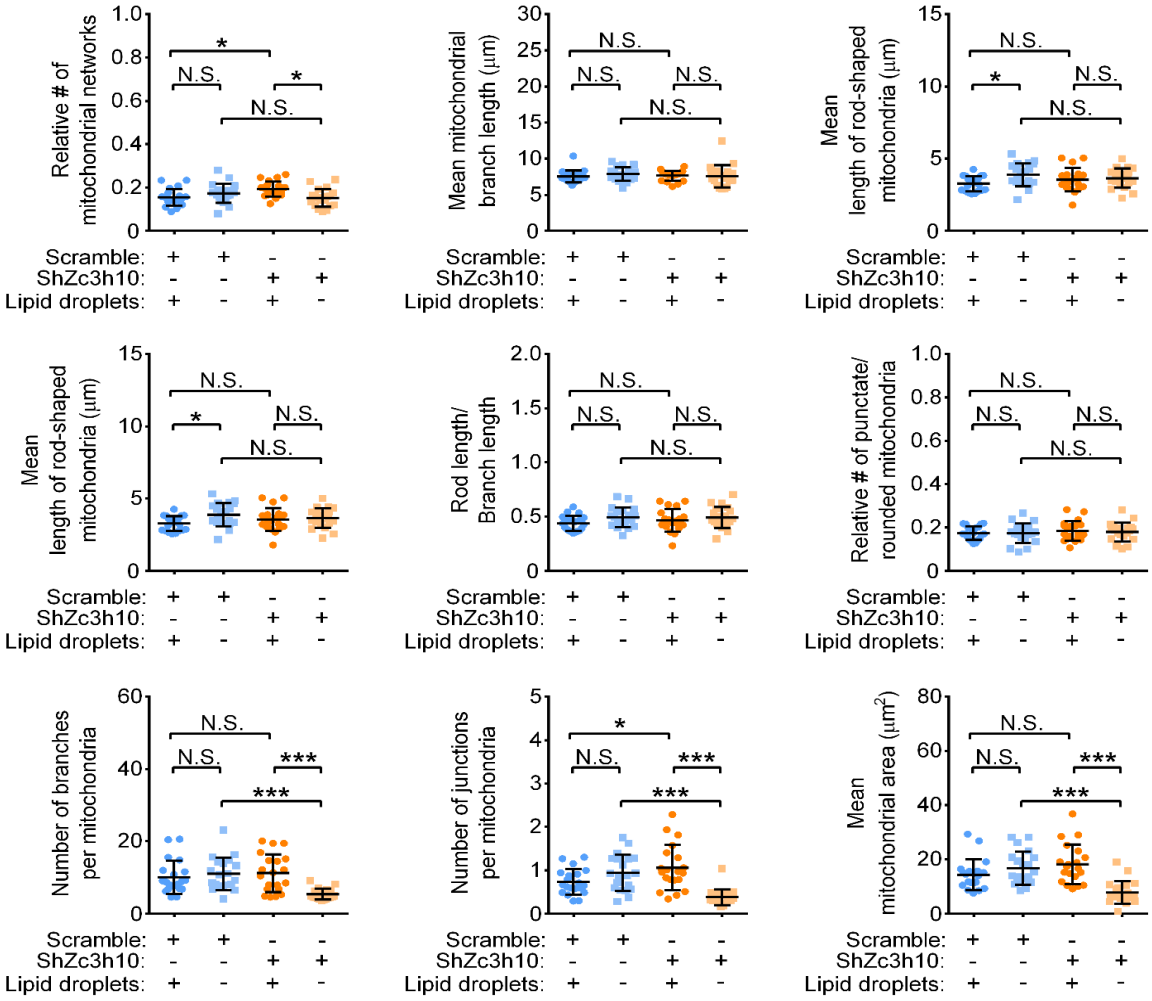

Figure Legend: Quantitative analysis of the mitochondrial network in Scramble and ShZc3h10 pre-adipocytes that were positive (Lipid droplet +) or negative for lipids (Lipid droplet -). n = 20 cells; statistical analysis was performed using One-Way ANOVA followed by Tukey's post-hoc test; *p<0.05, **p<0.01, ***p<0.001 vs Scramble. N.S.= Not Significant.

• *What is the functional consequence of all the phenotypes discussed? For example, the initial colocalization of mitochondria with F-actin and then dissociation over time and how Zc3h10 negatively regulates this association. When mitochondria are bound to F-actin during differentiation is there more or less fusion dynamics occurring? Is a fusion phenotype important for glutamine mediated lipogenesis in order to form lipid droplets? Any effort to link the phenotypes towards a more functional outcome important for adipocyte differentiation would improve this manuscript.*

Response: Before addressing the role of Zc3h10 in regulating F-actin/mitochondria networks, we first investigated how these networks change during the early phases of adipogenesis. The data in Supplementary Figure 2 indicates that F-actin/mitochondria contacts decline over time from day -2 to day 0 (Supplementary Fig. 2A). This decline was due to cell confluence (Supplementary Fig. 2B)

leading to F-actin stress fiber breakdown (decreased numbers of punctate/rounded filaments, junctions, and branches; increased number of rod-shaped filaments and mitochondrial length, Supplementary Fig. 2C-G). These effects occurred without affecting F-actin area (Supplementary Fig. 2H). During the same time frame, we observed increased mitochondrial fission, as evidenced by increased numbers of mitochondrial networks and punctuate/rounded mitochondria as well as a decreased number of rod-shaped mitochondria (Supplementary Fig. 2I-L). After hormonal stimulation of the adipogenic program (day 0 to day 1), F-actin/mitochondria contacts increased along with stress fiber breakdown, as evidenced by the increased number of F-actin networks together with the changes observed in the other parameters mentioned above (Supplementary Fig. 2A-G). In addition, F-actin re-organization toward the cell periphery started at day 1 and became more evident at 36 hours (Supplementary Fig. 2B), concomitantly, the contacts between F-actin and mitochondria detected at 36 hours compared to day 1 were decreased (Supplementary Fig. 2A). This phenotype was associated with increased numbers of mitochondrial junctions and branches along with greater mitochondrial area (Supplementary Fig. 2M-O). Taken together, our data suggest that there are two distinct phases of F-actin/mitochondria interaction during the transition from MSCs to pre-adipocytes. In the first phase, (before day 0) the reduction of contacts between F-actin/mitochondria promotes mitochondrial fission. In the second phase (from day 0), there is a priming and transient interaction between F-actin and mitochondria that promotes mitochondrial biogenesis and fusion.
Zc3h10-depletion led to decreased F-actin junctions, branches, and area suggesting accelerated F-actin breakdown (Fig. 2B). In addition, *Zc3h10* silencing impaired F-actin re-organization toward the cell periphery, leading to increased F-actin/mitochondria contacts (Fig. 2A and 3E). Of note, aberrant contacts between F-actin and mitochondria precluded mitochondrial biogenesis and fusion (Fig. 3A and B). Furthermore, the reduced levels of mitochondrial biogenesis and fusion observed in *Zc3h10*-silenced preadipocytes led to decreased M+3 MalonylCoA from [U-$^{13}C_5$]glutamine in both pre- and mature adipocytes, suggesting that mitochondrial fusion is required for glutamine-dependent lipogenesis (Supplementary Fig. 4D and Supplementary Fig. 5F).
Conversely, Zc3h10 overexpression led to increased F-actin re-organization toward the cell periphery (reduced numbers of punctate filaments and junctions, and an increased number of rod-shaped filaments, Fig. 2C and D) and precocious F-actin/mitochondria dissociation (Fig. 3F) facilitating mitochondrial biogenesis and fusion (reduced number of punctate/rounded mitochondria and increased number of mitochondrial branches and area, Fig. 3D).

• *Genetic experiments could be used to bolster your data. For example, does promoting mitochondrial fusion genetically or pharmacologically bypass defects in F-actin remodeling in shZc3h10 cells and promote lipid droplet formation?*

Response: The overexpression of Opa1 in *Zc3h10*-silenced pre-adipocytes increased lipid droplet size and improved the F-actin and mitochondrial phenotypes (Fig. 4G-J). However, the number of AdipoRed-positive cells was not rescued (Fig. 4K and L). Therefore, in this context, Opa1 only favors lipid droplet enlargement.

• *The PKA epistasis experiments are not convincing. Removing PKA activator from the differentiation cocktail, does not preclude PKA modulation by Zc3h10. The RNA finding that Zc3h10 regulates a PKA subunit doesn't support your point either. Please target PKA genetically or pharmacologically or consider probing for PKA activity in your different conditions to demonstrate that PKA activity is lower without IBMX and is not positively modulated by Zc3h10 expression.*

Response: We have removed the data related to IBMX withdrawal in the revised manuscript and have followed the reviewer's suggestion by performing experiments with the PKA inhibitor KT5720. PKA inhibition reduced lipid droplet size in Zc3h10-overexpressing cells and did not

change the number of adipocytes (Supplementary Fig. 5G and H). Taken together, these results indicate that Zc3h10 partially requires PKA signaling during adipogenesis.
Minor comments:

• *It is unclear how quantification is performed in this paper. For example, in Figure 1C, lipid droplet measurements only include 6 measurements per group. In the methods it states, "For lipid droplet size, images were first binarized and particles were then quantified." Considering each cell contains a dozen or more lipid droplets, how are we to interpret this data. Is this average lipid droplet size per cell? If so, it should be indicated on the axis. If not, then you need many more data points >30. This comment also holds true for quantification of F-actin networks. Please include more information regarding quantification methods for this paper.*

Response: We thank the reviewer for this point because it helped us to better present our data. Indeed, for lipid droplet size, we re-analyzed all the images and plotted the size of each lipid droplet (at least 1,000 lipid droplets from at least three different images per experimental group). As evidenced in the presented images for F-actin, we were not able to quantify its morphology per single cell (as we did for mitochondria) due to several contacts between cells. Therefore, the values in the graphs represent the mean value of six or seven different images containing at least 15 cells per experimental group. Finally, we added these details into the Materials and Methods section.

• *Please use blots with lower exposure for 4EBP1. Blots are overexposed hiding the normal ~4 band ladder pattern observed with phospho or total 4EBP1, which complicates quantitation. Also, in Fig 2B, Zc3h10 FLAG p-4EBP1 signal is quantified as 0.5 fold control despite roughly equivalent signal on the blot. I would double check this quantification. If it is a ratiometric quantification p-4ebp1/total 4ebp1 please indicate on axis or figure legends and methods.*

Response: We attempted to perform 4E-BP1 western blots using 15 to 20% SDS-PAGE, however, we were unable to improve the normal ~4 band ladder pattern. Therefore, we acquired data from 4E-BP1 western blots using lower exposures and obtained bands similar to those reported on the data sheets of the antibody manufacturer (Fig. 8D-F). All the quantifications were ratiometric, so we amended the appropriate Y-axis titles and the description of the western blot procedure in the Materials and Methods to reflect this process.

• *The goal in Figures 8 and 9 is interesting as you are asking questions regarding F-actin remodeling and its sufficiency in regulating mitochondrial and lipid droplet phenotypes. However, the data doesn't necessarily fit tightly and doesn't always repeat from previously in the manuscript. For example in Fig 9A, shZc3h10 cells are differentiating equivalent to controls (according to adipored staining) making it difficult to address a negative result from Y27632. Cell size data in Fig 9 is also not repeating from Fig 7 making data interpretation difficult.*

Response: To address the reviewer's concerns, we performed confocal microscopy experiments with inhibitors to quantify lipid droplet size at day 9 instead of at day 5, as reported in the previous version of the manuscript, to make the interpretation of our results more consistent (Fig. 7A-C). Furthermore, to better focus and improve the flow of our manuscript, in agreement with the editor, we have removed all data related to MCE, cell cycle, and cell shape. Indeed, the role of Zc3h10 in the cell cycle in general, and specifically, during adipogenesis, deserves an in-depth analysis. The reviewer's comments on this issue will help us in our future work in this direction.

• *A linear model from f-actin>mitochondria>lipid droplets is complicated by the above issues with controls and the Ml141 data where mitochondrial phenotypes are rescued from Zc3h10*

overexpression, but lipid droplet size and adipored cells are unchanged. Perhaps expand upon this latter discrepancy in the discussion.

Response: As stated in the previous point, we performed confocal microscopy experiments with inhibitors to quantify lipid droplet size at day 9 instead of at day 5, as reported in the previous version of the manuscript, to make the interpretation of our results more consistent. Indeed, at this later time point, ML141 treatment significantly decreased lipid droplet size compared to Zc3h10 overexpressing cells (Fig. 7B). Thus, our data support the hypothesis that Cdc42 inhibition prevents the effects associated with Zc3h10 overexpression.

• *In discussion, "Functional validation experiments demonstrate that Zc3h10 depletion in pre-adipocytes is associated to translation inhibition as evidenced by the hyperphosphorylated status of 4E-BP1." This is backwards, it should read "translation activation". Hyperphosphorylated 4E-BP1 releases from eIF4E allowing for cap-dependent mRNA translation.*

Response: We thank the reviewer for bringing this to our attention. This statement has been amended in the text.

• *Considering incorporating a model figure for this paper. This will help the reader and perhaps aid in the organization/emphasis of certain data in this manuscript.*

Response: As suggested by the reviewer, we have added a schematic representation of our major results in Figure 8K.

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

In this study entitled: Zc3h10 regulates adipogenesis by controlling translation and F-actin/mitochondria interaction, Audano and colleagues propose a physiological role for the zinc finger CCCH-type containing 10 (Zc3h10) TF in the context of white adipogenesis. Zc3h10 was previously identified by the same group as a mitochondrial regulator that is required during myogenesis (Audano et al., 2018), as well as a factor regulating the thermogenic program in brown adipose tissue (Yi et al., 2019). It was also found to be one of the most enriched genes in mammalian adipose tissue (albeit based on sheep breed transcript data) (Li et al., 2018). To study the molecular processes that are regulated by Zc3h10 during white adipogenesis, a wide range of assays was used, revealing that Zc3h10, via its transcriptional activities, represses protein translation and promotes cytoskeleton remodelling. This in turn determines mitochondrial function, metabolic activation and cell shape, enabling mitotic clonal expansion (MCE) and, ultimately, lipid loading. The authors thereby suggest that Zc3h10 is involved in the early stages of adipogenesis, given that several of the modulated processes are most active early during this differentiation process.
The employed experimental approaches are based on classical methods of cell and molecular biology including shRNA-mediated Zc3h10 depletion, its adenoviral overexpression, transcriptomics, metabolomics, oxygen consumption measurements, mitochondrial dynamics measurements, cell cycle analysis and others. The novelty of the study lies in the elucidation of the physiological role of Zc3h10 during white adipogenesis.
A strength of the study is certainly that multiple aspects of Zc3h10's involvement in adipogenesis are dissected by a myriad of approaches and assays. Overall, the manuscript and the science underneath it appear of reasonable to good quality. However, the multitude of experiments often leaves the reader lost with respect to the importance and relevance of the addressed features. Indeed, this data overload substantially clouds clarity on the findings, which is why a thorough structural and grammatical revision of the manuscript is highly recommended. The goal here is to

simplify the paper's structure, to provide a better rationale of why certain analyses / data are presented, and to interpret the data in a more biologically meaningful way.

Comments:
1. *The authors show the effects of both knockdown and overexpression of Zc3h10 in almost all the assays. It is however unclear why the consequences of these perturbations are not always consistent. For example, why does both knockdown and overexpression of Zc3h10 reduce the number of adipocytes (Figure 1B and E)? This question applies also to other assays, whose data is shown in Figures 2D/F, 3B/D, 5A, 6D/E. The authors should thus interpret the available data in a clearer way or at least provide sound explanations for these puzzling phenomena.*

Response: As shown in Figure 1B and 1E, both *Zc3h10* silencing and overexpression led to reduced cell numbers at day 9. These effects were due to *Zc3h10*-silenced cells being blocked in the G2/M phase and to precocious MCE in Zc3h10 overexpressing cells (Fig. 6D and 6E of the previous version of the manuscript). To better focus and improve the flow of our manuscript, in agreement with the editor, we have removed all data related to MCE, cell cycle, and cell shape in the present form of the manuscript. Indeed, the role of Zc3h10 in the cell cycle in general, and specifically, during adipogenesis, deserves an in-depth analysis. The reviewer's comments on this issue will help us in our future work in this direction.
The effects mediated by *Zc3h10* silencing and overexpression are not always consistent, and those parameters that go in the same direction, despite opposite genetic manipulation, have to be interpreted in the context of other experimental settings. For example, in Figure 2B and 2D of the present version of the manuscript, both *Zc3h10* knockdown and overexpressing cells show fewer F-actin junctions. In the case of *Zc3h10* knockdown cells, fewer junctions together with fewer branches and lower filament area mean that the F-actin is less complex and organized (Fig. 2A). In contrast, in Zc3h10 overexpressing cells fewer F-actin junctions and less punctate F-actin together with higher numbers of rod-shaped F-actin filaments mean that Zc3h10 boosts stress fiber breakdown, favoring F-actin reorganization in the cell periphery (cortical F-actin, Fig. 2C and D).
To convey our findings in a clearer way, in some cases, data is shown only for the silencing of *Zc3h10* since knockdown of an endogenous protein provides more biologically meaningful insights into its biological/physiological role than overexpression (i.e., removal of overexpression experiments related to oxygen consumption analyses; see new Fig. 5 and Supplementary Fig. 4).

2. An important weakness of the current study is that overarching statements are made based on overall rather subtle differences. While often reported as "significant", the absence of details on which type of statistical test is exactly used (e.g. the authors often mention "Student's t test", but which one?) and on whether these analyses are corrected for multiple testing makes the interpretation of what "significant" precisely means unclear. One of the reasons why only subtle differences are detected in a knockdown setting could be the sizeable Zc3h10 protein amount that remains in cells after the knockdown. To support their findings, the authors would do well to generate a full Zc3h10 knockout cell line (e.g. by CRISPR) and to repeat some of the principal analyses to test whether this makes the overall impact of the perturbation more meaningful.

Response: We thank the reviewer for these important points regarding our statistical analyses. In the new version of the manuscript we have included as many details as possible in both the Methods section and the Figure Legends to describe the statistical analyses performed.
We agree with the reviewer that CRISPR/Cas9-mediated genome editing is the gold standard for understanding the physiological role of a protein. However, a recent paper reported that for *Zc3h10*, this methodology yielded only heterozygote mutant cells and no homozygous knockouts in any of the cell lines tested (Treiber T. et al. Mol. Cell. 2017. 66 (2): 270-284). Moreover, in MSCs, Zc3h10 is barely expressed (Fig. 1M and Supplementary Fig. 1A), which makes the genome editing

more complicated. Finally, since the expression of Zc3h10 increases at 36 hours after differentiation induction, we think that silencing this gene at the time of differentiation induction helped us to better define its target genes. Moreover, we have now added functional data related to Zc3h10 depletion in mature adipocytes (following the protocol in Supplementary Fig. 1J) showing unchanged lipid accumulation (Oil Red O), basal respiration, and mtDNA content (Supplementary Fig. 1K-N). These results indicate that the effects of Zc3h10 on lipid accumulation and mitochondrial function depend on the stage of differentiation. More specifically, Zc3h10 regulates the beginning of the differentiation program. In this case, we expect that CRISPR/Cas9-mediated deletion of Zc3h10 would not have allowed us to characterize the time-specific role of Zc3h10 in adipogenesis that was achieved with our shRNA-mediated approach.

3. A point in case illustrating the subtle differences is the nascent mRNA analysis, which overall revealed few striking transcriptional differences, i.e. almost all differentially regulated pre-mRNAs were less than two-fold different. Downstream protein work validated some of the observations, yet it is surprising that the authors chose not to better integrate these data with other downstream analyses involving transcriptomic profiling of wildtype and knockdown mature adipocytes. This is especially relevant since, as one example, in earlier stages of adipogenesis, Zc3h10 knockdown appears to downregulate pre-mRNAs involved in actin filament-based processes, yet the opposite is true for mature mRNAs later in adipogenesis, even though even more Zc3h10 is being expressed then. A more profound data integration may allow the authors to reconcile or refute these contrasting observations...

Response: Given that our results clearly indicate that the major functions of Zc3h10 in the adipogenic program take place a few hours after differentiation induction and that Zc3h10 is a transcription factor, we investigated the effects of Zc3h10 depletion on nascent/pre-mRNA synthesis 36 hours after hormonal induction to capture the most immediate effects of Zc3h10 during adipogenesis. For this reason, and in line with the reviewer comment, we did not expect large differences in most of the differentially expressed pre-mRNAs. Nevertheless, the validation analyses on F-actin morphology and translation activity confirmed data obtained by nascent mRNA transcriptome profiling.
In addition, to avoid overwhelming data in the present version of the manuscript, the transcriptomic microarray analysis performed in Zc3h10-depleted mature adipocytes (day 9 after differentiation) has been removed.

4*. It is laudable that the authors provide both the negative and positive data. But instead of simply showing all the data, it would be even more valuable if the authors could guide the reader in better understanding the biological meaning of their observations. For example, in Figures 2D and 2E, which property of F-actin does each of the parameters stand for? The same for Figures 3B and D.*

Response: Based on the reviewer's comments, we have integrated explanations of the biological meanings of the experiments represented in Fig. 2, 3 and Supplementary Fig. 2 into the present version of the manuscript in the Results section to help the reader understand the interpretation of the data.

5. Data shown in Figure 5A demonstrates that there is more oxygen consumption in a Zc3h10 overexpression context when oligomycin is added. How do the authors explain this proton leakage? Are there more mitochondria that are damaged in these cells?

Response: As stated above, this data has been removed from the present version of the manuscript. However, it has been reported that during 3T3-L1 adipogenic differentiation, state 3 respiration (uncoupled respiration mediated by oligomycin) is increased in mature adipocytes compared to

undifferentiated cells (Ducluzeau PH et al. J. Physiol. Biochem. 2011, 67:285-296). Moreover, increased mitochondrial respiration is due to ATP production and maintenance of mitochondrial membrane potential during adipogenesis. For this reason and considering that Zc3h10 overexpression increases uncoupled oligomycin respiration, we conclude that Zc3h10 is relevant to sustain mitochondrial membrane potential in pre-adipocytes. Moreover, the uncoupling protein Ucp1 has been reported as a Zc3h10 target gene in brown and white adipose tissue (Yi, D., et al. Cell Rep. 2019; 29, 2621-2633.e4), suggesting that Zc3h10 may directly regulate uncoupled respiration also in white adipocytes. Consistently, we also found that Ucp1 mRNA levels were reduced in Zc3h10 depleted adipocytes (see figure below).

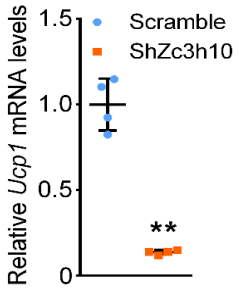

Figure legend: Relative *Ucp1* mRNA levels in Scramble and ShZc3h10 mature adipocytes.

6. The authors claim that Zc3h10 controls the early stages of white adipogenesis. However, this conclusion seems to be only supported by an observation of Zc3h10-depleted adipocytes showing mitochondrial dysfunction in their mature state. How can the authors confidently conclude that Zc3h10 is exclusively relevant in the early stages of differentiation without targeting this TF at later stages of adipogenesis?

Response: As stated above, we have now added functional data relative to Zc3h10 depletion in mature adipocytes (following the protocol reported in Supplementary Fig. 1J) showing unchanged lipid accumulation (Oil Red O), basal respiration, and mtDNA, content (Supplementary Fig. 1K-N). These results indicate that Zc3h10 regulates lipid accumulation and mitochondrial function during the early stages of differentiation, but not in mature adipocytes.

7. The observation that Zc3h10 may directly impact the cell cycle obviously has important consequences in terms of how this factor may influence adipogenesis, since the cell cycle and early differentiation are heavily intertwined. Can this factor be directly found on the mitotic spindle? And do the authors think that all other effects stem from the factor's role in the cell cycle? (chicken / egg question).

Response: To better focus and improve the flow of our manuscript, in agreement with the editor, we have removed all data related to MCE, cell cycle, and cell shape. Indeed, the role of Zc3h10 in the cell cycle in general, and specifically, during adipogenesis, deserves an in-depth analysis. The reviewer's comments on this issue will help us in our future work in this direction.

8. The methodology that is used to quantify in vitro adipogenesis, one of the main readouts employed in the study, is not at all clear.
The authors claim that the "Lipid accumulation and cell shape analyses" are conducted using flow cytometry.
The cytofluorometer settings for both side scatters (SSC) and forward scatters (FSC) to analyse cell shape were dependent on the analytic sensitivity of the machine. The voltages and compensation between scatters were set to the degree so that most control cells at day 0 were located above the

scale of 105 for both the SSC and the FSC. Cell shape analysis was performed by measuring the median FSC- A of the whole range of the cells.
The way these results are represented and annotated in the manuscript leaves the reader to believe that the number of differentiated cells is inferred from the microscopic images, presented alongside (Fig. 1A and B, D and E, G and I, J and L, etc). Using flow cytometry measurements for in vitro adipogenesis quantification is not referenced and no supporting data is presented to demonstrate how this quantification is performed.

Concerning the measurements of lipid droplet sizes, this time based on the microscopy images, the authors included a sparse comment in the methods section:
Confocal microscopy image processing and analysis
For lipid droplet size, images were first binarized and particles were then quantified.
These two parameters, 1) the relative number of differentiated cells (adipored+) and 2) the lipid droplet size, are widely used throughout the study and serve as a principal basis for the main conclusions that are presented in the manuscript. A thorough documentation of how these parameters were obtained is absolutely necessary to support the proposed observations.

This is especially relevant since accurate quantification of in vitro adipogenesis is highly challenging and numerous methods have been described, starting from the classical Oil Red O staining of fixed cells and subsequent imaging of cells, inferring a ratio of lipid and nucleic acid-specific dye intensities over colorimetric measurements of adipocyte lysates to imaging live differentiating cells using specific/high throughput imaging platforms and employing mathematical models to analyse the degree of adipogenic lipid accumulation or/and size distribution of lipid-containing intracellular vesicles as a read-out of assayed conditions (Eom et al., 2018; Kraus et al., 2016; Varinli et al., 2015; Yuan et al., 2019).

Response: We understand the reviewer's concerns on the importance of machine/technical settings in flow cytometry analyses. However, previous studies have demonstrated that flow cytometry is a valuable tool to quantify adipocytes in 3T3-L1 cultures (Lee YH, et al. J Lipid Res. 2004;45(6):1162-1167. doi:10.1194/jlr.D300028-JLR200). Moreover, flow cytometry measurements for *in vitro* adipogenesis quantification has been previously described (Higuchi M. et al. Stem Cells Dev. 2013; 22 (6): 878-888). However, to better focus and improve the flow of our manuscript, in agreement with the editor, we have removed all data related to MCE, cell cycle, and cell shape. Indeed, the role of Zc3h10 in the cell cycle in general, and specifically, during adipogenesis, deserves an in-depth analysis. The reviewer's comments on this issue will help us in our future work in this direction.
Reviewer 2 also brought up the point of lipid quantification (first minor point). Therefore, for lipid droplet size we re-analyzed all the images and plotted the size of each lipid droplet (at least 1,000 lipid droplets from at least three different images per experimental group). The details for this approach have been included in the Materials and Methods section of the revised manuscript.

9. A similar comment applies to assays addressing F-actin morphology and mitochondrial dynamics. Zc3h10 was shown to affect actin filament-based processes and actin cytoskeleton organisation, which in turn affects mitochondrial dynamics. The morphology of F-actin, including the shape and length of the filaments, the number of punctuate/rounded filaments as well as the number and morphology of junctions and branches, were heavily used as a readout of the morphological changes that are affected by Zc3h10.
However, the methodology describing the image acquisition and analysis of the F-actin and mitochondrial networks is yet again inadequate. Even though the image treatment algorithm developed for this type of data is this time referenced (Valente et al., 2017), there is a complete lack of description of how the method needed to be adapted to the presented study. Intermediate images

of the pipeline leading to image skeletonisation, presumably yielding the numerical values, are also missing (except representative binary images (i.e. Fig. 2C, E, G, H, Fig. 3A, C)).

Response: We have added more details about the image analyses for the F-actin and mitochondrial quantifications in the Materials and Methods section of the revised manuscript. These include all the steps in the algorithm and a representative image of each step.

10. In general, the methods section thus lacks a detailed description of many of the utilized, experimental procedures.

Response: We have revised the Materials and Methods section to better convey the experimental procedures used in our manuscript.

November 24, 2020

RE: JCB Manuscript #202003173R-A

Dr. Nico Mitro
University of Milan
Pharmacological and Biomolecular Sciences
Via Balzaretti 9
Milano 20133
Italy

Dear Dr. Mitro:

Thank you for submitting your revised manuscript entitled "Zc3h10 regulates adipogenesis by controlling translation and F-actin/mitochondria interaction". As you will see the reviewers agree that your refocused and revised manuscript is now suitable for publication in JCB, therefore we would be happy to publish your paper pending final revisions necessary to meet our formatting guidelines (see details below). In your final revision, please also provide a response to the remaining reviewer concerns and make any necessary text edits to clarify your text.

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

A. MANUSCRIPT ORGANIZATION AND FORMATTING:

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 title page, abstract, introduction, results, discussion, acknowledgments, and figure legends. Count does not include 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. *

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.

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 (e.g. film or model of digital imager) 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/Tools may have up to 5 supplemental display items (figures and tables).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. At resubmission of your final files, please consider

providing an ORCID ID for as many contributing authors as possible.

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.

B. FINAL FILES:

Please upload the following materials to our online submission system. These items are required prior to acceptance. If you have any questions, contact JCB's Managing Editor, Lindsey Hollander (lhollander@rockefeller.edu).

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

Sincerely,

Johan Auwerx, MD, PhD
Monitoring Editor

Andrea L. Marat, PhD
Senior Scientific Editor

Journal of Cell Biology

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

The authors have addressed most of my critics and concerns. I think that the manuscript has significantly improved, and the data presented justifies the publication of the article.

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

Overall, the authors have addressed the critiques raised by the reviewers, and there is a more focused approached on how Zc3h10 controls adipocyte mitochondrial dynamics and metabolic function depending on the stage of differentiation. There are several important questions that remain, and should be discussed-

1- It is unclear why the effects of manipulating Zc3h10 depend on the differentiation state- Does Zc3h10 control the translation program in differentiated adipocytes? Is actin dynamics different in these stages?

2- The mitochondrial rescue in Zc3h10 depleted cells through Rho manipulation, is a nice piece of data supporting their model. However, one would predicts based on the actin dynamics that other organelles are also affected?

3- The 4EBP depletion experiments are puzzling, based on the TOP motif present on the Zc3h10 mRNA targets? How the authors explain this result?

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

Audano and colleagues have properly revised their manuscript "Zc3h10 regulates adipogenesis by controlling translation and F-actin/mitochondria Interaction" in my opinion. The workflow has been streamlined, while the cell cycle-related parts have been removed and other parts re-organised. Although a deeper understanding of the intertwined nature of the cell cycle and adipogenesis is clearly interesting, not including the respective data in the current manuscript is a good decision as a clear demonstration of cell cycle regulation will need much more analyses and render the already overcrowded manuscript difficult to follow. The authors have also enhanced the data interpretation and the methodology description of the F-action and mitochondria remodeling, which solidified one of the main conclusions of the manuscript. Nevertheless, the manuscript remains difficult to read and thus for this study to have a meaningful impact, a further streamlining of the results and text is recommended.

JOURNAL OF CELL BIOLOGY MANUSCRIPT #202003173R-A Audano M. et. al.

Response to Reviewers

We thank the reviewers and the editors for their insightful comments. We have carefully addressed their suggestions as detailed below in the point-by point response. We have also thoroughly revised the text to enhance the flow of our manuscript as well as the clarity and presentation of our findings and to fit in the requirements of the journal. We are indebted to the reviewers and the editors for their aid. We believe that we have addressed all concerns, as detailed below:

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

The authors have addressed most of my critics and concerns. I think that the manuscript has significantly improved, and the data presented justifies the publication of the article.

Response: we thank the reviewer since his/her previous suggestions helped us to shape the new version of the manuscript.

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

Overall, the authors have addressed the critiques raised by the reviewers, and there is a more focused approached on how Zc3h10 controls adipocyte mitochondrial dynamics and metabolic function depending on the stage of differentiation. There are several important questions that remain, and should be discussed-

1- It is unclear why the effects of manipulating Zc3h10 depend on the differentiation state- Does Zc3h10 control the translation program in differentiated adipocytes? Is actin dynamics different in these stages?

Response: we thank the reviewer for raising these points. In the first version of our manuscript, we reported the genome wide expression analyses of Zc3h10-depleted mature adipocytes. The translation program was not among the most differentially modulated pathways; therefore, we conclude that translation is a pathway controlled by Zc3h10 mainly in the early phases of adipogenic program.
Concerning the actin dynamics in mature adipocytes, it should be underlined that actin is barely detectable or even undetectable in control, ShZc3h10 and Zc3h10 Flag cells, therefore it is not possible to provide quantitative data at this stage.

2- The mitochondrial rescue in Zc3h10 depleted cells through Rho manipulation, is a nice piece of data supporting their model. However, one would predicts based on the actin dynamics that other organelles are also affected?

Response: we agree with the reviewer and this topic will be investigated in future studies.

3- The 4EBP depletion experiments are puzzling, based on the TOP motif present on the Zc3h10 mRNA targets? How the authors explain this result?

Response: our data indicate that Zc3h10 controls adipogenesis through two independent pathways, namely protein translation and F-actin/mitochondria interactions. We also demonstrated that upregulated genes in the absence of Zc3h10 (involved in protein translation) are enriched for TOP

motif. Many of these genes are physiologically and directly inhibited by the activity of Zc3h10 at the transcriptional level. In addition, being TOP motif target of 4E-BP1 in the cytoplasm, the translation of these genes is also inhibited. As result, the expression of translation-related proteins is inhibited by the combined action of Zc3h10/4E-BP1, thus favoring adipogenesis.

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

Audano and colleagues have properly revised their manuscript "Zc3h10 regulates adipogenesis by controlling translation and F-actin/mitochondria Interaction" in my opinion. The workflow has been streamlined, while the cell cycle-related parts have been removed and other parts re-organised. Although a deeper understanding of the intertwined nature of the cell cycle and adipogenesis is clearly interesting, not including the respective data in the current manuscript is a good decision as a clear demonstration of cell cycle regulation will need much more analyses and render the already overcrowded manuscript difficult to follow. The authors have also enhanced the data interpretation and the methodology description of the F-action and mitochondria remodeling, which solidified one of the main conclusions of the manuscript. Nevertheless, the manuscript remains difficult to read and thus for this study to have a meaningful impact, a further streamlining of the results and text is recommended.

Response: we thank the reviewer since his/her previous suggestions helped us to shape the new version of the manuscript and now we also amended the text to fit in the requirements of the journal.