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Lipin1 deficiency causes sarcoplasmic reticulum stress and chaperone-responsive myopathy

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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 3rd May 2018

Thank you for the submission of your manuscript (EMBOJ-2017-98772) to The EMBO Journal. My apologies for the extended duration of the review process of the manuscript due to delayed referee feedback. Your manuscript has been sent to four referees, and we have received reports from all of them, which I enclose below.

As you will see, the referees acknowledge the potential high interest and novelty of your work, although they also express a number of major issues that will have to be addressed before they can support publication of your manuscript in The EMBO Journal. In more detail, referee #2 is concerned about the hierarchy between the observed ER stress, altered lipid metabolism and mitochondrial fragmentation, which need to be characterized in greater depth in his-her view. Referee #4 agrees in that a more detailed analysis of the cellular events and rescue by TUDCA would be required (Ref #4, pts 2,3,4, 6). Referee #3 states concerns about discrepancies with earlier genetic data on lipin1 loss-of-function models and asks you to integrate those. Finally, referee #1 points to issues related to experimental design and missing controls for the lipidomics/metabolomics data, that would need to be conclusively addressed to achieve the level of robustness needed for The EMBO Journal.

I judge the comments of the referees to be generally reasonable and given their overall interest, we are in principle happy to invite you to revise your manuscript experimentally to address the referees' comments. Please note however, that we would need strong support from the referees on such a revised version of the manuscript to move towards publication. I agree that it would be essential to consolidate the causalities between the distinct cellular phenotypes at Lipin1 deficiency and add mechanistic insights into their nature.

REFEREE REPORTS:

Referee #1:

This review focuses on lipidomic/metabolomic analysis aspects of the submitted work.

Analysis of phospholipids was performed by shotgun approach to assess the effect of Lipin1 deficiency in the muscle of a murine model. The approach is similar to commonly adopted shotgun approach, with some deviations which may limit the conclusiveness of the results:
(1) Since QqQ instrument was used at unit mass resolution, how could isobaric lipids be separated (a challenge of shotgun approach even for accurate mass instruments)? Ether phospholipids are abundant in the muscle, yet how can they be reliably covered using the approach.
(2) The B&D extraction is sub-optimal for PA and PG species (i.e. they cannot be extracted with high efficiency). A separate extraction protocol would be needed for these classes of lipids. How was the extraction efficiency controlled for?
(3) Using the internal standards with 17:0/20:4 acyl chains is a bit unusual. Have the authors checked the abundance of endogenous lipids containing this acyl composition (they are there)?
The method is not quantitative, but at best semi-quantitative, therefore discussion of % composition of lipids is not meaningful. The authors should also state how the lipid levels were calibrated using the internal/external(?) standards.

Targeted analysis of polar metabolites is problematic, because there is no reference to the protocol providing all the necessary detail about the method (extraction, analytical conditions, standard compounds used, calibration, QC, MRM transitions etc), while the description is insufficient to evaluate the method. The method is not standard, as one can rarely see method, which would measure in a single analysis together e.g. nucleotides, acetyl-CoA, bile acids and amino acids. Theoretically this is possible, but the question is how reliable the concentration values are? Again, have the authors checked the extraction efficiency and how were the values calibrated? How were the analytes uniquely identified?

The authors apply MetaboAnalyst for pathway analysis as a 'black box' solution, with too little detail provided. With 30 metabolites included in the analysis, how representative are the pathways (i.e. what was the fraction of metabolites represented in any given pathway listed)? There are nearly as many pathways listed in Figure 4D as there were metabolites used in the analysis.

Referee #2:

Genetic mutations causing loss of function of LPIN1 gene lead to rhabdomyolysis in children, although metabolic alterations and therapeutic interventions are still elusive. In this manuscript, Rashid, T. et al demonstrated that muscle specific deletion of Lipin1 causes severe myopathy in mice, with excessive accumulation of both neutral- and phospho-lipids, which the authors attribute to excessive fatty acid synthesis and impaired fatty acid oxidation (FAO), accompanied by a defect in acyl chain elongation and desaturation and activation of the lipogenic SREBP1c/SREBP2 factors. The authors performed lipidomics and metabolomics analyses to gain insight into the mechanisms for the severe myopathy. They propose that ER stress may be a primary cause for the disease, as a major UPR response is detected in Lipin 1 deficient muscles and attenuation of ER stress by TUDCA treatment improves muscle strength, reduces lipid accumulation and improves overall muscle fiber morphology. The relevance of the study lies in the fact that by identifying ER stress as a major cause for the pathology, it opens the possibility for new therapies targeting to restore ER function in patients with lipin1 mutations. The manuscript is generally well written and demonstrate interesting findings with potential relevance for the treatment of patients with genetic mutations in the LPIN1 gene. However, most of the data presented is descriptive in nature. Although the authors were able to demonstrate that alleviation of ER stress can ameliorate some aspects of the pathology, they were unable to restore the mitochondrial phenotype. The mechanisms for ER stress and UPR activation remain to be determined and better characterization of the mitochondrial phenotype is warranted.

Major Concerns:
1- The authors report that mtDNA is dramatically reduced in Lipin1 deficient mice. TEM also demonstrates changes in mitochondrial morphology, including changes in the cristae structure, and downregulation of mitochondrial encoded genes, which was not ameliorated by attenuating ER stress. Could this suggest that changes in mitochondria are an upstream event and that ER stress is secondary to mitochondrial stress? What is the cause for mitochondrial dysfunction in this model? Better characterization of mitochondrial function is warranted. The authors should measure mitochondrial oxygen consumption, ATP synthesis and mtROS. Additionally, proteins involved in mitochondrial dynamics should be investigated. Is AMPK activity increased?

2- Lipotoxicity has been shown to interfere with lysosomal acidification in the heart. In the present manuscript, TEM suggest autophagy and lysosomal function may be impaired in Lipn1 KO mice. This should be further investigated, as impaired autophagy/mitophagy could result in accumulation of damaged mitochondria. Indeed, autophagy-deficient muscles have impaired mitochondrial function and activation of the UPR.

3- Studies in primary myotubes lacking Lipin1 would be useful to elucidate the mechanisms for mitochondrial dysfunction and ER stress described in the manuscript. For example, can the mitochondrial phenotype be ameliorated by increasing autophagic flux in these cells? If so, will be this sufficient to prevent ER stress and limit lipid accumulation.

4- Mitochondrial and ER stress is often associated with loss of muscle mass, and atrophy of muscle fibers. More sensitive assays should be applied to measure total lean mass and muscle fiber diameter.

5- The authors report an increase in FGF21 mRNA expression and protein levels in Lipin1 deficient muscle. Were FGF21 levels elevated in the circulation? Muscle-derived FGF21 has been shown to have potent effects on systemic metabolism, preventing obesity and improving insulin sensitivity. Were body mass and fasting glucose levels changed in these animals?

6- The data suggest that glycolytic muscles, such as TA, are more susceptible to fiber damage in response to Lipin1 deficiency? Why is that? Also, the oxidative fibers are the ones accumulating lipids in Lipin1 deficient muscles, but they look healthier overall. With that in mind, please, discuss how the increase in lipid accumulation would contribute to your muscle phenotype? This is critical to understand the mechanisms for the myopathy in this model. What is the mitochondrial phenotype in TA and gastrocnemius muscle?

Minor:

1- In methods, please, specify the protocol for tamoxifen administration (how it was administered, dosage, age of animals, duration). Also, indicate the age in which the experiments were performed. In the legends, I was able to find information about the ages, which ranged from 3-6 months of age. Why were different ages utilized for different assays? Does the phenotype progress with age?

2- Still in methods, please specify the age in which TUDCA treatment was initiated? Was the treatment meant to prevent muscle dysfunction or reverse it?

3- Lipn1 protein levels should be measured in skeletal muscle of KO mice.

Referee #3:

Summative comments
The manuscript submitted by Rashid and colleagues proposes a novel mechanism by which loss of function of the Lpin1 phosphatidic acid phosphatase (PAP) in murine muscle results in ER stress and myopathy. Muscle-specific loss of function exhibits reduced PAP1 activity, histological and

functional evidence of myopathy, and dysregulation of lipid homeostasis manifest by modest increase in PA abundance, dramatic increase in total phospholipid (PL) and triglyceride (TG) levels, and altered phospholipid acyl chain composition. The authors go on to show increased activation of SREBPs in Lipin1 deficient-muscle and suggest enforced SREBP-dependent lipogenesis is therefore responsible for the observed accumulation of lipids. The authors demonstrate activation of multiple ER stress markers in Lipin1 deficient gastrocnemius, a phenotype accompanied by sarcoplasmic reticulum (SR) fragmentation, a reduction in mitochondrial DNA (mtDNA) content, and evidence of mitochondrial fragmentation. Finally, the authors treat mice with the anti-apoptotic bile acid chaperone TUDCA and demonstrate reductions in ER stress, lipid accumulation, myonecrosis, and functional impairment.

Overall, the presented work is interesting but important questions remain surrounding the mechanisms of ER stress and lipid accumulation, particularly in light of prior work in a similar mouse model with substantially different findings. In addition, several of the reported findings are not particularly novel: myopathy with abnormal mitochondrial morphology has previously been reported in mice harboring Lpin1 mutations and SREBP regulation is a well-documented function of Lipin1. This manuscript's primary novel findings are (1) the nature of the lipid dysregulation (increased PLs and TGs) and (2) reversible ER stress. Unfortunately, the mechanistic basis for each of these findings goes largely unaddressed. Nevertheless, the therapeutic potential of chemical chaperones in a clinically-relevant myopathy model is of significant interest and would be suitable for publication if additional mechanistic data were provided.

Major points

1. Why are PC, PE, and TG levels increased upon Lpin1 mutation?

Since PC and PE are predominantly generated through a PAP-dependent pathway, the finding is somewhat counterintuitive despite the Lpin yeast homolog Smp2's role as a negative (transcriptional) regulator of phospholipid biosynthesis. The authors speculate in the discussion that this might reflect alternative biosynthesis via the CDP-DAG pathway, but this point would need to be addressed experimentally to be convincing. Similarly, the authors speculate on but do not address the mechanism by which TG levels are increased in mutant mice. This is also a counterintuitive result, as most non-enterocytes synthesize TGs via the glycerol-phosphate pathway, which would be expected to be impaired upon PAP loss of function and reduced availability of DAG.

These are particularly key points because prior published work (Zhang, et al Cell Metab 2014) in LipinFld/Fld homozygous loss-of-function mice also revealed a myopathy accompanied by increased abundance of lipid droplets and aberrant mitochondria. However, contrary to the findings presented here, a significant reduction in TG levels, an increase in cholesterol levels, and no significant difference in PC levels were noted. Prior work in Lpin1 null muscle also reports a decrease rather than the increase reported here in fatty acid synthetic genes and an increase in mtDNA rather than the decrease reported in the current manuscript. The authors need to address these significant discrepancies given their central position in the manuscript's claims; might they reflect differences in the mouse models, such as the contributions in Lpin1fEx2-3/ fEx2-3;HSA-Cre of non-myocytes with retained Lipin1 function? Are LipinFld/Fld mice available in order to assess whether the ER stress and PL accumulation phenotypes presented here are consistent with previously published work?

2. The causes of ER stress in Lpin1fEx2-3/ fEx2-3;HSA-Cre mice remain unclear.

In the manuscript text the authors suggest that ER stress may drive SREBP cleavage and, in doing so, explain accumulation of lipid species. In the discussion, however, the authors suggest that the ER stress is itself the result of altered lipid metabolism. It is certainly possible that Lipin1 LOF directly causes "an overall disturbance of ER function" as suggested, but experimental evidence to support this claim is lacking.

Previous studies (Peterson, et al Cell 2011) identified nuclear Lpin1 as an important negative regulator of SREBP target expression by modulating SREBP protein's nuclear localization; other data support a role of Lipin1 as a more general co-regulator of transcription factor (TF) function. One model to explain the data in the manuscript might therefore be that SREBP de-repression in the setting of Lpin1 loss of function leads to unregulated lipid biosynthesis, which drives both ER stress and the observed lipidomic and metabolomic abnormalities. ER stress, in turn, might further amplify

post-translational SREBP activation via IRE1 and other UPR responses. Alternatively, SREBP-independent effects of Lpin1 loss-of-function on other TFs may be responsible some portion of the observed effects. No data are presented to address these important issues, but they would be tractable either with further in vivo experiments or in complementary tissue culture systems.

Minor points
- Fig1 and elsewhere: the authors need to clarify the genotype of "control" mice used throughout the manuscript. Are these animals HSA-Cre alone, Lpin1fEx2-3/ fEx2-3 alone, or WT?

- Fig 1B: To what do the authors attribute the increase in PAP2 activity in Lpin1fEx2-3/ fEx2-3;HSA-Cre animals?

- Fig 2A: DAG levels (in addition to PA levels) should be measured in Lpin1fEx2-3/ fEx2-3;HSA-Cre animals.

- Fig 3 and associated text: The statement "our data demonstrate that the absence of Lipin1 in skeletal muscle turns on an SREBP-dependent program promoting storage of triglycerides" is not fully supported by the presented evidence, as the SREBP-independent consequences of Lipin1 deficiency on lipid homeostasis are not assessed. Does enforced SREBP activation alone phenocopy the Lipin1 deficiency phenotype, and to what extent? Additional experiments would need to be performed to justify this conclusion.

- Fig 3: The authors conclude that the oil red O staining represents TG accumulation but do not address whether it may instead (or in addition) represent cholesterol accumulation, which might be expected in light of SREBP activation and presumably depleted pools of DAG for TG synthesis.

- Fig 5A: Associated text claims a statistically significant increase in all 3 arms of the UPR response (PERK, ATF6, and IRE1) but the Western blotting is not convincing for a biological difference in p-eIF2A levels, suggesting the PERK pathway may not be particularly engaged under these conditions. Given prior data in cancer cell models (He, et al. FASEB 2017) that the PERK pathway was not significantly activated upon Lpin1 depletion, the authors should examine downstream effectors of the PERK-CHOP and IRE1a-JNK pathways in this context with additional Western blotting.

- Fig 7: Previous work in cancer and muscle cell systems has implicated a central role for dysregulation of autophagy upon Lpin loss-of-function. Are these effects noted in Lpin1fEx2-3/ fEx2-3;HSA-Cre animals and does TUDCA reverse them as well?

- Fig 7: Does TUDCA treatment reverse the ultrastructural ER and mitochondrial defects associated with Lpin1 loss-of-function?

Non-essential suggestions
- Fig 2C: This figure is difficult to interpret (specifically the abundance of PC in control mice) as currently drawn owing to the broken axis. The axis should be, at a minimum, relabeled after the break as in Fig 2B.

- Extended View Fig 1 and elsewhere: At several points through the manuscript the authors present data from various muscle groups (gastrocnemius, tibialis anterior, extensor digitorum longus). Given the significant differences in myopathy and lipid profiles between these muscle groups (see for example Extended View 1A), authors should justify the use of particular muscles for particular experiments

- Extended View Fig 1D is used to support the claim that fiber type composition is unchanged upon Lipin1 deletion, but data are shown only from EDL muscle which (based upon Extended View Fig 1A) is among the less affected muscles in Lpin1fEx2-3/ fEx2-3;HSA-Cre mice. Further, the EDL is composed (per the authors) of predominantly fast-twitch fibers whereas TG accumulation is most pronounced (Fig 3A) in oxidative fibers. Are the findings similar in GC and TA?

- The figure legend refers to Fig 7G, which does not appear to exist - perhaps having been replaced

with Extended View Fig 4B?

Referee #4:

This is an interesting work addressing the role of Lipin1 in skeletal muscle. The authors generated a muscle specific Lipin1 knockout mouse line and characterised the phenotype of these mice. They found that ablation of Lipin1 results in amyopathic phenotype with important weakness. The metabolomic analyses revealed an alteration in lipid homeostasis because absence of Lipin triggered ER stress and Srebp1/2 activation. Importantly, administration of the chemical chaperon TUDCA greatly ameliorate the muscle phenotype. This is an excellent work that finally shed a light of the mechanistic insights of the human disease due to homozygous or heterozygous loss of functions mutations of LIPN1 gene. The characterization of the mice has been well performed and the identification of a problem in ER resulted in a successful treatment that can be translated to humans since ursodeoxycolate acid and derivatives are used in medical practice. Few points should be addressed to better dissect the mechanistic insights.
Point1. Does triglycerides and lipid accumulation happen also in soleus or diaphragm muscles, which are rich of mitochondria and display a beta oxidative metabolism? Or is peculiar of beta oxidative fibers of fast glycolytic muscles?
Point2. Accumulation of Lipids as well as induction of FGF21 and GDF15 are hallmarks of mitochondrial dysfunction. The authors analysed the mitochondrial morphology, mtDNA and expression of several mitochondrial encoded genes and found that are altered. However, a better characterization of mitochondria is required. Is respiration and complex activity affected? The increased electron-dense contacts between mitochondria were interpreted as a fission problem by the authors. However this interestingly observation need to be sustained by biochemical analyses. Is the fission machinery increased (DRP1, Fis, Mff) and/or the fusion proteins (OPA1. Mtf1/2) decrease in knockout mice? The decrease of fusion or increased fission/mitophagy may explain the reduced mtDNA. Better EM pictures of mitochondrial morphology showing cristae shape are requested. An alteration in SR-mitochondria tethering may also cause calcium changes, mitochondrial dysfunction and SR stress. Better EM images of triads are also required.
Point3. Similar to point 2, the ultrastructural observation of an autophagy impairment should be better sustained by biochemical analyses. Please check the LC3II levels or LC3 puncta as well as the expression of some mitophagy markers such as Bnip3, Nix, Bcl2l13. It has been reported that PA stimulates mTOR pathway resulting in an autophagy inhibition. Please check the status of Akt-mTOR axis in knockout mice. It is possible that the primary effect of Lipin inhibition is on mitochondria/mitophagy that triggers an ER stress response and consequently phospholipids biogenesis.
Point4. The mechanism of ER stress should be better characterised. SR and cytosolic calcium levels and the presence of oxidative stress (resulting from mitochondrial dysfunction) may explain why different UPR pathways are simultaneously activated in KO mice.
Point5. The accumulation of glucose, citrate and the formation of ketones suggest an impairment of TCA cycle due probably to underutilization of NADH from the respiratory chain. The necrotic effect might be consequent to stress induced energy crisis. How is the ATP level in resting and exercised KO mice? Are these mice exercise intolerant?
Point6. It would be interesting to know how much of the metabolite and lipid profiles is rescued by TUDCA treatment. Although this point is not necessary for the publication it would add interesting insights of the mechanism of action. Indeed, TUDCA is a cholesterol derived molecule and the beneficial effects might rely on its action on membranes independently of the chaperone effect.

We thank the four reviewers for their comments and we appreciate that they found broad interests in our work. We think we largely addressed the reviewer's comments and improved the manuscript. In this revised version, we provide new functional data delineating the pathophysiological alterations in Lipin1 deficient myopathies and possible therapeutic targets. Briefly, we address four main points. First, we provide further analysis of mitochondrial and autophagy impairments. Second, we compare a variety of pharmacological treatments, from chemical chaperones to autophagy activators, anti-oxidants, PPARα activators. Third, we extend our observations to two additional animal models of whole body Lipin1 deficiency, the FLD mice and the Hubr rats. Fourth, we localize Lipin1 activity in the muscle fibers. In addition, we include a thorough panel of quantifications and controls to interpret the data on metabolomics and biochemical analysis, as requested by the reviewers. There is a large amount of new data that are included in the 9 main figures and 10 expanded view figures of this revised manuscript.

Below is our response to the reviewer comments.

Referee #1:

This review focuses on lipidomic/metabolomic analysis aspects of the submitted work. Analysis of phospholipids was performed by shotgun approach to assess the effect of Lipin1 deficiency in the muscle of a murine model. The approach is similar to commonly adopted shutgun approach, with some deviations which may limit the conclusiveness of the results: (1) Since QqQ instrument was used at unit mass resolution, how could isobaric lipids be separated (a challenge of shotgun approach even for accurate mass instruments)? Ether phospholipids are abundant in the muscle, yet how can they be reliably covered using the approach.

The reviewer is right that isobaric lipids are not resolved in our shotgun approach using a QQQ instrument. However, the limitation does not affect our conclusions, as we quantify the total amount of phospholipids in a class. To deal with the limitation, we avoid any over interpretation based on the quantity of single lipid species.

Ether phospholipids were included in our target list (see Table R1). The species detected reliably (>0.5% of total signal intensity in a lipid class) were quantified and counted. In general ether lipids are not abundant in our samples (see below point 3).

Table R1: Target list

m/z	Lipid Name	788.6	PC 36:1	868.6	PC 42:3	750.5	PE O-38:6	830.5	PE 42:1
796.6	PC 37:4	790.6	PC 36:0	870.6	PC 42:2	752.5	PE O-38:5	836.5	PE O-44:5
676.6	PC 28:1	792.6	PC O-38:6	872.6	PC 42:1	756.5	PE O-38:3	848.5	PE 44:6
678.6	PC 28:0	794.6	PC O-38:5	754.5	PE 37:4	758.5	PE O-38:2	850.5	PE 44:5
690.6	PC O-30:1	798.6	PC O-38:3	636.5	PE 28:0	760.5	PE O-38:1	852.5	PE 44:4
692.6	PC O-30:0	800.6	PC O-38:2	650.5	PE O-30:0	762.5	PE O-38:0	854.5	PE 44:3
702.6	PC 30:2	802.6	PC O-38:1	662.5	PE 30:1	764.5	PE 38:6	862.4	PE O-46:6
704.6	PC 30:1	804.6	PC O-38:0	664.5	PE 30:0	766.5	PE 38:5	864.5	PE O-46:5
706.6	PC 30:0	806.6	PC 38:6	674.5	PE O-32:2	768.5	PE 38:4	890.5	PI 37:4
714.6	PC O-32:3	808.6	PC 38:5	676.5	PE O-32:1	770.5	PE 38:3	812.5	PI O-32:1
716.6	PC O-32:2	810.6	PC 38:4	678.5	PE O-32:0	772.5	PE 38:2	824.5	PI 32:2
718.6	PC O-32:1	812.6	PC 38:3	686.5	PE 32:3	774.5	PE 38:1	826.5	PI 32:1
720.6	PC O-32:0	814.6	PC 38:2	688.5	PE 32:2	776.5	PE 38:0	828.5	PI 32:0
728.6	PC 32:3	816.6	PC 38:1	690.5	PE 32:1	778.5	PE O-40:6	838.5	PI O-34:2
730.6	PC 32:2	818.6	PC 38:0	692.5	PE 32:0	780.5	PE O-40:5	840.5	PI O-34:1
732.6	PC 32:1	820.6	PC O-40:6	700.5	PE O-34:3	782.5	PE O-40:4	842.5	PI O-34:0
734.6	PC 32:0	822.6	PC O-40:5	702.5	PE O-34:2	784.5	PE O-40:3	850.5	PI 34:3
740.6	PC O-34:4	824.6	PC O-40:4	704.5	PE O-34:1	786.5	PE O-40:2	852.5	PI 34:2
742.6	PC O-34:3	826.6	PC O-40:3	706.5	PE O-34:0	788.5	PE O-40:1	854.5	PI 34:1
744.6	PC O-34:2	828.6	PC O-40:2	710.5	PE 34:5	790.5	PE O-40:0	856.5	PI 34:0
746.6	PC O-34:1	830.6	PC O-40:1	712.5	PE 34:4	792.5	PE 40:6	864.5	PI O-36:3
748.6	PC O-34:0	832.6	PC O-40:0	714.5	PE 34:3	794.5	PE 40:5	866.5	PI O-36:2
754.6	PC 34:4	834.6	PC 40:6	716.5	PE 34:2	796.5	PE 40:4	868.5	PI O-36:1
756.6	PC 34:3	836.6	PC 40:5	718.5	PE 34:1	798.5	PE 40:3	874.5	PI 36:5
758.6	PC 34:2	838.6	PC 40:4	720.5	PE 34:0	800.5	PE 40:2	876.5	PI 36:4
760.6	PC 34:1	840.6	PC 40:3	722.5	PE O-36:6	802.5	PE 40:1	878.5	PI 36:3
762.6	PC 34:0	842.6	PC 40:2	724.5	PE O-36:5	804.5	PE 40:0	880.5	PI 36:2
764.6	PC O-36:6	844.6	PC 40:1	726.5	PE O-36:4	806.5	PE O-42:6	882.5	PI 36:1
766.6	PC O-36:5	846.6	PC 40:0	728.5	PE O-36:3	808.5	PE O-42:5	884.5	PI 36:0
768.6	PC O-36:4	848.6	PC O-42:6	730.5	PE O-36:2	810.5	PE O-42:4	888.5	PI O-38:5
770.6	PC O-36:3	850.6	PC O-42:5	732.5	PE O-36:1	812.5	PE O-42:3	892.5	PI O-38:3
772.6	PC O-36:2	852.6	PC O-42:4	734.5	PE O-36:0	814.5	PE O-42:2	894.5	PI O-38:2
774.6	PC O-36:1	854.6	PC O-42:3	736.5	PE 36:6	816.5	PE O-42:1	896.5	PI O-38:1
776.6	PC O-36:0	856.6	PC O-42:2	738.5	PE 36:5	818.5	PE O-42:0	900.5	PI 38:6
778.6	PC 36:6	858.6	PC O-42:1	740.5	PE 36:4	820.5	PE 42:6	902.5	PI 38:5
780.6	PC 36:5	860.6	PC O-42:0	742.5	PE 36:3	822.5	PE 42:5	904.5	PI 38:4
782.6	PC 36:4	862.6	PC 42:6	744.5	PE 36:2	824.5	PE 42:4	906.5	PI 38:3
784.6	PC 36:3	864.6	PC 42:5	746.5	PE 36:1	826.5	PE 42:3	908.5	PI 38:2
786.6	PC 36:2	866.6	PC 42:4	748.5	PE 36:0	828.5	PE 42:2	910.5	PI 38:1

914.5	PI O-40:6	762.5	PS 34:1	834.5	PS 40:7	664.5	PA 32:1	712.5	PG 28:0
916.5	PI O-40:5	764.5	PS 34:0	836.5	PS 40:6	666.5	PA 32:0	724.5	PG O-32:1
918.5	PI O-40:4	774.5	PS O-36:2	838.5	PS 40:5	676.5	PA O-34:2	736.5	PG 32:2
920.5	PI O-40:3	776.5	PS O-36:1	840.5	PS 40:4	678.5	PA O-34:1	738.5	PG 32:1
922.5	PI O-40:2	784.5	PS 36:4	842.5	PS 40:3	680.5	PA O-34:0	740.5	PG 32:0
926.5	PI 40:7	786.5	PS 36:3	844.5	PS 40:2	690.5	PA 34:2	752.5	PG O-34:1
928.5	PI 40:6	788.5	PS 36:2	846.5	PS 40:1	692.5	PA 34:1	764.5	PG 34:2
930.5	PI 40:5	790.5	PS 36:1	864.5	PS 42:6	694.5	PA 34:0	766.5	PG 34:1
932.5	PI 40:4	792.5	PS 36:0	866.5	PS 42:5	704.5	PA O-36:2	768.5	PG 34:0
934.5	PI 40:3	800.5	PS O-38:3	870.5	PS 42:3	706.5	PA O-36:1	780.5	PG O-36:1
936.5	PI 40:2	802.5	PS O-38:2	872.5	PS 42:2	716.5	PA 36:3	790.5	PG 36:3
956.5	PI 42:6	804.5	PS O-38:1	874.5	PS 42:1	718.5	PA 36:2	792.5	PG 36:2
958.5	PI 42:5	808.5	PS 38:6	728.6	PA 37:4	720.5	PA 36:1	794.5	PG 36:1
960.5	PI 42:4	810.5	PS 38:5	610.4	PA 28:0	722.5	PA 36:0	816.5	PG 38:4
798.5	PS 37:4	812.5	PS 38:4	636.4	PA 30:1	742.5	PA 38:4	868.5	PG 42:6
734.5	PS 32:1	814.5	PS 38:3	638.5	PA 30:0	744.5	PA 38:3	870.5	PG 42:5
732.5	PS 32:0	816.5	PS 38:2	650.5	PA O-32:1	746.5	PA 38:2		
748.5	PS O-34:1	818.5	PS 38:1	652.5	PA O-32:0	794.5	PA 42:6		
760.5	PS 34:2	828.5	PS O-40:3	662.5	PA 32:2	802.5	PG 37:4		

(2) The B&D extraction is sub-optimal for PA and PG species (i.e. they cannot be extracted with high efficiency). A separate extraction protocol would be needed for these classes of lipids. How was the extraction efficiency controlled for?

The BD extraction protocol used in the study was a modified version that allows efficient extraction of PA and PG. We have checked signal intensities (ion counts) from internal standards 1) when samples are injected to MS directly and 2) when they are analyzed after they go through the extraction protocol. In all lipid classes, no apparent loss of signal could be seen (Fig. R1). Moreover, known amount of internal standards for every lipid classes were infused into the samples before extraction and biological lipids are quantified using the signal intensity of the internal standards. Therefore, the eventual loss during the extraction is normalized and does not affect quantitative performance even if the extraction was sub-optimal.

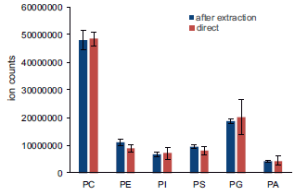

Fig. R1. Extraction efficiency. Signal intensities for the indicated phospholipids after direct injection to MS (red bars) or after the extraction protocol (blue bars).

(3) Using the internal standards with 17:0/20:4 acyl chains is a bit unusual. Have the authors checked the abundance of endogenous lipids containing this acyl composition (they are there)?
The method is not quantitative, but at best semi-quantitative, therefore discussion of % composition of lipids is not meaningful. The authors should also state how the lipid levels were calibrated using the internal/external(?) standards.

The lipids with 17:0/20:4 acyl chains are commonly used internal standards (IS) for the quantification of membrane lipids in mammalian cells/tissues, as experimentally validated by the lipid MAPS consortium (Moore, Caufield et al., 2007). One concern remains that the 17:0/20:4 species are isobaric to the ether lipid species with acyl chains of 38:4 in total (mainly 18:0/20:4 in mammalian tissue) at unit resolution that could be abundant in muscle and affect quantitative performance. Therefore, we checked signal intensity from O-38:4 species at the mass of IS when ISs are not infused into samples. In the analyses of PS, PG and PA, no signal over the background was detected at the mass. Signals of PC-O-38:4, PE-O-38:4 and PI-O-38:4 were detected but the signal intensity was 4.5 % of those of the corresponding IS at most (Fig. R2). Moreover, the intensity of these O-38:4 species are less than 0.5% of total intensity in the class. We anyway omit those very minor species from quantification to avoid counting signals from minor contaminations or noise. Therefore, we conclude that the effect of the overlap between ether lipids and IS on quantitative performance is negligible. We stated more precisely how lipids are quantified using the internal standards in methods.

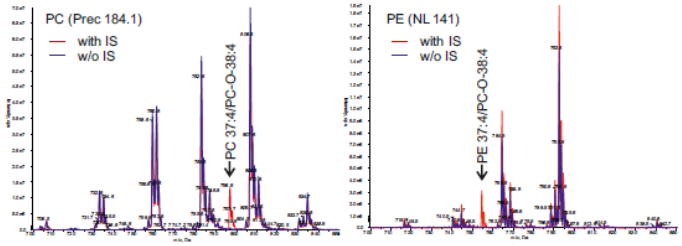

Fig. R2. Internal standards and its overlap with biological lipids. Signal intensities of the indicated phospholipid in the biological material without (purple line) or with internal standard (red line).

Targeted analysis of polar metabolites is problematic, because there is no reference to the protocol providing all the necessary detail about the method (extraction, analytical conditions, standard compounds used, calibration, QC, MRM transitions etc), while the description is insufficient to evaluate the method. The method is not standard, as one can rarely see method which would measure in a single analysis together e.g. nucleotides, acetyl-CoA, bile acids and amino acids. Theoretically this is possible, but the question is how reliable the concentration values are? Again, have the authors checked the extraction efficiency and how were the values calibrated? How were the analytes uniquely identified?

To address the reviewer comments, we significantly modified the Materials and method section by adding more details and references on the used method of extraction and analyses of metabolites. In brief, the use of ZIC-pHILIC columns at high pH9.2 is widely used and referenced approach in metabolomics field. In combination with the Orbitrap full scan mass spectrometer which is operated in polarity switching mode, it gives an opportunity to capture a wide range of metabolites. We use it together with the method of metabolite extraction established in Glasgow University and Beatson Institute for Cancer Research (Mackay, Zheng et al., 2015). This method offers a relatively simple and quick protocol for metabolite extraction that is adapted for different biological samples. We have established this method in our metabolomics facility in close collaboration with the Beatson Institute. In our validated compound database, we were able identify over 200 metabolites in various samples by matching mass and retention time with commercial standard compounds. The metabolites include amino acids, organic acids, sugars, phosphates (glycolysis and pentose phosphate pathways), nucleotides, cofactors (such as CoA, NADH), free fatty acids. Importantly, this approach using ZIC-pHILIC column and Orbitrap was also recently used and further validated on even more elaborate samples such as sub-cellular organelles in several papers by the team of David Sabatini at MIT for characterization of the mitochondria and lysosomal metabolomes (Abu-Remaileh, Wyant et al., 2017, Chen, Freinkman et al., 2017, Chen, Freinkman et al., 2016).

Furthermore, we provide a representative LC-MS extracted ion chromatograms for all significantly changing metabolites presented in Fig. 5B and 5C of the revised manuscript (Figure R3), which shows correct peak shape and good selectivity. The

retention time for each compound is indicated, demonstrating the efficacy of chromatographic separation.

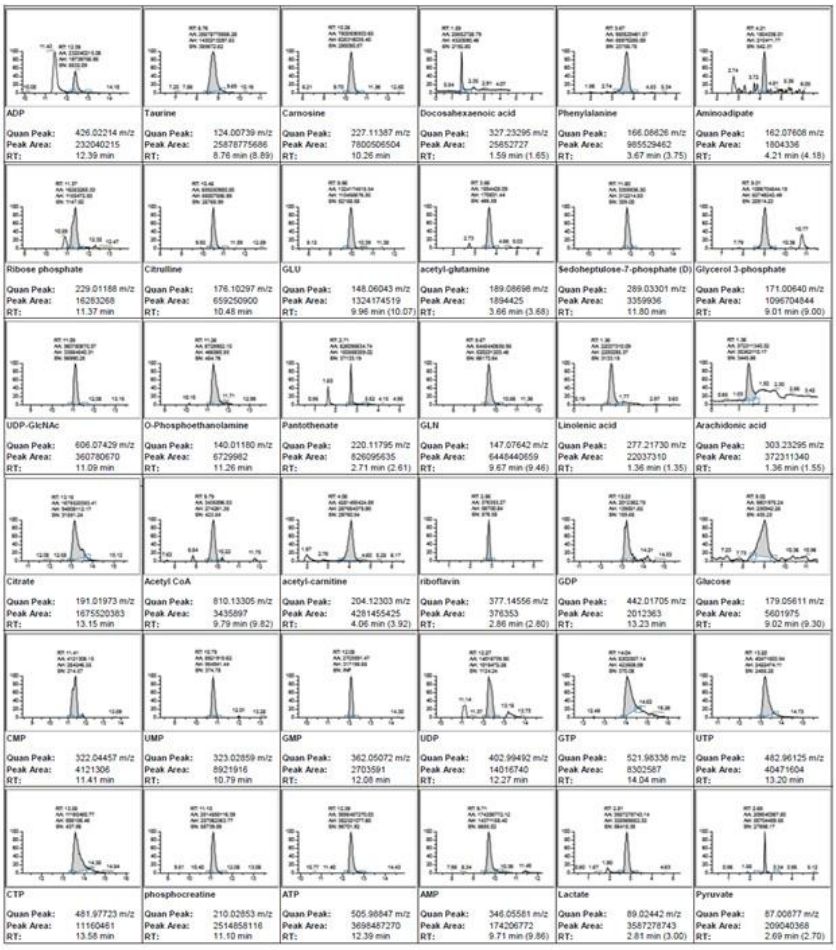

Fig. R3. Representative LC- MS extracted ion chromatogram (XIC) with peak areas for selected identified compounds with mass accuracy of 5ppm form gastrocnemius muscle extract.

The authors apply MetaboAnalyst for pathway analysis as a 'black box' solution, with too little detail provided. With 30 metabolites included in the analysis, how representative are the pathways (i.e. what was the fraction of metabolites represented in any given pathway listed)? There are nearly as many pathways listed in Figure 4D as there were metabolites used in the analysis.

We apologize for not providing enough details. In our metabolic analyses, a total of 124 metabolites were identified, including amino acids, organic acids, sugars, phosphates (glycolysis and pentose phosphate pathways), nucleotides, cofactors and free fatty acids. In Fig. 5A, we exclusively present the list of metabolites significantly changing. Conversely, in the MetaboAnalyst software, the values of all metabolites are taken into account. We agree this is not an essential piece of data, it was used to

convey the message of important alterations of the lipid biosynthesis pathway. Considering the large amount of new data in this revised version, we decided to remove the panel 4C of the previous version.

Referee #2:

Genetic mutations causing loss of function of LPIN1 gene lead to rhabdomyolysis in children, although metabolic alterations and therapeutic interventions are still elusive. In this manuscript, Rashid, T. et al demonstrated that muscle specific deletion of Lipin1 causes severe myopathy in mice, with excessive accumulation of both neutral- and phospho-lipids, which the authors attribute to excessive fatty acid synthesis and impaired fatty acid oxidation (FAO), accompanied by a defect in acyl chain elongation and desaturation and activation of the lipogenic SREBP1c/SREBP2 factors. The authors performed lipidomics and metabolomics analyses to gain insight into the mechanisms for the severe myopathy. They propose that ER stress may be a primary cause for the disease, as a major UPR response is detected in Lipin 1 deficient muscles and attenuation of ER stress by TUDCA treatment improves muscle strength, reduces lipid accumulation and improves overall muscle fiber morphology. The relevance of the study lies in the fact that by identifying ER stress as a major cause for the pathology, it opens the possibility for new therapies targeting to restore ER function in patients with lipin1 mutations. The manuscript is generally well-written and demonstrate interesting findings with potential relevance for the treatment of patients with genetic mutations in the LPIN1 gene. However, most of the data presented is descriptive in nature. Although the authors were able to demonstrate that alleviation of ER stress can ameliorate some aspects of the pathology, they were unable to restore the mitochondrial phenotype. The mechanisms for ER stress and UPR activation remain to be determined and better characterization of the mitochondrial phenotype is warranted.
Major Concerns:

1- The authors report that mtDNA is dramatically reduced in Lipin1 deficient mice. TEM also demonstrates changes in mitochondrial morphology, including changes in the cristae structure, and downregulation of mitochondrial encoded genes, which was not ameliorated by attenuating ER stress. Could this suggest that changes in mitochondria are an upstream event and that ER stress is secondary to mitochondrial stress? What is the cause for mitochondrial dysfunction in this model? Better characterization of mitochondrial function is warranted. The authors should measure mitochondrial oxygen consumption, ATP synthesis and mtROS. Additionally, proteins involved in mitochondrial dynamics should be investigated. Is AMPK activity increased?

This is an important point that led us to a thorough characterization of mitochondrial function. All the data and conclusions are for fast twitch muscles, since we score for pharmacological rescue using strength and necrosis parameters in this common type of muscle. However, in this revised version we also included some data on slow twitch soleus muscles. We performed the analysis at different levels:

- Expression: Despite the reduction in mitochondrial DNA, we could not detect differences in mitochondrial proteins by western blot analysis (new Extended View 6A). These data suggest alterations in mitochondrial nucleoid segregation and release, rather than mitochondrial mass and protein expression.

- Activities: Polarography experiments also failed to reveal differences in specific activities of respiratory complexes from I to V in mutants vs control muscles. This was performed in isolated mitochondria from *HSA-Cre;Lipin1*$^{floxed/floxed}$ mice (new Extended View 6B) and permeabilized fibers from Hubr rats (Fig. R4).

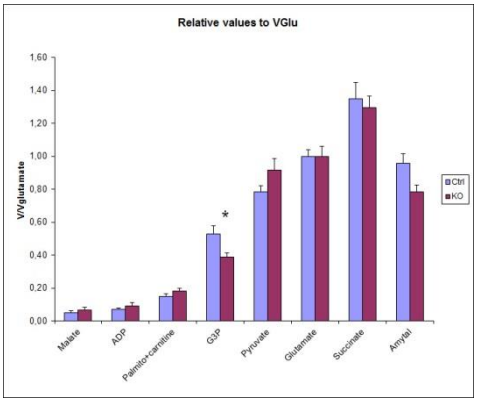

Fig. R4. Single muscle fibers were isolated from GC muscle of Hubr rats (KO) and control. Respiratory activities of mitochondria were assessed in oxygraphic chambers. Histograms are means ± SEM from at least 3 rats. *p < 0.05 versus wild type genotype. Respiration rates of 10–15 mg of skinned fibers were measured using the indicated substrates and expressed as relative values to respiration rates in presence of 5mM glutamate as substrate.

- From the above analysis, the only difference was a slight decrease in GQDR activity corresponding to mitochondrial glycerol 3 phosphate dehydrogenase (GPD2) and in the G3P utilization (Extended View 6C and Fig. R4). This was consistent to a decrease in GPD2 protein levels (Extended View 6E) and an increase in glycerol 3-phosphate/ dihydroxyacetone phosphate (DHAP) ratio (Extended View 6D). Interestingly, GPD2 connects lipid metabolism to mitochondrial respiration, pointing to a specific role of Lipin1 on this pathway. Future studies should address the functional relevance of this pathway in Lipin1 deficiency.

- ATP levels and AMPK activation: we assayed ATP, ADP, AMP, phosphocreatine levels and failed to detect a general energy stress (new Fig. 5B and 5C). Consistently, AMPK and ACC phosphorylation did not differ (Extended View 6F).

- Fusion and fission machinery: Mitofusins, DRP1 expression was assayed by western blot analysis (Extended View 7A). These GTPases have opposing roles in mediating fusion and fission of the mitochondrial outer membrane, respectively. They are also localized in contact sites between mitochondria and ER. The increased expression of both Mitofusin and Drp1 is consistent with an increase in the ER volume and contact sites with mitochondria.

- ROS production and protein carbonylation: as a measure of ROS production and oxidative stress, protein carbonylation was assayed in mutant muscles and no significant difference was detected as compared to controls (Extended View 6G).

- Anti-oxidant treatment: Since previous studies on Opa1 mutant muscles showed that mitochondrial alterations could increase Fgf21 levels and induce a myopathy that was rescued by anti-oxidant treatment in addition to TUDCA, we treated mice with Trolox. However, in lipin1 mutants Trolox did not ameliorate ER stress and histological defects (Fig. R5). Taken together, our data indicate that lipin1 mutations have distinctive features as compared to mitochondrial myopathies and do not benefit to anti-oxidant therapies.

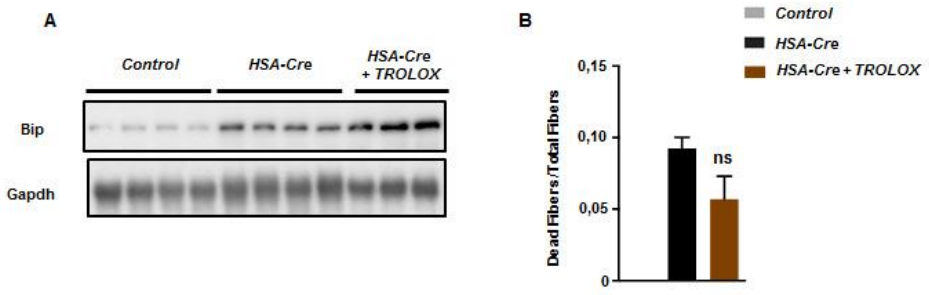

Fig. R5. One month Trolox treatment did not ameliorate ER stress, as assessed by Bip expression (left panel) and histopathology, as assessed by IgG staining of necrotic fibers (right panel).

2- Lipotoxicity has been shown to interfere with lysosomal acidification in the heart. In the present manuscript, TEM suggest autophagy and lysosomal function may be impaired in Lipn1 KO mice. This should be further investigated, as impaired autophagy/mitophagy could result in accumulation of damaged mitochondria. Indeed, autophagy-deficient muscles have impaired mitochondrial function and activation of the UPR.

Consistent with previous studies, we agree that autophagy is defective in Lipin1 mutants. In this revised version, we showed that p62, Lamp2 and LC3 were accumulated in Lipin1 mutants (Extended View 8A). Of note, this correlated with mTOR activation, a known negative regulator of autophagy. Hence, Lipin1 mutant mice were treated with the rapamycin derivative temsirolimus (Extended View 9). While mTOR inhibition was effective in activating autophagy, it was not sufficient to relieve ER stress markers. Importantly, the force and histological defects did not significantly improve.

3- Studies in primary myotubes lacking Lipin1 would be useful to elucidate the mechanisms for mitochondrial dysfunction and ER stress described in the manuscript. For example, can the mitochondrial phenotype be ameliorated by increasing autophagic flux in these cells? If so, will be this sufficient to prevent ER stress and limit lipid accumulation.

Lipin1 deficient myotubes were prepared by adenoviral expression of Cre recombinase followed by 6 day differentiation protocol. However, this model did not recapitulate the severity of the ER-mitochondria alterations observed *in vivo*. The differentiation state of the cells or the need of extended time periods for the accumulation of defects may explain these findings. In this revised version, we extensively characterized autophagy and mitochondria *in vivo* (see above). To evaluate the functional involvement of autophagy and mitochondria defects we treated mice with rapamycin derivative and trolox. It is likely that autophagy defects may concur to the disease state, as suggested by a number of studies which we confirm. However, using different pharmacological treatments (temsirolimus, trolox, bezafibrate, TUDCA) we were able to differentially affect autophagy and ER stress. The novelty of our study points to the correlation between ER stress severity and disease.

4- Mitochondrial and ER stress is often associated with loss of muscle mass, and atrophy of muscle fibers. More sensitive assays should be applied to measure total lean mass and muscle fiber diameter.

We measured cross sectional area (CSA) and observed no difference (Extended View 1E). The lack of atrophy may be explained by the up-regulation of the mTOR pathway, a known anabolic signal for muscles (Extended View 8A).

5- The authors report an increase in FGF21 mRNA expression and protein levels in Lipin1 deficient muscle. Were FGF21 levels elevated in the circulation? Muscle-derived FGF21 has been shown to have potent effects on systemic metabolism, preventing obesity and improving insulin sensitivity. Were body mass and fasting glucose levels changed in these animals?

Whole body weight and fasting glucose levels were not changed in resting conditions (Extended View 1C and 1D). Following the reviewer suggestion, we measured FGF21 levels in the blood (Extended View 4C). There was an increase in circulating levels of Fgf21 in mutant mice, though the induction was less marked as compared to muscle tissues. In future studies, it would be interesting to put the mice on high fat diet and assay glycaemia, ER stress and the myopathy phenotypes.

6- The data suggest that glycolytic muscles, such as TA, are more susceptible to fiber damage in response to Lipin1 deficiency? Why is that? Also, the oxidative fibers are the ones accumulating lipids in Lipin1 deficient muscles, but they look healthier overall. With that in mind, please, discuss how the increase in lipid accumulation would contribute to your muscle phenotype? This is critical to understand the mechanisms for the myopathy in this model. What is the mitochondrial phenotype in TA and gastrocnemius muscle?

We thank the reviewer for this opportunity to clarify our work. In this revised version, different muscles underwent a battery of histopathology assays: hematoxylin-eosine for centronucleated fibers, IgG staining for necrotic fibers, OilRedO for lipid accumulation, EM analysis for sarcomere/mitochondria/autophagy/ER structures, ER stress markers by western blot. As expected, regeneration and centronucleated fibers are more prominent in glycolytic muscles (TA, GC vs. soleus), while lipid accumulation is more prominent in oxidative fibers. However, by EM we see severe pathological signs in both fast twitch EDL and slow twitch soleus (new Fig. 6C and Extended View 5B). By EM and gene expression studies, we reveal ER stress in all the analyzed muscle types. Sometimes, the choice of one muscle versus another is

dictated by the type of assay: small muscles (EDL and soleus) are more convenient for EM analysis, while bigger muscles are more convenient for biochemical studies. Anyway, the main focus and the conclusions of this study are on the fast twitch muscles, as in this common type of muscle we score for force and necrosis after pharmacological treatments.

Minor:

1- In methods, please, specify the protocol for tamoxifen administration (how it was administered, dosage, age of animals, duration). Also, indicate the age in which the experiments were performed. In the legends, I was able to find information about the ages, which ranged from 3-6 months of age. Why were different ages utilized for different assays? Does the phenotype progress with age?

We apologize for not being clear in the previous version. The mice were not treated with tamoxifen, as we used a constitutive expression of the Cre recombinase under the HSA promoter. The phenotype is stable and consistent in the 3-6 month old aged mice. Age-matched controls were used in every experiments but the choice of the age largely depended on the mouse availability.

2- Still in methods, please specify the age in which TUDCA treatment was initiated? Was the treatment meant to prevent muscle dysfunction or reverse it?

As mentioned in this revised version, TUDCA treatment was started in 5 month-old mice and therefore was able to reverse the phenotype.

3- Lipn1 protein levels should be measured in skeletal muscle of KO mice.

Lipin1 levels were measured by western blot analysis (Extended View 1A). As expected, a truncated band was detected in mutant muscles lacking PAP1 activity (Fig. 1B). A recent study by the Finck group demonstrated a myopathy phenotype in mice carrying this truncated version that was equivalent to mice displaying undetectable protein (Schweitzer, Collier et al., 2018).

Referee #3:

Summative comments:

The manuscript submitted by Rashid and colleagues proposes a novel mechanism by which loss of function of the Lpin1 phosphatidic acid phosphatase (PAP) in murine muscle results in ER stress and myopathy. Muscle-specific loss of function exhibits reduced PAP1 activity, histological and functional evidence of myopathy, and dysregulation of lipid homeostasis manifest by modest increase in PA abundance, dramatic increase in total phospholipid (PL) and triglyceride (TG) levels, and altered phospholipid acyl chain composition. The authors go on to show increased activation of SREBPs in Lipin1 deficient-muscle and suggest enforced SREBP-dependent lipogenesis is therefore responsible for the observed accumulation of lipids. The authors demonstrate activation of multiple ER stress markers in Lipin1 deficient gastrocnemius, a phenotype accompanied by sarcoplasmic reticulum (SR) fragmentation, a reduction in mitochondrial DNA (mtDNA) content, and evidence of mitochondrial fragmentation. Finally, the authors treat mice with the anti-apoptotic bile acid chaperone TUDCA and demonstrate reductions in ER stress, lipid accumulation, myonecrosis, and functional impairment.

Overall, the presented work is interesting but important questions remain surrounding the mechanisms of ER stress and lipid accumulation, particularly in light of prior work in a similar mouse model with substantially different findings. In addition, several of the reported findings are not particularly novel: myopathy with abnormal mitochondrial morphology has previously been reported in mice harboring Lpin1 mutations and SREBP regulation is a well-documented function of Lipin1. This manuscript's primary novel findings are (1) the nature of the lipid dysregulation (increased PLs and TGs) and (2) reversible ER stress. Unfortunately, the mechanistic basis for each of these findings goes largely unaddressed. Nevertheless, the therapeutic potential of chemical chaperones in a clinically-relevant myopathy model is of significant interest and would be suitable for publication if additional mechanistic data were provided.

Major points

1. Why are PC, PE, and TG levels increased upon Lpin1 mutation? Since PC and PE are predominantly generated through a PAP-dependent pathway, the finding is somewhat counterintuitive despite the Lpin yeast homolog Smp2's role as a negative (transcriptional) regulator of phospholipid biosynthesis. The authors speculate in the discussion that this might reflect alternative biosynthesis via the CDP-DAG pathway, but this point would need to be addressed experimentally to be convincing. Similarly, the authors speculate on but do not address the mechanism by which TG levels are increased in mutant mice. This is also a counterintuitive result, as most non-enterocytes synthesize TGs via the glycerol-phosphate pathway, which would be expected to be impaired upon PAP loss of function and reduced availability of DAG.

We thank the reviewer for raising this important point that led to the new Fig. 5C-F. To quantify TG, DAG and cholesterol separately, we performed shotgun qMS. Significant increase of TAGs in Lipin1KO (HSA-Cre) is evident by qMS. Moreover,

cholesterol and cholesteryl esters are also more abundant in LipinKO. The extent of increase is largely proportional to that of phospholipids. Of note, DAG is also induced in mutant muscles despite the decreased PAP1 activity and consistent with a recent report (Schweitzer et al., 2018). Since DAG serves as intermediate lipid and is readily converted to TAG or phospholipids, these results are consistent with the global induction of lipid synthesis in Lipin KO muscles. It is likely that lipid uptake from circulation and lipid synthesis are up-regulated in the mutant muscle, at least in part due to SREBP induction and ER stress.

These are particularly key points because prior published work (Zhang, et al Cell Metab 2014) in LipinFld/Fld homozygous loss-of-function mice also revealed a myopathy accompanied by increased abundance of lipid droplets and aberrant mitochondria. However, contrary to the findings presented here, a significant reduction in TG levels, an increase in cholesterol levels, and no significant difference in PC levels were noted. Prior work in Lpin1 null muscle also reports a decrease rather than the increase reported here in fatty acid synthetic genes and an increase in mtDNA rather than the decrease reported in the current manuscript. The authors need to address these significant discrepancies given their central position in the manuscript's claims; might they reflect differences in the mouse models, such as the contributions in Lpin1fEx2-3/ fEx2-3;HSA-Cre of non-myocytes with retained Lipin1 function? Are LipinFld/Fld mice available in order to assess whether the ER stress and PL accumulation phenotypes presented here are consistent with previously published work?

Following the suggestion by the reviewer, we extended our work to two additional models of Lipin1 deficiency: the Fld mice lacking detectable Lipin1 protein in the whole body; and the Hubr rats carrying a catalytic dead protein as a consequence of point mutations. In these two distinct animal models, we confirm ER stress, mTOR activation, autophagy defects and TAG accumulation (Extended View 8B, 8C and 8D).

2. The causes of ER stress in Lpin1fEx2-3/ fEx2-3;HSA-Cre mice remain unclear. In the manuscript text the authors suggest that ER stress may drive SREBP cleavage and, in doing so, explain accumulation of lipid species. In the discussion, however, the authors suggest that the ER stress is itself the result of altered lipid metabolism. It is certainly possible that Lipin1 LOF directly causes "an overall disturbance of ER function" as suggested, but experimental evidence to support this claim is lacking.

Previous studies (Peterson, et al Cell 2011) identified nuclear Lpin1 as an important negative regulator of SREBP target expression by modulating SREBP protein's nuclear localization; other data support a role of Lipin1 as a more general co-regulator of transcription factor (TF) function. One model to explain the data in the manuscript might therefore be that SREBP de-

repression in the setting of Lpin1 loss of function leads to unregulated lipid biosynthesis which drives both ER stress and the observed lipidomic and metabolomic abnormalities. ER stress, in turn, might further amplify post-translational SREBP activation via IRE1 and other UPR responses. Alternatively, SREBP-independent effects of Lpin1 loss-of-function on other TFs may be responsible some portion of the observed effects. No data are presented to address these important issues, but they would be tractable either with further in vivo experiments or in complementary tissue culture systems.

We agree with the reviewer that the causes of ER stress are complex and we could not dissect a single factor. In addition to the dramatic changes in lipid composition, saturation and elongation, in this revised version we also provide evidence of mTOR activation (new Figure 8A and Extended View 8A) and we detect Calcium Units by EM (Extended View 5A). Lipid alterations, mTOR-dependent protein synthesis, deregulation of Calcium fluxes are known factors contributing to ER stress. In this revised version we compared the efficacy of different pharmacological treatments: i) TUDCA alleviates ER stress with no effect on mTOR and autophagy (new Fig. 8); ii) temsirolimus alters mTOR/autophagy with no effect on ER stress markers (new Extended View 9); iii) bezafibrate promotes FAO and alleviates ER stress with mild effect on SREBP cleavage. Since TUDCA and bezafibrate, but not temsirolimus, have beneficial effect on muscle histology and strength, our findings suggest that lipid composition is an important factor for ER stress and myopathy in Lipin1 deficiency.

Minor points

- Fig1 and elsewhere: the authors need to clarify the genotype of "control" mice used throughout the manuscript. Are these animals HSA-Cre alone, Lpin1fEx2-3/ fEx2-3 alone, or WT?

Figure legend was changed, control mice were floxed alone.

- Fig 1B: To what do the authors attribute the increase in PAP2 activity in Lpin1fEx2-3/ fEx2-3;HSA-Cre animals?

The increased PAP2 activity is likely due to broad specificity Phospholipid Phosphatase (PLPP), as the expression of PLPP2 mRNA is upregulated in mutant

muscles (Extended View 1B). These enzymes mediate lipid signalling, and are less involved in the bulk production of phospholipids and triglycerides. However, at this stage we cannot exclude their role in the adaptation of lipid homeostasis in mutant muscles.

- Fig 2A: DAG levels (in addition to PA levels) should be measured in Lpin1fEx2-3/ fEx2-3;HSA-Cre animals.

As outlined above, we thank both reviewer #2 and 3 for this important point that revealed the paradoxical up-regulation of DAG levels in mutant muscles (new Fig. 3F). This explains the up-regulation of both TAG and phospholipids.

- Fig 3 and associated text: The statement "our data demonstrate that the absence of Lipin1 in skeletal muscle turns on an SREBP-dependent program promoting storage of triglycerides" is not fully supported by the presented evidence, as the SREBP-independent consequences of Lipin1 deficiency on lipid homeostasis are not assessed. Does enforced SREBP activation alone phenocopy the Lipin1 deficiency phenotype, and to what extent? Additional experiments would need to be performed to justify this conclusion.

We modified the text to blunt our statements. We think that SREBP-dependent lipid uptake and synthesis concur in the overall lipid accumulation in mutant muscles. ER stress at least in part promotes SREBP cleavage, as TUDCA treatment decreases it. Clearly other players may concur in the established link between lipidosis, ER stress and myopathy. In this revised version, we show that PPARa agonists, such as bezafibrate, also alleviate ER stress and the myopathy phenotype (new Fig. 9).

- Fig 3: The authors conclude that the oil red O staining represents TG accumulation but do not address whether it may instead (or in addition) represent cholesterol accumulation, which might be expected in light of SREBP activation and presumably depleted pools of DAG for TG synthesis.

As outlined above, in the new Fig. 3 we present evidence for TAG, DAG and cholesterol accumulation.

- Fig 5A: Associated text claims a statistically significant increase in all 3 arms of the UPR response (PERK, ATF6, and IRE1) but the Western blotting is not convincing for a biological

difference in p-eIF2A levels, suggesting the PERK pathway may not be particularly engaged under these conditions. Given prior data in cancer cell models (He, et al. FASEB 2017) that the PERK pathway was not significantly activated upon Lpin1 depletion, the authors should examine downstream effectors of the PERK-CHOP and IRE1a-JNK pathways in this context with additional Western blotting.

The reviewer is correct as the PERK-CHOP pathway is not induced as much as the XBP1 and ATF6 pathways. In the new Fig. 6A, we included the assay of CHOP, as well as quantification to show some degree of specificity in the UPR induction. This is interesting as PERK mainly senses proteostasis while IRE1 has been shown to sense lipostasis.

- Fig 7: Previous work in cancer and muscle cell systems has implicated a central role for dysregulation of autophagy upon Lpin loss-of-function. Are these effects noted in Lpin1fEx2-3/ fEx2-3;HSA-Cre animals and does TUDCA reverse them as well?

Also see point #2 of reviewer #2. We confirmed autophagy defects by assaying p62, Lamp2 and LC3 levels (Extended View 8A). This may be in part due to mTOR activation, a known negative regulator of autophagy. As shown in the new Fig. 8, TUDCA treatment did not reverse the LC3 marker of stalled autophagy. In addition, in Extended View 9, we show that mTOR inhibition was effective in activating autophagy, though it was not sufficient to relieve ER stress markers. These treatments somehow disconnect ER stress and autophagy defects.

- Fig 7: Does TUDCA treatment reverse the ultrastructural ER and mitochondrial defects associated with Lpin1 loss-of-function?

Unfortunately, the samples from this cohort of mice were not available for EM analysis. In this revision, our priority was to include additional treatments to answer specific points on autophagy, antioxidant, master regulators of lipid metabolism, raised by this and other reviewers. As outlined in our response to point #1-reviewer #2, in this revised version we did an extensive characterization of the mitochondrial alterations. We failed to detect changes in respiratory complexes, high energy phosphate and carbonylated proteins. Anti-oxidant Trolox treatment did not improve

ER stress and myopathy. Taken together, our data suggest that mitochondria and autophagy defects are not major causes of ER stress in these muscles

Non-essential suggestions

- Fig 2C: This figure is difficult to interpret (specifically the abundance of PC in control mice) as currently drawn owing to the broken axis. The axis should be, at a minimum, relabeled after the break as in Fig 2B.

We modified the figure according to the suggestion by the reviewer.

- Extended View Fig 1 and elsewhere: At several points through the manuscript the authors present data from various muscle groups (gastrocnemius, tibialis anterior, extensor digitorum longus). Given the significant differences in myopathy and lipid profiles between these muscle groups (see for example Extended View 1A), authors should justify the use of particular muscles for particular experiments

Please see our response to point #6 of reviewer #2

- Extended View Fig 1D is used to support the claim that fiber type composition is unchanged upon Lipin1 deletion, but data are shown only from EDL muscle which (based upon Extended View Fig 1A) is among the less affected muscles in Lpin1fEx2-3/ fEx2-3;HSA-Cre mice. Further, the EDL is composed (per the authors) of predominantly fast-twitch fibers whereas TG accumulation is most pronounced (Fig 3A) in oxidative fibers. Are the findings similar in GC and TA?

Please see our response to point #6 of reviewer #2

- The figure legend refers to Fig 7G, which does not appear to exist - perhaps having been replaced with Extended View Fig 4B?

We corrected the figure legend

Referee #4:

This is an interesting work addressing the role of Lipin1 in skeletal muscle. The authors generated a muscle specific Lipin1 knockout mouse line and characterised the phenotype of these mice. They found that ablation of Lipin1 results in amyopathic phenotype with important weakness. The metabolomic analyses revealed an alteration in lipid homeostasis because absence of Lipin triggered ER stress and Srebp1/2 activation. Importantly, administration of the chemical chaperon TUDCA greatly ameliorate the muscle phenotype. This is an excellent work that finally shed a light of the mechanistic insights of the human disease due to homozygous or heterozygous loss of functions mutations of LIPN1 gene. The characterization of the mice has been well performed and the identification of a problem in ER resulted in a successful treatment that can be translated to humans since ursodeoxycolate acid and derivatives are used in medical practice. Few points should be addressed to better dissect the mechanistic insights.

Point1. Does triglycerides and lipid accumulation happen also in soleus or diaphragm muscles, which are rich of mitochondria and display a beta oxidative metabolism? Or is peculiar of beta oxidative fibers of fast glycolytic muscles?

Following the suggestion by the reviewer, we performed EM analysis in soleus muscles revealing massive lipid droplets and alterations of SR-mitochondrial structures (Extended View 5B).

Point2. Accumulation of Lipids as well as induction of FGF21 and GDF15 are hallmarks of mitochondrial dysfunction. The authors analysed the mitochondrial morphology, mtDNA and expression of several mitochondrial encoded genes and found that are altered. However, a better characterization of mitochondria is required. Is respiration and complex activity affected? The increased electron-dense contacts between mitochondria was interpreted as a fission problem by the authors. However this interestingly observation need to be sustained by biochemical analyses. Is the fission machinery increased (DRP1, Fis, Mff) and/or the fusion proteins (OPA1. Mtf1/2) decrease in knockout mice? The decrease of fusion or increased fission/mitophagy may explain the reduced mtDNA. Better EM pictures of mitochondrial morphology showing cristae shape are requested. An alteration in SR-mitochondria tethering may also cause calcium changes, mitochondrial dysfunction and SR stress. Better EM images of triads are also required.

This is an important point also raised by reviewer #2. As stated above (point 1 of reviewer 2), we performed extensive characterization of mitochondrial respiration, fusion, fission, carbonylated proteins. We also provide better pictures of mitochondria in soleus and triads (Extended View 5). The overall structure of the triad is maintained. However, the massive increase in SR volume may cause a distortion of their orientation.

Point3. Similar to point 2, the ultrastructural observation of an autophagy impairment should be better sustained by biochemical analyses. Please check the LC3II levels or LC3 puncta as well as the expression of some mitophagy markers such as Bnip3, Nix, Bcl2l13. It has been reported that PA stimulates mTOR pathway resulting in an autophagy inhibition. Please check the status of Akt-mTOR axis in knockout mice. It is possible that the primary effect of Lipin inhibition is on mitochondria/mitophagy that triggers an ER stress response and consequently phospholipids biogenesis.

We thank the reviewer for this important point that revealed a striking up-regulation of mTOR activity in Lipin1 mutant muscles that concurs to the alteration of autophagy flux (Extended View 8). This led to the new treatments with mTOR inhibitors that failed to alleviate ER stress and the myopathy (Extended View 9)

Point4. The mechanism of ER stress should be better characterised. SR and cytosolic calcium levels and the presence of oxidative stress (resulting from mitochondrial dysfunction) may explain why different UPR pathways are simultaneously activated in KO mice.

Please see our response to the point #2 of reviewer #3. This reviewer also mentioned the specificity of the UPR response. We show that PERK-CHOP pathway is not induced as much as the XBP1 and ATF6 pathways. In the new Fig. 6A, we included the assay of CHOP, as well as quantification to show some degree of specificity in the UPR induction

Point5. The accumulation of glucose, citrate and the formation of ketones suggest an impairment of TCA cycle due probably to underutilization of NADH from the respiratory chain. The necrotic effect might be consequent to stress induced energy crisis. How is the ATP level in resting and exercised KO mice? Are these mice exercise intolerant?

As shown in the new Fig. 5 and Extended View 6F, we measured high energy phosphates and AMPK activation. We did not reveal an energy stress in resting conditions. The reviewer is correct that it would be interesting to challenge the mice in stress conditions. In the Hubr rat model of Lipin1 deficiency, we measured the Franck-Starling effect of inotropy in the heart. The drop in phosphocreatine after balloon insertion in perfused hearts was 14.9% in control animals, but 25.1% in mutant animals. Due to the difference in tissue model, we would prefer not to include these data in the present manuscript.

Point6. It would be interesting to know how much of the metabolite and lipid profiles is rescued by TUDCA treatment. Although this point is not necessary for the publication it would add interesting insights of the mechanism of action. Indeed, TUDCA is a cholesterol derived molecule and the beneficial effects might rely on its action on membranes independently of the chaperone effect.

In this revised version, we provided evidence that TUDCA did not alter autophagy and mTOR activation (new Fig. 8). In addition, we included an additional pharmacological treatment that alleviates ER stress and ameliorates the phenotype. In the new Fig. 9, we showed that the PPARα agonist bezafibrate decreases lipid droplets by activating FAO without affecting SREBP cleavage. This is sufficient to decrease ER

stress markers, necrotic fibers and muscle weakness. We think these data provide additional evidence for a link between lipidosis, ER stress and the myopathy due to Lipin1 deficiency.

Additional references

Abu-Remaileh M, Wyant GA, Kim C, Laqtom NN, Abbasi M, Chan SH, Freinkman E, Sabatini DM (2017) Lysosomal metabolomics reveals V-ATPase- and mTOR-dependent regulation of amino acid efflux from lysosomes. Science 358: 807-813

Chen WW, Freinkman E, Sabatini DM (2017) Rapid immunopurification of mitochondria for metabolite profiling and absolute quantification of matrix metabolites. Nat Protoc 12: 2215-2231

Chen WW, Freinkman E, Wang T, Birsoy K, Sabatini DM (2016) Absolute Quantification of Matrix Metabolites Reveals the Dynamics of Mitochondrial Metabolism. Cell 166: 1324-1337 e11

Mackay GM, Zheng L, van den Broek NJ, Gottlieb E (2015) Analysis of Cell Metabolism Using LC-MS and Isotope Tracers. Methods in enzymology 561: 171-96

Moore JD, Caufield WV, Shaw WA (2007) Quantitation and standardization of lipid internal standards for mass spectroscopy. Methods in enzymology 432: 351-67

Schweitzer GG, Collier SL, Chen Z, McCommis KS, Pittman SK, Yoshino J, Matkovich SJ, Hsu FF, Chrast R, Eaton JM, Harris TE, Weihl CC, Finck BN (2018) Loss of lipin 1-mediated phosphatidic acid phosphohydrolase activity in muscle leads to skeletal myopathy in mice. FASEB journal : official publication of the Federation of American Societies for Experimental Biology: fj201800361R

2nd Editorial Decision 14th Sep 2018

Thank you for submitting the revised version of your manuscript. Your revised study has now been re-evaluated by three of the original referees whose comments are enclosed. Please note that referee #3 was not able this time to look back into the work, but I have carefully assessed your response to his/her criticism editorially.

As you will see, all referees find that the study has improved and their concerns have been largely addressed. While we notice that referee #2 remains more critical, in light of the strong support of referees #1 and 4, we have now decided to proceed with publication of this work in The EMBO Journal as soon as possible. Thus, we ask you to revise your manuscript regarding the remaining points raised by referees #2 and #4 and evaluate, whether you would be able to address the issues experimentally or, alternatively, relativise your statements and introduce caveats where appropriate.

REFEREE REPORTS:

Referee #1:

The author have provided detailed and satisfactory response to my, mainly methodological, concerns. The additional information provided is helpful for understanding the methods, and I have no further comments.

Referee #2:

In this revised version of the manuscript, the authors included a series of pharmacological treatments to try to dissect the mechanisms for the severe phenotype observed in lipin1-deficient muscles. In summary, the new data complements the original manuscript and ratifies a role for ER stress and impaired lipid oxidation on the lipidosis and myopathy reported in HSA-Cre;Lipin1floxed/floxed mice. The authors were able to address most of my concerns. Although mechanistic insights regarding the cause for ER stress in this model are still missing, the authors present new data favoring a major alteration of lipostasis rather than proteostasis as a trigger of ER stress. They also speculate that defects on fatty acyl chain desaturation and elongation, which are altered in Lipin1 deficient muscles could directly limit the reducing capacity of the ER, thereby resulting in ER stress in Lipin1 deficient muscles.
However, despite the authors' efforts to include additional mitochondrial phenotyping, the data provided in the revised manuscript is still inconclusive. Especially in lieu of the new data, demonstrating that induction of FAO with bezafibrate significantly alleviates ER stress and improves the muscle phenotype of lipin1-deficient mice, a more rigorous analysis of mitochondrial oxidative capacity is critical. It is likely that the ability of mitochondria to oxidize fatty acids is impaired in this model. Whether this is an intrinsic problem with the mitochondria OXPHOS machinery or a defect with upstream enzymes of mitochondrial fatty acid oxidation such as CPT1, CPT2 or MCAD it is not clear. Specific recommendations regarding more appropriate methods to assess mitochondrial respiratory capacity and, particularly, fatty acid oxidation are provided. These studies will considerably strengthen the author's conclusion.

Specific Comments

MITOCHONDRIAL PHENOTYPING
BLUE NATIVE PAGE would have been a more appropriate method to assess the native form of OXPHOS proteins, as well as super complexes formation. Also, densitometric quantification of immunoblots are missing for many of the western blot data reported in the revised manuscript.

High resolution respirometry in isolated mitochondria or muscle fibers from HSA-Cre;Lipin1floxed/floxed mice using different type of substrates (particularly palmitoyl-carnitine) would have been the appropriate assay to directly measure mitochondrial oxidative capacity. At this point, I am not completely convinced that mitochondrial function is overall unaffected. This is especially puzzling, because pharmacological treatment with PPAR alpha agonist, which increases fatty acid oxidation has beneficial effects on ER stress, muscle histological parameters and strength

in lipin1-deficient muscles. This implies that defective FAO by the mitochondria is implicated in the phenotype. I suspect that a more sensitive analysis would have detected a reduction in palmitoyl-carnitine-supported respiration. This data would strengthen the conclusions. It is possible that improving mitochondrial fatty acid oxidation is just as effective and relevant as directly alleviating ER stress in this model.

Although total ATP levels were unchanged, ATP synthesis rates per se were not actually measured in Lipin1-deficient muscles. Nonetheless, the ACC and AMPK data suggest that mitochondrial bioenergetics is largely unaffected in these muscles.

Given the dramatic morphological changes to mitochondria cristae structure, data for OPA1, a fusion protein that also plays crucial roles on cristae integrity and mitochondrial quality control is important to show. Recent studies in OPA1-deficient muscles share similar features with this manuscript, including ER stress and FGF21 induction.

AUTOPHAGY
Although the effects of temsirolimus on S6 phosphorylation was very potent, the effects on autophagy markers are not as evident. Densitometric analysis of the immunoblots are missing. The authors should be careful excluding a role for autophagy at this point.

ADDITIONAL COMMENT
With regards to TUDCA treatment to reduce ER stress. This is particularly interesting for the therapeutic potential of treating ER stress in patients with rhabdomyolysis. On that note, have alterations in ER function ever been reported in patient with rhabdomyolysis? If so, this should be included in the discussion.

Referee #4:

The paper is improved and the addition of another therapeutic approach that ameliorates lipin1 ko phenotype is relevant for the tranlsational implications. Few points should be corrected. The authors claimed that mTOR inhibitor temsirolimus failed to ameliorate the phenotype despite autophagy flux was ameliorated and therefore, concluded with the sentence "...do not support a role of mTOR and autophagy as therapeutic targets". While I agree that mTOR is not a target die to the strong inhibition, the effect on autophagy are minor if there are effects. In fact, the LC3II levels (extended view 9A) does not really changes after mTOR inhibtion and doubts that are significant different from untreated lipin KO. I suggest that authors soften the conclusions on autophagy. In extended view 10A the FGF21 induction is around 100 fold in KO mice while in extended view 10C the upregulation of FGF21 is 7 fold in lipin KO mice. How the authors explain this difference in the same mice? In page 15 the authors refer to Fig 7C and D for the description of lipid droplets and triglyceride levels. The correct panels are 8C and 8D. Finally, the amelioration of lipin1 KO mice after bezafibrate is important. It would be of interest to show whether also mitochondrial genes are restored after the bezafibrate administration. This piece of data would at least support an effect on mitochondria that would justify the FAO rescue.

2nd Revision - authors' response 21st Sep 2018

We thank the four reviewers for their interest and appreciation of our work. Below is our response to the final questions raised by Referee #2 and 4.

Referee #2:
MITOCHONDRIAL PHENOTYPING
BLUE NATIVE PAGE would have been a more appropriate method to assess the native form of OXPHOS proteins, as well as super complexes formation. Also, densitometric quantification of immunoblots are missing for many of the western blot data reported in the revised manuscript. High resolution respirometry in isolated mitochondria or muscle fibers from HSA-Cre;Lipin1floxed/floxed mice using different type of substrates (particularly palmitoyl-carnitine) would have been the appropriate assay to directly measure mitochondrial oxidative capacity. At this

point, I am not completely convinced that mitochondrial function is overall unaffected. This is especially puzzling, because pharmacological treatment with PPAR alpha agonist, which increases fatty acid oxidation has beneficial effects on ER stress, muscle histological parameters and strength in lipin1-deficient muscles. This implies that defective FAO by the mitochondria is implicated in the phenotype. I suspect that a more sensitive analysis would have detected a reduction in palmitoyl-carnitine-supported respiration. This data would strengthen the conclusions. It is possible that improving mitochondrial fatty acid oxidation is just as effective and relevant as directly alleviating ER stress in this model.

Although total ATP levels were unchanged, ATP synthesis rates per se were not actually measured in Lipin1-deficient muscles. Nonetheless, the ACC and AMPK data suggest that mitochondrial bioenergetics is largely unaffected in these muscles.

Given the dramatic morphological changes to mitochondria cristae structure, data for OPA1, a fusion protein that also plays crucial roles on cristae integrity and mitochondrial quality control is important to show. Recent studies in OPA1-deficient muscles share similar features with this manuscript, including ER stress and FGF21 induction.

In the new extended view 7B, we performed an immunoblot analysis to detect the different Opa1 cleavage forms. Consistent with published works, we were able to detect five forms, labelled from a to e. The bands c and e are usually low abundant in healthy mitochondria, while they are actively produced by the Oma1 protease in stressed mitochondria. Our data show that Oma1 protease is not active in the Lipin1 deficient muscles, suggesting that mitochondria maintain a proper control of membrane potential over the inner membrane. As for the FAO defect, we provide evidence in Figure 4C that this is at least in part due to a transcriptional control of the Mcad and Cpt1b genes. We also performed flux experiments in Lipin1 deficient myotubes to measure FAO (Figure R1). Flux analysis by mass spectrometry after C12-palmitate labeling demonstrated lower levels of acylcarnitines in Lipin1 deficient myotubes, as compared to wild type and rescued cells Stimulation with the PPAR agonist bezafibrate caused a two fold increase in acylcarnitine production in the different genotypes and brought the flux of beta oxidation in mutant cells to a level equivalent to untreated wild type controls. Taken together, our data suggest a muscle cell autonomous defect in fatty acid beta oxidation that is accompanied by a decrease in the expression of beta oxidation enzymes.

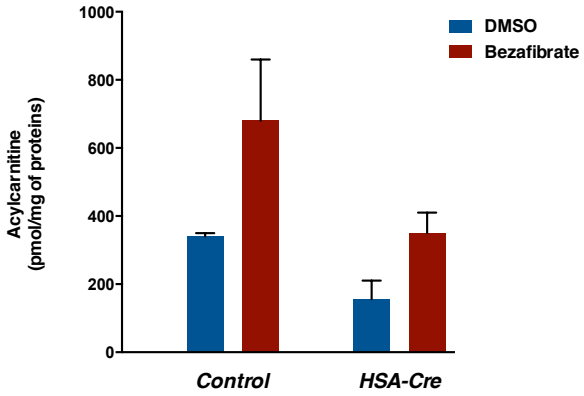

Fig. R1. Fatty acid oxidation (FAO) measurements. Quantification of acylcarnitines levels in differentiated myotubes from control and *Lpin*$^{fld/fld}$ (*Ko*) mice treated either with vehicle (DMSO) or 300mM Bezafibrate.

AUTOPHAGY

Although the effects of temsirolimus on S6 phosphorylation was very potent, the effects on autophagy markers are not as evident. Densitometric analysis of the immunoblots are missing. The authors should be careful excluding a role for autophagy at this point.

In the new Extended View 9, we quantified the immunoblot by densitometric analysis. Lamp2 and LC3-II are significantly decreased by temsirolimus treatment. We agree that temsirolimus is more potent on S6K activity than autophagy, as shown by several groups. It is possible that a better rescue of autophagy may be beneficial and we stated this point in the discussion of this

revised version (page 14 lines 23-25 and page 15 lanes 4-7). The novelty of our manuscript is the ER stress component that correlates very well with the disease.

ADDITIONAL COMMENT

With regards to TUDCA treatment to reduce ER stress. This is particularly interesting for the therapeutic potential of treating ER stress in patients with rhabdomyolysis. On that note, have alterations in ER function ever been reported in patient with rhabdomyolysis? If so, this should be included in the discussion.

This is an extremely interesting point that we are currently addressing in human patients. No systematic analysis of ER markers in rhabdomyolysis patients has been reported in the literature.

Referee #4:

The paper is improved and the addition of another therapeutic approach that ameliorates lipin1 ko phenotype is relevant for the tranlsational implications. Few points should be corrected. The authors claimed that mTOR inhibitor temsirolimus failed to ameliorate the phenotype despite autophagy flux was ameliorated and therefore, concluded with the sentence "...do not support a role of mTOR and autophagy as therapeutic targets". While I agree that mTOR is not a target die to the strong inhibition, the effect on autophagy are minor if there are effects. In fact, the LC3II levels (extended view 9A) does not really changes after mTOR inhibtion and doubts that are significant different from untreated lipin KO. I suggest that authors soften the conclusions on autophagy. In extended view 10A the FGF21 induction is around 100 fold in KO mice while in extended view 10C the upregulation of FGF21 is 7 fold in lipin KO mice. How the authors explain this difference in the same mice? In page 15 the authors refer to Fig 7C and D for the description of lipid droplets and triglyceride levels. The correct panels are 8C and 8D. Finally, the amelioration of lipin1 KO mice after bezafibrate is important. It would be of interest to show whether also mitochondrial genes are restored after the bezafibrate administration. This piece of data would at least support an effect on mitochondria that would justify the FAO rescue.

As for the autophagy, please see our response to reviewer #2.
The difference in fold induction of FGF21 was due to the very low FGF21 signal in wild type controls. It was barely detectable in the TUDCA group, thus explaining the 100 fold induction. The inhibition of FGF21 expression in ko mice by the TUDCA and bezafibrate treatments is a more meaningful and reliable measurement. This measurement is similar for both groups. We corrected the mislabeling of the figure 8.
As for the FAO, please also see our response to reviewer #2. We find that Mcad and Cpt1b gene expression correlates well with the FAO rate. They are decreased in the Lipin1 mutants and rescued by bezafibrate treatment. Our main goal in this experiment was to show the effect on ER stress markers by promoting FAO. We clarified this point in the discussion (page 18 lines 21-23, page 19 line 23).

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PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Mario Pende
Journal Submitted to: EMBO Journal
Manuscript Number: 99576R

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 $\chi 2$ 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?	For each experiment, the sample size was decided to balance sensitivity and specificity. For in vivo studies, at least three animals for each genotype were analyzed, in most of the experiments were 5.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	For animals studies, we kept the number of animals to a minimum, as we have implemented a protocol to perform multiple assays of protein and RNA expression, in distinct tissues from the same animal. At least three animals for each genotype were analysed, in most of the cases were 5.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	NA (No animals were excluded a posteriori from the 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.	A randomization procedure was used to minimize the effects of subjective bias when allocating animals or samples to treatment.
For animal studies, include a statement about randomization even if no randomization was used.	As stated in point 3, we used a randomize procedure to minimize the effects of subjective bias Mice from different breeding pairs were analysed when possible to increase randomization.
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.	For animal studies, animals were randomly chosen to prevent selection bias.
4.b. For animal studies, include a statement about blinding even if no blinding was done	For animal studies, animals were numbered and randomly chosen to prevent selection bias.
5. For every figure, are statistical tests justified as appropriate?	For every figure, we justified the tests used for statistical analysis, as indicated in figure legends and Materials and Methods.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Each group sample follow a normal distribution.
Is there an estimate of variation within each group of data?	Yes. Variation of each group of data was estimated calculating its standard deviation and standard error.
Is the variance similar between the groups that are being statistically compared?	Yes.

C- Reagents

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
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http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
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http://datadryad.org

http://figshare.com

http://www.ncbi.nlm.nih.gov/gap

http://www.ebi.ac.uk/ega

http://biomodels.net/

http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

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).	All antibodies used are commercially available and have been listed in Materials and Methods.
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.	Each mutant was compared with Cre negative mittermates of the same genetic background. All studies were done in 3-7 month-old male animals and were approved by the Direction Départementale des Services Vétérinaires, Préfecture de Police, Paris, France (authorization number 75-1313) and the ethical committee of Paris Descartes University. Mice were maintained at 22°C with a 12-hour dark/12-hour light cycle and fed a standard chow diet (Teklad global protein diet: 20% protein, 75% carbohydrate and 5% fat)
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	All animal studies were approved by the Direction Départementale des Services Vétérinaires, Préfecture de Police, Paris, France (authorization number 75-1313) and the ethical committee of Paris Descartes University
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.	The animal studies comply the ARRIVE guidelines.

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 targeted metabolomics mass spectrometry data from this publication have been deposited to the EMBL-EBI MetaboLights database (DOI: 10.1093/nar/gks1004. PubMed PMID: 23109552) with the identifier MTBLS750.
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).	NA
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
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

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