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Seipin regulates lipid homeostasis by ensuring calcium-dependent mitochondrial metabolism

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 18th July 2017

Thank you for submitting your manuscript for consideration by the EMBO Journal. It has now been seen by three referees whose comments are shown below.

As you will see, referee #2 thinks that the observed effects could be indirect and that the advance and insight provided is not sufficient for further consideration here. Referee #1 and #3 note that the steady-state analysis performed is not informative enough and that the overall insight into cause and consequence provided remains too limited at this stage.
During further cross-commenting among all referees, they all agreed that the outcome of addressing the issues raised is rather unclear, precluding at this stage further consideration of your work for publication here. Given this input, I am afraid I see no other choice but to return your manuscript to you with the message that we cannot offer to publish it in The EMBO Journal. Having said this, should addressing the concerns of the referees still support your main conclusions, I would be prepared to take a fresh look at your manuscript again in the future. In this case I would have to treat the manuscript as a new submission though, taking novelty into account.

Thank you in any case for the opportunity to consider this manuscript. I am sorry we cannot be more positive on this occasion, but we hope nevertheless that you will find our referees' comments helpful.

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REFEREE REPORTS

Referee #1:

Ding et al. address the mechanism of action of seipin in promoting lipogenesis in fat tissues. In mammals loss of function of seipin prevents adipogenesis. The protein is also required for proper assembly of lipid droplets. In this manuscript the model system is the Drosophila larval fat body, where it has been shown that disruption of the seipin gene causes a large drop in fat accumulation, mimicking the situation in mammals. In 2014 the group showed that seipin binds to the ER calcium import pump SERCA, that the absence of seipin interferes with pump activity, and that disruption of SERCA phenocopies disruption of seipin.

To better understand how an ER calcium pump is tied to fat accumulation, the authors first performed proteomic analysis of larval fat bodies from control and seipin KO animals and found that several glycolytic enzymes were low, while some TCA cycle enzymes were higher than wild type. To determine if low glycolytic intermediates could be responsible for low fat accumulation, they showed that knockdown of pyruvate kinase indeed led to low fat in the tissue, and overexpression rescued the seipin mutant. However, corresponding mRNAs were not different, so the effect was post-transcriptional on enzyme protein levels. Next they looked at metabolites and found, unexpectedly, that some glycolytic intermediates were high in the seipin mutant, while TCA enzymes were low (although two were high), suggesting a bottleneck in the mitochondria. In fact, mitochondria in the seipin mutant tissues were functionally compromised and often found in phagolysosomes. As the group previously showed a defect in the SERCA pump in the absence of seipin, they next looked at mitochondrial calcium levels and found them to be lower in the seipin mutant. Interestingly, mitochondrial calcium, fat accumulation, and citrate levels were all rescued by blocking Ca efflux from mitochondria or increasing ER calcium stores by blocking the ryanodine receptor. In summary, the authors argue that the loss of fat from seipin-deficient adipose is due to low Ca in mitochondria leading to poor citrate production, which in turn decreases the amount of the lipogenic precursor acetyl-CoA.

I found the argument that fat is decreased due to poor mitochondria and low citrate in the tissue intriguing. As predicted, feeding animals citrate or pyruvate can complement the seipin mutation. Moreover, increasing the ER calcium concentration by genetic means also reverses the effect on citrate, suggesting that mitochondrial calcium derived from the ER is critical for citrate production.

All the data consists of steady-state measurements, and these have given the authors very suggestive results. What is missing in my opinion, is a direct test that glucose flux into fatty acids is decreased in the seipin tissue. If animals are fed isotopic glucose (C13- or C14-tagged) for short times, is there indeed a difference in the rate of citrate formation, fatty acid synthesis, and TAG generation? Is the flux indeed reversed with genetically raising ER calcium? Conversely, what are the rates of lipolysis of the droplet in the two strains?

Besides this major criticism, there are some other concerns, mostly editorial:

(1) There are no methods I can find for the comparative proteomics shown in Fig. 1. Elements of these data are confusing:
a. What is the standard to which protein levels are compared? As both wild-type and seipin show changes, how are these data normalized? This is not clear.
b. What is Mutant1 and Mutant2? Control1 and Control2? Different seipin deletion clones? Different days of organ removal of the same strain? The data points don't seem to pair well between Mutant1 and Mutant2 in Fig. 1A. More information is needed. What statistic went into the p value determination?
c. There are many more significant proteomic differences besides enzymes in glycolysis and TCA. A supplementary table should be provided with the proteins that are most altered.
(2) There is a statement in the text on page 3 of the Results: "We targeted candidate calcium-responsive transcription factors," but I don't see any data for this. Does this refer to Fig. 2D?
(3) Regarding mitochondrial health, it is stunning that so many mitochondria are in phagolysosomes. Are these counted in the respiratory activity assay? The rhod-2 sensor assay? Naturally, if

mitochondria are being digested, their respiratory activity would be severely compromised.
(4) Relatedly, I wonder if the poor mitochondria are related not so much by poor flux of calcium from the ER as poor beta-oxidation of fatty acids, as the pools of TG are so much reduced. Poor lipid stores could also account for low ATP and increased mitophagy. In other words, I'm concerned that it is low stores of fat that lead to poor mitochondrial health than the other way around. Can this be addressed experimentally?
(5) Using the fluorescent probe the authors find larger mitochondria in controls than in the seipin mutant. This was not the case in the electron micrographs. Could the data from fluorescence be due to oversaturation of signal, giving a false impression of mitochondrial size?
(6) Feeding animals citrate or pyruvate: is there a negative control? Does feeding them glucose or glycerol not give the same result? I'm somewhat surprised that administered citrate or pyruvate are not inter-converted in a liver-like organ. Perhaps these larvae lack such function.

Referee #2:

This manuscript by Ding and colleagues focus on the interplay between mitochondrial calcium levels and Seipin-mediated lipid homeostasis. Through a combination of transcriptomics, proteomics, metabolomics and conventional molecular genetics the authors analyse the phenotypes of seipin mutants in drosophila fat bodies. It was found that glycolytic enzymes were present at lower levels even if their transcripts were normal or even elevated. Moreover, glycolytic metabolites such as pyruvate and lactate strongly accumulated in seipin mutants. Concomitantly, the authors found that seipin mutant, while maintaining normal levels of TCA cycle enzymes, showed strong depletion of its metabolites, some of which (citrate) is shuttled to cytoplasm for acyl-CoA production. Based on rescue experiments using pyruvate and citrate as well as overexpression and knock down approaches the authors come up with a model in which low mitochondrial Ca, as a consequence of low ER Ca due to SERCA impaired function, is the main cause of lipodystrophy of Seipin mutant fat bodies. I have major conceptual problems with this interpretation. Even if some of the metabolomic and proteomic phenotypes are remarkably strong, in this reviewer's opinion there is no evidence that they are a direct consequence of Seipin modulation of SERCA in the ER. Moreover, several of the experiments do not seem fully convincing or may have alternative interpretations (see below). Thus, I consider this is a very preliminary set of observations, many of which are confusing and over interpreted, and that do not provide an advance to the current understanding of Seipin function.
1- In fig 4 it is shown that seipin phenotypes are suppressed by exogenous pyruvate supplementation. This is weird as, in comparison to controls, Seipin mutants appear to have already massive pyruvate levels (Fig3A). This is interpreted as the extra pyruvate is necessary to rescue the endogenous citrate levels, which is not satisfactory. Moreover, at the concentrations used, the effects of citrate and pyruvate are exclusively detected in the seipin mutants while controls appear completely irresponsive to the extra metabolites. It is not clear why that is and how direct the effects are.
2- Previous work in different model systems, including Drosophila cell lines (Wang et al. eLife 2016) performed detailed lipid analysis in seipin mutant. Altogether these experiments argue strongly against a defect in fatty acid and/or lipid synthesis. However, the authors did not measure the levels of Acyl-CoA or activity of Fatty acid synthase.
3- Zhou et al (MCB, 2016) previously studied the impact of seipin mutations on the mitochondrial function of mice brown adipocytes. In this context, it was found that seipin mutation increases OCR while MitoTracker labelling was indistinguishable from control cells. Now the authors found that in fly larvae fat bodies seipin appears to a different impact. It would then be important to understand the basis of the differences. Are there cell type specificities that may underlie the discrepancies?
4- It is assumed that mitochondrial "encapsulation" phenotype described in Figure 5 correspond to mitochondria in autophagosomes. However, no autophagy markers were monitored or measurements of autophagic flux were performed to convincingly support this observation.

Referee #3:

In this paper, Ding at al. investigate the Seipin mutation in Drosophila fat cells and report that the

observed drop in lipid droplets (LD) is due to a decrease in lipogenesis. According to the authors, low mitochondrial calcium, would lead to decreased PDH activity and consequently to low levels of citrate, which plays an important role in lipogenesis. The decrease in calcium uptake into mitochondria would be due to decreased levels of calcium in the ER, an observation which the authors previously made using Seipin-deficient fat cells. Overall the results are sound and interesting.
Specific comments :
-The authors analyze LD at steady state. How can they distinguish between a decrease in the biogenesis of LD and an increase in the use of lipids, stored in LD, for beta oxidation and ATP production? Both scenarios would have the same consequence, i.e. a drop in LD. Can the authors discuss this.
-The export of citrate from the mitochondria into the cytosol contributes to lipogenesis by increasing acetyl CoA levels in the cytosol. What is the level of acetyl CoA in the cytosol of Seipin-deficient cells?
-Would a knockout of the MCU lead to a decrease in LD?
-Would activation of the PDH, using for example Dichloroacetate, increase the amount of LD?
-Would inhibition of the mitochondrial pyruvate carrier lead to a drop in LD?
-The authors report increased mitophagy in Seipin-deficient cells. Do they have an explanation for this observation? Does mitophagy contribute to the phenotype?

1st Revision - authors' response 06th April 2018

Point-by-Point response

Referee #1:

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To better understand how an ER calcium pump is tied to fat accumulation, the authors first performed proteomic analysis of larval fat bodies from control and seipin KO animals and found that several glycolytic enzymes were low, while some TCA cycle enzymes were higher than wild type. To determine if low glycolytic intermediates could be responsible for low fat accumulation, they showed that knockdown of pyruvate kinase indeed led to low fat in the tissue, and overexpression rescued the seipin mutant. However, corresponding mRNAs were not different, so the effect was post-transcriptional on enzyme protein levels. Next they looked at metabolites and found, unexpectedly, that some glycolytic intermediates were high in the seipin mutant, while TCA enzymes were low (although two were high), suggesting a bottleneck in the mitochondria. In fact, mitochondria in the seipin mutant tissues were functionally compromised and often found in phagolysosomes. As the group previously showed a defect in the SERCA pump in the absence of seipin, they next looked at mitochondrial calcium levels and found them to be lower in the seipin mutant. Interestingly, mitochondrial calcium, fat accumulation, and citrate levels were all rescued by blocking Ca efflux from mitochondria or increasing ER calcium stores by blocking the ryanodine receptor. In summary, the authors argue that the loss of fat from seipin-deficient adipose is due to low Ca in mitochondria leading to poor citrate production, which in turn decreases the amount of the lipogenic precursor acetyl-CoA.

I found the argument that fat is decreased due to poor mitochondria and low citrate in the tissue intriguing. As predicted, feeding animals citrate or pyruvate can complement the seipin mutation. Moreover, increasing the ER calcium concentration by genetic means also reverses the effect on citrate, suggesting that mitochondrial calcium derived from the ER is critical for citrate production.

"All the data consists of steady-state measurements, and these have given the authors very suggestive results. What is missing in my opinion, is a direct test that glucose flux into fatty acids is decreased in the seipin tissue. If animals are fed isotopic glucose (C13- or C14-tagged) for short times, is there indeed a difference in the rate of citrate formation, fatty acid synthesis, and TAG generation? Is the flux indeed reversed with genetically raising ER calcium? Conversely, what are the rates of lipolysis of the droplet in the two strains?"
We appreciate this insightful comment very much. As suggested, we have performed an isotopic flux tracing experiment using a short pulse of U-^{13}C-glucose. We incubated the isolated larval fat tissues in medium containing isotope-labeled glucose. The results indicate that the glucose flux into the mitochondrial TCA cycle products is lower in *dSeipin* mutants compared to controls, while the kinetics of glycolysis are not significantly changed (Figure 3). Moreover, we found that genetic inhibition of mitochondrial Ca^{2+} release can partially restore the metabolic flux (Figure 6E). We didn't find isotope tracer in Acyl-CoA or TAG fragments. This may be because our tracing period is not long enough or our assay is not sensitive enough.

As suggested, we have measured the lipolysis activities in *dSeipin* mutant and wild-type larval fat bodies. There is no significant difference between wild type and mutants, indicating that the lipodystrophic phenotype of *dSeipin* mutants is unlikely caused by hyperactive lipolysis (Figure S1).

Minor points:
"(1) There are no methods I can find for the comparative proteomics shown in Fig. 1. Elements of these data are confusing:
a. What is the standard to which protein levels are compared? As both wild-type and seipin show changes, how are these data normalized? This is not clear.
b. What is Mutant1 and Mutant2? Control1 and Control2? Different seipin deletion clones? Different days of organ removal of the same strain? The data points don't seem to pair well between Mutant1 and Mutant2 in Fig. 1A. More information is needed. What statistic went into the p value determination?
c. There are many more significant proteomic differences besides enzymes in glycolysis and TCA. A supplementary table should be provided with the proteins that are most altered."
We apologize for these oversights. The iTraq data were shown in our previously published paper (Bi *et al*, 2014). The detailed information about the proteomic differences can be found in the supplemental material of that paper. In this present study, we performed further data-mining. To make it more understandable, we added the information about the data analysis in this revised version.

The volcano plot shows the logarithm-transformed ratios of all quantitative proteins in different samples (x-axis, WT2:WT1, red circle; Mutant1:WT1, orange yellow; Mutant2:WT1, yellow) versus the probability that the difference from the

unity ratio (1:1) is random (y-axis). We used the target-decoy method to estimate the false discovery rate (FDR). The channels WT1 and WT2 are two equivalent biological replicates of the wild type control and the ratio of WT2:WT1 was assigned as the decoy. The channels Mutant1 and Mutant2 are two equivalent biological replicates for the *dSeipin* mutant, and the ratios of Mutant1:WT1 and Mutant2:WT1 were assigned as the targets.

The target-decoy method is likely to be a more reliable FDR estimator as it is sensitive to the specific nature of the data set and does not rely on the accuracy of p-values - only that they rank more likely changes ahead of less likely changes. Therefore, we can be more confident about the changes of proteins in two replicates of *dSeipin* mutants compared with wild type with a p-value less than most of the p-values of WT2:WT1 (for example, 0.001).

"(2) There is a statement in the text on page 3 of the Results: "We targeted candidate calcium-responsive transcription factors," but I don't see any data for this. Does this refer to Fig. 2D?"
Sorry, it was our mistake. We removed this sentence in the revised version.

"(3) Regarding mitochondrial health, it is stunning that so many mitochondria are in phagolysosomes. Are these counted in the respiratory activity assay? The rhod-2 sensor assay? Naturally, if mitochondria are being digested, their respiratory activity would be severely compromised."
This is a good point. In the seahorse assay and other mitochondrion-related experiments, we didn't isolate the active/normal mitochondria from the cells. We used intact tissue to perform the measurements of mitochondrial parameters. The results from metabolic flux, mitophagy and citrate rescue all indicate that defective mitochondria contribute to the lipid storage phenotype.

"(4) Relatedly, I wonder if the poor mitochondria are related not so much by poor flux of calcium from the ER as poor beta-oxidation of fatty acids, as the pools of TG are so much reduced. Poor lipid stores could also account for low ATP and increased mitophagy. In other words, I'm concerned that it is low stores of fat that lead to poor mitochondrial health than the other way around. Can this be addressed experimentally?"
This is a very brilliant opinion. Based on the following results, we think that the mitochondrial defects in *dSeipin* mutants are not due to poor lipid storage. First, the lipid storage defect of *dSeipin* mutants can be rescued by specifically restoring the mitochondrial calcium (Figure 6). Second, citrate treatment rescued the lipid storage defects, but it didn't rescue the mitochondrial defects of *dSeipin* mutants (Figure 5G and 5H).

"(5) Using the fluorescent probe the authors find larger mitochondria in controls than in the seipin mutant. This was not the case in the electron micrographs. Could the data from fluorescence be due to oversaturation of signal, giving a false impression of mitochondrial size?"
Similar staining results can be found in published papers (Di Cara *et al*, 2013； Frei *et al*, 2005). Moreover, we used 100nM Mitotracker red for the staining, which is only 1/3 of the concentration used in previous publications (Di Cara *et al*, 2013； Frei *et al*, 2005) and our observation was done with relatively low exposure settings. Mitotracker signal is specifically retained by mitochondrial membrane

potential. It is possible that mitochondrial membrane potential is low in *dSeipin* mutants, resulting in less fluorescent probe retained in mitochondria within the staining period, and therefore small puncta.

"(6) Feeding animals citrate or pyruvate: is there a negative control? Does feeding them glucose or glycerol not give the same result? I'm somewhat surprised that administered citrate or pyruvate are not inter-converted in a liver-like organ. Perhaps these larvae lack such function."
This suggestion is reasonable and also insightful. As suggested, we used glucose as the candidate negative control. We found that adding 3% or 10% more glucose did not rescue the lipid storage defect of *dSeipin* mutants (Figure S5). This in part reflects the specific rescuing activities of pyruvate and citrate in our study.

Referee #2:
This manuscript by Ding and colleagues focus on the interplay between mitochondrial calcium levels and Seipin-mediated lipid homeostasis. Through a combination of transcriptomics, proteomics, metabolomics and conventional molecular genetics the authors analyse the phenotypes of seipin mutants in drosophila fat bodies. It was found that glycolytic enzymes were present at lower levels even if their transcripts were normal or even elevated. Moreover, glycolytic metabolites such as pyruvate and lactate strongly accumulated in seipin mutants. Concomitantly, the authors found that seipin mutant, while maintaining normal levels of TCA cycle enzymes, showed strong depletion of its metabolites, some of which (citrate) is shuttled to cytoplasm for acyl-CoA production. Based on rescue experiments using pyruvate and citrate as well as overexpression and knock down approaches the authors come up with a model in which low mitochondrial Ca, as a consequence of low ER Ca due to SERCA impaired function, is the main cause of lipodystrophy of Seipin mutant fat bodies. I have major conceptual problems with this interpretation. Even if some of the metabolomic and proteomic phenotypes are remarkably strong, in this reviewer's opinion there is no evidence that they are a direct consequence of Seipin modulation of SERCA in the ER. Moreover, several of the experiments do not seem fully convincing or may have alternative interpretations (see below). Thus, I consider this is a very preliminary set of observations, many of which are confusing and over interpreted, and that do not provide an advance to the current understanding of Seipin function.

"1- In fig 4 it is shown that seipin phenotypes are suppressed by exogenous pyruvate supplementation. This is weird as, in comparison to controls, Seipin mutants appear to have already massive pyruvate levels (Fig3A). This is interpreted as the extra pyruvate is necessary to rescue the endogenous citrate levels, which is not satisfactory. Moreover, at the concentrations used, the effects of citrate and pyruvate are exclusively detected in the seipin mutants while controls appear completely irresponsive to the extra metabolites. It is not clear why that is and how direct the effects are."
We understand the reviewer's viewpoint. It is interesting that pyruvate supplementation has rescuing effect in *dSeipin* mutants, which already show accumulation of cellular pyruvate. It is possible that although mitochondrial metabolic activity is partly impaired in *dSeipin* mutants, it is not fully abolished. Therefore, providing more substrates might generate more TCA cycle products to support the downstream metabolic processes. Indeed, the newly included metabolic

flux results suggest that the metabolic flux from glucose to the TCA cycle is reduced, but not abolished, in *dSeipin* mutants (Figure 3). Furthermore, we have now performed Acetyl-CoA measurements, and we found that the reduced intracellular Acetyl-CoA in *dSeipin* mutants can be rescued by pyruvate or citrate supplementation (Figure 4D). This result provides further evidence that additional pyruvate can effectively replenish the downstream metabolites for lipogenesis.

Under lab culture conditions, larvae are reared in nutrient-rich/excess medium, so wild-type larvae may have already reached their maximal lipid storage capacity. Therefore, it is reasonable to find that exogenous metabolite supplementation does not have profound effects on TAG content or lipid droplets in wild type. In line with that, *Drosophila plin1* mutants only display adult-onset obesity, despite exhibiting super-large LDs in larval fat body (Beller *et al*, 2010).

"2- Previous work in different model systems, including Drosophila cell lines (Wang et al. eLife 2016) performed detailed lipid analysis in seipin mutant. Altogether these experiments argue strongly against a defect in fatty acid and/or lipid synthesis. However, the authors did not measure the levels of Acyl-CoA or activity of Fatty acid synthase."
Indeed, both Wang *et al*. (eLife 2016) and us (Tian *et al*, 2011) reported previously that there is no defect in fatty acid and/or lipid synthesis in a *Drosophila* cell line (Wang *et al*, 2016) and salivary gland (Tian *et al*, 2011). The difference is likely due to cell type specificities. Both *Drosophila* cell lines and salivary gland store very little fat normally, while large amount of lipids are synthesized and stored in larval fat body. We have discussed the cell type specificities issue in the discussion. In addition, we have now performed steady-state Acetyl-CoA measurement, and we found that the intracellular Acetyl-CoA level is reduced in *dSeipin* mutants. In addition, although we were unable to determine the synthetic rates of Acyl-CoA and fatty acid, we found that the level of Acetyl-CoA (the precursor of Acyl-CoA) is low in *dSeipin* mutants compared to controls in the metabolic flux assay (Figure 3B).

"3- Zhou et al (MCB, 2016) previously studied the impact of seipin mutations on the mitochondrial function of mice brown adipocytes. In this context, it was found that seipin mutation increases OCR while MitoTracker labelling was indistinguishable from control cells. Now the authors found that in fly larvae fat bodies seipin appears to a different impact. It would then be important to understand the basis of the differences. Are there cell type specificities that may underlie the discrepancies?"
We agree with the reviewer. The discrepancies are likely due to cell type specificities. Many studies in the mouse Seipin model (Chen *et al*, 2014； Jiang *et al*, 2014； Liu *et al*, 2014； Zhou *et al*, 2014) and our previous work in the *Drosophila* Seipin model (Bi *et al*, 2014； Tian *et al*, 2011) suggested that Seipin functions via distinct mechanisms in different tissues. These findings are also consistent with the fact that BSCL2 patients manifest distinct lipid storage phenotypes in adipose tissue and non-adipose tissues. The difference in the interacting partner of Seipin may explain the discrepancies. We have discussed these points in the discussion section of the revised manuscript.

"4- It is assumed that mitochondrial "encapsulation" phenotype described in Figure 5 correspond to mitochondria in autophagosomes. However, no autophagy markers were monitored or measurements of autophagic flux were performed to convincingly support this observation."
Great suggestion! To validate the TEM observation, we performed immunostaining using the autophagosome-specific marker Atg8a and the mitochondrial marker ATP5A. We examined the mitophagy incidence by colocalization analysis. We found that there was significant colocalization of ATP5A-positive mitochondria and Atg8a-positive autophagosomes in *dSeipin* mutant fat cells, indicating that mitophagy is elevated in *dSeipin* mutants. The results are consistent with our TEM observation and are included in our revised version (Figure 5).

Referee #3:
In this paper, Ding at al. investigate the Seipin mutation in Drosophila fat cells and report that the observed drop in lipid droplets (LD) is due to a decrease in lipogenesis. According to the authors, low mitochondrial calcium, would lead to decreased PDH activity and consequently to low levels of citrate, which plays an important role in lipogenesis. The decrease in calcium uptake into mitochondria would be due to decreased levels of calcium in the ER, an observation which the authors previously made using Seipin-deficient fat cells. Overall the results are sound and interesting.
Specific comments :

"-The authors analyze LD at steady state. How can they distinguish between a decrease in the biogenesis of LD and an increase in the use of lipids, stored in LD, for beta oxidation and ATP production? Both scenarios would have the same consequence, i.e. a drop in LD. Can the authors discuss this."
This is an excellent comment shared with referees #1 and 2#. We have performed an isotopic flux tracing experiment using a short pulse of U-^{13}C-glucose. We incubated isolated larval fat tissues in medium containing isotope-labeled glucose. The results indicate that the glucose flux into mitochondrial TCA cycle products is lower in *dSeipin* mutants than in controls, while the kinetics of glycolysis are not significantly changed (Figure 3). Moreover, we found that genetic inhibition of mitochondrial Ca^{2+} release partially restored the metabolic flux (Figure 6G and H). We also measured the activities of TAG hydrolase in *dSeipin* mutant and wild-type larval fat bodies. There is no significant difference between wild type and mutants, indicating that the lipodystrophic phenotype of *dSeipin* mutant is probably not caused by hyperactive lipolysis (Figure S1).

"-The export of citrate from the mitochondria into the cytosol contributes to lipogenesis by increasing acetyl CoA levels in the cytosol. What is the level of acetyl CoA in the cytosol of Seipin-deficient cells?"
We appreciate this suggestion by the reviewer. In the revision, we measured the level of Acetyl-CoA in *dSeipin* mutants under various rescuing conditions. We found that the Acetyl-CoA level is indeed low in *dSeipin* mutants and could be recovered by genetically rebalancing the mitochondrial calcium level or by dietary restoration of the substrates for lipogenesis (Figure 4D and 6G).

"-Would a knockout of the MCU lead to a decrease in LD?"

We performed larval fat body-specific knockdown of *MCU*, but the RNAi showed no LD phenotype. In addition, it didn't affect mitochondrial calcium. It is possible that MCU is not the limiting factor for larval fat tissue mitochondrial calcium influx. Alternatively, the RNAi may not be efficient enough to result in functional loss of MCU and impaired mitochondrial calcium homeostasis.
We also genetically reduced the mitochondrial calcium by knocking down the ERMES factors that scaffold the ER and mitochondrion. These manipulations phenocopy the *dSeipin* mutant (Figure 6C-E), indicating that impaired mito-calcium uptake can cause lipodystrophy.

"-Would activation of the PDH, using for example Dichloroacetate, increase the amount of LD?"
This is a great suggestion. Dichloroacetate activates PDH via a Ca^{2+}-independent mechanism. We added Dichloroacetate in the medium and found that this treatment rescued the lipodystrophy phenotype of *dSeipin* mutant fat cells (Figure S7).

"-Would inhibition of the mitochondrial pyruvate carrier lead to a drop in LD?"
The impacts of MPC on organismal metabolism have been addressed beautifully by Bricker and colleagues (Bricker *et al*, 2012). Their results are very similar with our metabolomic data. *dMPC1* mutants display impaired pyruvate metabolism, with an accumulation of glycolytic metabolites and a depletion of TCA cycle intermediates and ATP. This metabolic alteration is indeed associated with a reduction of lipid storage.

(Figures for referees not shown)

"-The authors report increased mitophagy in Seipin-deficient cells. Do they have an explanation for this observation? Does mitophagy contribute to the phenotype?"
This question is shared with referee #1. We found that dietary supplements can restore the lipid phenotype without rescuing mitophagy (Figure 5G and 5H). Therefore, it is likely that the mitochondrial defects in *dSeipin* mutant are not due to the lipid storage phenotype. Based on a previous report regarding the relationship between mitochondrial Ca^{2+} and mitophagy (Rimessi *et al*, 2013), we hypothesized that increased mitophagy in *dSeipin* mutants is likely the consequence of impaired mitochondrial Ca^{2+} homeostasis. It is hard to fully separate the defective mitochondrial metabolism and mitophagy. It is possible that defective mitochondrial metabolism and subsequently mitophagy both contribute to the lipid storage phenotype.

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2nd Editorial Decision 30th May 2018

Thank you for submitting a revised version of your manuscript (EMBO-J2017-97572R).
Your study has been seen by the three original referees and we have now received their comments, which are enclosed below for your information.

As you can see, while referee #1 and #3 are fully satisfied with the new data, referee #2 raises few minor issues that need to be addressed before formal acceptance here. In particular, s/he asks to further discuss the effect of reduced levels of glycolytic enzymes and citrate on glycolysis.

I am therefore formally returning the manuscript to you for a final round of minor revision.

REFEREE REPORTS.

Referee #1:

I have scrutinized carefully all the reviewers' comments and the authors' rebuttal. Overall, I think authors have strengthened their data to further support their hypothesis that intrinsic mitochondrial defects in metabolism significantly contribute to the lipogenesis defect in seipin-deleted cells. I am satisfied with their responses to my questions.

My only request is that they include in the manuscript the statement they make in their rebuttal: "It is possible that defective mitochondrial metabolism and subsequently mitophagy both contribute to

the lipid storage phenotype." There is a lot of mitophagy in their seipin-deficient flies, and I think it is important to explicitly state that this may be contributing to the lipodystrophy in the animals.

Referee #2:

The revised manuscript by Ding and colleagues has two major new pieces of data, the metabolomics flux experiments and the characterization of mitochondrial defects (Figure 5). The data is still not completely compelling but it appears to support the main hypothesis of the authors (mitochondrial calcium imbalance reduces the available amounts of citrate/Acyl-CoA for lipogenesis). In fact, some of the data remains puzzling to me. For example, the authors show that many glycolytic enzymes are down regulated, likely inhibited by a product inhibition mechanism due to the large accumulation of intermediates (such as pyruvate and lactate). However, despite the meager levels of the enzymes dSeipin mutants, flux analysis shows that production of glycolysis intermediates are not significantly altered or even slightly increased in these cells. How is it possible to have glycolysis with such downregulation of key enzymes of the pathway?
Similarly, the flux analysis shows obvious defects in citrate and isocitrate processing but not in pyruvate, which appears indistinguishable between wt and mutant fat bodies. Wouldn't this condition favor citrate accumulation (instead of depletion)? How would this fit on the model proposed?
The data on Fig 5G is not terribly convincing - it is not clear to me what we are looking at. Also, disruption of ER-mitocondria contacts has pleiotropic defects (such as lipid composition) and is not exclusive to calcium changes.

Minor points:
- "ectopic expression of CG7069 also rescues the larval lipid storage defect of dSeipin mutants". There is a rescue albeit not to the same extent as pyk and ERR- this should be acknowledge.

- Figure S7 is not very clear- Mitochondria almost looks like ER.

Referee #3:

The authors have addressed my previous concerns. The paper has been substantially strengthened.

2nd Revision - authors' response 8th June 2018

Point-by-Point response

Referee #1:
I have scrutinized carefully all the reviewers' comments and the authors' rebuttal. Overall, I think authors have strengthened their data to further support their hypothesis that intrinsic mitochondrial defects in metabolism significantly contribute to the lipogenesis defect in seipin-deleted cells. I am satisfied with their responses to my questions.
My only request is that they include in the manuscript the statement they make in their rebuttal: "It is possible that defective mitochondrial metabolism and subsequently mitophagy both contribute to the lipid storage phenotype." There is a lot of mitophagy in their seipin-deficient flies, and I think it is important to explicitly state that this may be contributing to the lipodystrophy in the animals.
> Thanks. As suggested, this statement has been integrated into the revised version (Page 12, line 21-22).

Referee #2:
The revised manuscript by Ding and colleagues has two major new pieces of data, the metabolomics flux experiments and the characterization of mitochondrial defects (Figure 5). The data is still not completely compelling but it appears to support the main hypothesis of the authors (mitochondrial calcium imbalance reduces the available amounts of citrate/Acyl-CoA for lipogenesis). In fact, some of the data remains puzzling to me. For example, the authors show that many glycolytic enzymes are down regulated, likely inhibited by a product inhibition mechanism due to the large accumulation of intermediates (such as pyruvate and lactate). However, despite the meager levels of the enzymes dSeipin mutants, flux analysis shows that production of glycolysis intermediates are not significantly altered or even slightly increased in these cells. How is it possible to have glycolysis with such downregulation of key enzymes of the pathway?
> The difference may be due to the fact that the larvae used in the flux assay and the iTraq experiment (in which we found that many glycolytic enzymes are down-regulated) are at different stages. The flux assay requires that lipogenic metabolism (from glucose to TAG) is still active in the tissues. Therefore, we used fat bodies from early L3 larvae to perform this experiment. The samples in the iTraq experiments were from wandering stage (later L3) larvae. It is likely that the reduced protein level of glycolytic enzymes we observed in wandering stage larvae only happens after long-term defects in metabolic flux, which cause the large accumulation of intermediates (such as pyruvate and lactate). In the revision, we have now clearly stated the stage of the animals used in the flux assay (Page 10, line 2).

Similarly, the flux analysis shows obvious defects in citrate and isocitrate processing but not in pyruvate, which appears indistinguishable between wt and mutant fat bodies. Wouldn't this condition favor citrate accumulation (instead of depletion)? How would this fit on the model proposed?
> The flux assay also indicates the reduced production of Acetyl-CoA (Figure 3B), which is the substrate for citrate synthesis. Therefore, the depletion of citrate is a reasonable outcome, and is consistent with our model. Regarding the levels of pyruvate in wild type and mutants, we observed a slightly increased level of pyruvate in the flux assay in mutants (Figure 3B), although not as dramatic as the reduction of Acetyl-CoA. It is possible that other products derived from pyruvate, such as lactate (which is not analyzed in our flux assay) may also be increased, and this is supported by the steady-state data (Figure 2A).

The data on Fig 5G is not terribly convincing - it is not clear to me what we are looking at. Also, disruption of ER-mitochondria contacts has pleiotropic defects (such as lipid composition) and is not exclusive to calcium changes.
> Thanks for this comment. To make Fig 5G clearer, we have split the different color channels to show the elevated mitophagy (more colocalization of ATP5A, the mitochondrial marker, and ATG8a, the autophagy marker) in *dSeipin* mutants.
> We agree that disruption of ERMES has pleiotropic defects besides the calcium changes. The purpose of the genetic manipulations is to show the positive correlation between the mito-Ca^{2+} and lipid storage.

Minor points:

- "ectopic expression of CG7069 also rescues the larval lipid storage defect of dSeipin mutants". There is a rescue albeit not to the same extent as pyk and ERR- this should be acknowledge.
> We agree. We have added this note in the revision (Page 7, line 2-3).

- Figure S7 is not very clear- Mitochondria almost looks like ER.
> To improve the image quality, we replaced the MitoTracker Red dye staining by anti-ATP5A antibody staining to label mitochondria. The new imaging data clearly highlights the mitochondria and depicts the partial colocalization of CG18660-EGFP and mitochondria.

Referee #3:
The authors have addressed my previous concerns. The paper has been substantially strengthened.
> Thanks.

3rd Editorial Decision 15th June 2018

Thank you for submitting a revised version of your manuscript and addressing the last concerns from referee #2 in your point-by-point letter.

I have looked at your response and find that all experimental concerns have been sufficiently addressed. However, before we can go on to officially accept the manuscript there are a few editorial issues concerning text and figures that I need you to address.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Xun Huang
Journal Submitted to: EMBO Journal
Manuscript Number: EMBOJ-2017-97572R1

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	The sample sizes are clearly described in the figure legends.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No sample was excluded from analysis
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	In GESA, randmization procedure was taken by computer when resample the RNA-seq Bam files.
For animal studies, include a statement about randomization even if no randomization was used.	NA
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	the original information of the samples for MS analysis was blinding of the investigator
4.b. For animal studies, include a statement about blinding even if no blinding was done	NA
5. For every figure, are statistical tests justified as appropriate?	Yes, statistical tests were justified as appropriate and clearly indicated in figure legends.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes. Data was checked for normalcy. When normal, t test or ANOVA test was taken.When not normal, the Mann-Whitney test was taken.Homogeneity of variance was also considered when choosing the post-hoc test. Detailed stastic methods were indicated in figure legends.
Is there an estimate of variation within each group of data?	Yes, the standard error of the mean for each data set are indicated in the figures.
Is the variance similar between the groups that are being statistically compared?	Yes.

C- Reagents

USEFUL LINKS FOR COMPLETING THIS FORM

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6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Antibody information is clearly described in the EXPERIMENTAL PROCEDURES part.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	NA

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	Strains of Drosophila and husbandry details are clearly described.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	NA
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	NA

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	The raw sequencing data of RNA-seq have been submitted to the Genome Sequence Archive (GSA) database with the accession number PRJCA000907
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	We included metabolomic dataset in Appendix Table S1.
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
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G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	NA