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# The MASTL/PP2A cell cycle kinase-phosphatase module restrains PI3K-Akt activity in an mTORC1-dependent manner

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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. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

Thank you for submitting your manuscript on MASTL-mTOR interplay to The EMBO Journal, and my sincere apologies for the delay in its evaluation caused by late referee reports. We have now received the comments of three expert referees, copied below for your information. As you will see, the reviewers all acknowledge the interest of your findings and the overall technical quality of this work, but do raise a number of experimental, presentational and interpretational concerns that would need to be addressed prior to publication. Since most of these points appear very specific and straightforward, we would be happy to consider this work further for The EMBO Journal, pending satisfactory revision along the lines suggested by the referees.

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Referee #1:

The authors of this paper characterise a new signalling network that controls the activity of the PP2A/B55 phosphatase complex which regulates the dephosphorylation of Akt. The authors' data suggests that the MASTL protein kinase phosphorylates 2 proteins termed ENSA and ARPP19 that act as inhibitors of the PP2A/B55 complex. This stimulates the dephosphorylation of Akt thereby sustaining the mTORC1 and S6K1 dependent phosphorylation of IRS1. The authors also present some data that MASTL is directly phosphorylated and activated by mTORC1, and this reduces PP2A/B55 mediated dephosphorylation of IRS1 by the above pathway. They also show that loss of MASTL increases glucose uptake and glucose tolerance in the mouse model.

Overall, the data are strong and the results convincing, and this study would be of significant interest to researchers working on glucose tolerance, mitosis, and cancer PI3K/Akt signalling pathways as well as mTORC1 biology.

Below I outline some key points that I believe need attention in a revised version of this study.

1. Can the authors add bar charts with statistics at the bottom of figs.1B, 1C and 1D? The bar chart in fig.1E does not correspond to the immunoblot shown above, that contains 2 time points of glucose stimulation. It is not clear what the time point corresponds to in the bar chart shown.

2. The authors undertake lot of studies using MASTL knockout cells in figures 1 and 2. To confirm that the effects on Akt and other phosphorylation pathways that are examined are not a clonal or CRISPR off target effect, it would be important that the authors perform a rescue experiment, demonstrating the effects of re-expressing wild type and kinase inactive MASTL in MASTL deficient cells, to demonstrate that expression of wild type MASTL reduces Akt phosphorylation but not kinase inactive MASTL.

3. In fig.3, a control immunoblot showing that knockdown of the shRNA against MASTL reduces expression of the MASTL kinase should be shown in the main figure?

4. Similarly, in fig.4 immunoblotting evidence should be presented to show that MASTL-deficient cells lack the MASTL kinase in the main figure.

5. The data shown in fig.3B most right-hand column does not look very persuasive. 2 of the 3 data points shown are in the same level as the control samples, with only one of the control data points being slightly lower. This does not look like the sort of experiment that would be easily reproduced independently.

6. In figs.7A and 7b the authors study phosphorylation of MASTL at a residue termed Threonine194 that does not appear to be one of the key phosphorylation sites that mTORC1 or S6K1 phosphorylates. What is the relevance of studying this site? Why is it reduced by rapamycin if it is not phosphorylated by mTORC1 and S6K1? Is it a phosphorylation site that was identified in the mass spectrometry phospho-proteomic analysis?

7. The authors perform detailed phospho-proteomic analysis to map key phosphorylation sites that are phosphorylated by mTORC1 and S6K1. Has the primary data for this mass spec analysis been deposited in PRIDE database or another equivalent depository? Only a high-level summary of the mass spectrometry data is presented in the paper, and it is hard to evaluate the strength of this mass spec analysis. The mass spectrometry data should be better presented.

8. The authors mutate Ser861 in MASTL in experiments shown in Figure 7F-H, but this site does not appear to be a phosphorylation site according to the mass spectrometry data. The danger is that mutating a conserved Serine to an alanine can impact the activity of the kinase in a phosphorylation independent manner. The author's data does not necessarily mean that phosphorylation of this site is essential for regulating activity.

9. Have the authors attempted to mutate the 864/861 sites to aspartic or glutamic acid to mimic phosphorylation, either individually or together? This could provide more evidence that phosphorylation of these sites, activates the MASTL kinase if activation was observed

Referee #2:

This is an interesting study by the Malumbres lab that presents a new link between cell cycle phosphatase control via MASTL and the mTor-AKT signalling cascade. The main message of the paper is that MASTL contributes to a negative feedback system that ensures a transient AKT activation following exposure to nutrients. The mechanisms of this signalling cascade are well worked out in the study and supported by experiments in human cell lines as well as in vivo studies in conditional MASTL knockout mice. The authors place MASTL downstream of mTor and present experiments that suggest that MASTL acts in this cascade via ENSA/ARPP19 dependent inhibition of PP2A-B55. Thus, MASTL activation following nutrient stimulation results in an increase in IRS1 and GRB10 phosphorylation and negative regulation of Insulin receptor signalling. There is a well-worked out link between TOR signalling and MASTL in budding and fission yeast but, to my knowledge, this study is the first to functionally link these two kinases in mammalian cells and implicate MASTL in metabolic control in mice.
The experiments in this study follow a logical progression, are mostly well executed and support the arguments of the authors. Overall, I believe this study represents a significant step forward in our understanding of MASTL and its functions outside of mitosis. I would recommend to publish this study in EMBOJ, after the following concerns have been addressed.

1) My major concern in the interpretation of the presented results lies in the differentiation between direct effect of MASTL depletion in interphase and potential indirect knock-on defects of aberrant mitotic progression, following loss of this essential mitotic kinase. The authors present data in MDA-MD-231 cells and show that at 72 hours depletion, MASTL is absent, but no cell cycle phenotypes are detected. This is shown in Figure EV1A for shRNA depletion. However, a previous study from the same lab (Alvarez-Fernandez et al. 2017) showed that these cells are sensitive to MASTL depletion.
I would suggest that the authors strengthen these data by performing a time-course analysis of MASTL depletion comparing depletion levels and cell cycle phenotypes both in the shRNA and the gRNA/Cas9 depleted cells. The FACS results in this experiment should also be quantified properly showing the standard deviation of three biological repeats.

2) Another important point of this study is the proposed direct activation of MASTL by mTOR phosphorylation of S878. The critical experiment here is the kinase activity measurement for MASTL. The in vitro experiments (MASTL pulldowns and ARPP19 immunoblots) are shown in EV4A. The observed changes in ARPP19 phosphorylation (an increase after TSC2 depletion and a decrease following Rapamycin treatment) are not convincing. This should be down at least three times and the significance of the differences analysed by a t-test. To confirm these data, the authors should also blot cell extracts with pS67 ENSA antibodies to test, if an increase in kinase activity can be directly measured.

Minor comments:

The text contains many small errors and imprecise descriptions. This should be corrected carefully before publication. Examples for this on page 5:
"Starvation of MDA-MB-231 cells for either growth factors or nutrients inhibited mTORC1 activity in control cells infected with a scrambled sequence"

Sequences were not transfected, this should be changed to shRNAs
"in the presence of specific small guide RNAs (sgRNAs)"
This should be "single guide RNA"

Figure 3
A - The image colours do not correspond with the colours reported in the figure legend. Also, "magenta" typo. The figure legend mentions a "histogram" and 800 cells scored per condition. The Figure does not show a histogram (but rather a swarm blot) and does not show data points for 800 cells. It is unclear what is being quantified and what statistical method was used to generate reported p-values.

B - It should be made clear which data points originate from the same experiment (technical repeats). Statistical test should be only applied on independent results.

Figure 4
A - The figure legend states that in all tissues, except pancreas, n = 3. However, only two data points are shown for eWAT and muscle tissues. Additionally, two datapoints reported for the eWAT tissue are conflicting.

Figure 5
B - Representative blot does not reflect the quantification. Ectopic expression of phosphomimetic ENSA-S67D and ARPP19-S62D in cells expressing MASTL appears to significantly increase AKT-T308 phosphorylation. In MASTL-depleted cells expression of ENSA-S67D and ARPP19-S62D reduces AKT-T308 phosphorylation.

Figure 6
D - Heterogeneity S6K1-T389 phosphorylation in MASTL-depleted tissues is not discussed.

Referee #3:

In the submitted manuscript, Sanz-Castillo investigate the mechanism that limits AKT activity. They show that Greatwall/MASTL suppresses PI3K-AKT activity via mTORC1- / S6K1-dependent phosphorylation of IRS1 and GRB10. Notably, ENSA/ARPP19, the downstream substrates of Greatwall/MASTL are involved in this process. Specifically, the authors can show that a phosphate-mimetic version of ENSA/ARPP19 rescues loss of Greatwall/MASTL. MASTL/Greatwall is directly phosphorylated by mTORC1 and S6K1. Thus, a mitotic-independent function of the Gwl-ENSA/ARPP-B55 axis modulates glucose response and negative feedback loops triggered by mTORC1-S6K1 activity. This is an interesting finding and I suggest publication, once the authors have addressed the following, major points:

These major points are key for the conclusions drawn by the authors and therefore must be rigorously addressed.

Figure 1:
- could the authors explain the effect of glucose re-addition in 1B?
- in Fig. 1E, glucose stimulation induces negative feedback, which leads to AKT inactivation. In 1E, MASTL depletion prevents AKT inactivation consistent with the idea that MASTL is involved in the negative feedback mechanism. However, in 1C and 1D, Akt gets partially inactivated by glucose even in the absence of MASTL, the difference to Ctrl KD is only minor. In 1B, there is no difference regarding P-T308-AKT at all between Ctrl and MASTL KD at all (although downstream targets like S6K1 show differences). Could the authors please clarify this discrepancy? It is an important data piece for the model.
- Fig. 1B: why does the level of P-T308-AKT not correlate with P-T389-S6K1, e.g., compare P-T308-AKT in lane 3 and 4 with the respective lanes of P-T389-S6K1

Figure 2A + 2B:
TSC2 knockdown triggers activation of the feedback loop and AKT inhibition. Why did the authors stimulate TSC2 depleted cells with insulin? As shown in 2A, starvation + insulin by itself triggers negative feedback after 30min resulting in P-T308-ATK dephosphorylation. The leftmost lane of 2B would (according to the figure legend) correspond to the 30min timepoint of 2A (-Dox). Why is there a strong P-T308 signal in 2B, and no signal in 2A?

Figure 4D:
Regarding glucose clearance in the presence/absence of PI3Ki in WT and MASTL KO cells. It is not really explained why PI3Ki has stronger effect in MASTL KO. If the effect goes via AKT there should be the same phenotype upon PI3Ki treatment.

Figure 4E:
There is a discrepancy between 3B and 4E. In 4E, there is a significant difference in GLUT4PM localization under fasting conditions, but no difference under refeeding conditions. In Fig. 3B, it seems to be the opposite, no difference upon starvation but upon insulin stimulation. Please clarify.

Figure 4F:
There is a discrepancy between 4F (glucose uptake) and 4E (GLUT4 PM localization). In EDL muscles, there is always an increase in glucose uptake in MASTL knockout cells (basal and insulin stimulated). GLUT4 PM localization is only affected under fasting conditions. In SOLEUS muscles it is even the opposite, i.e., no significant effect under fasting conditions, strong effect under insulin stimulation. Please explain.

Fig 5B:
The quantifications of P-T308-AKT (fold change) does not really correlate with the WB shown above. Please clarify.

Fig 5B:
ENSA/ARPP19 are the direct downstream substrates of MASTL. Phospho-mimetic ENSA/Arpp19 should act in a dominant manner. This implies that the presence or absence of MASTL should not play a role with respect to P-T308 phosphorylation of AKT, i.e., P-T308 levels should be the same in lanes 2 and 4. If anything, P-T308 levels should be lower in lane 2 because here in the presence of MASTL there is endogenous phosphorylated ENSA/ARPP19 in addition to the ectopic mimetic versions. Please, clarify.

Fig 5C:
Along the same line: If PP2A is inhibited by OA / fostriecin, then why does it still make a difference if MASTL is present or not. This indicates that the mechanism is more complex than proposed by the authors. Importantly, these inhibitors are far from being PP2A-specific, i.e., the authors have to consider PP4 and PP6 inhibition.

Fig 6D:
The authors argue that total levels of IRS1 were higher in MASTL-null tissues compared to the control. This seems to be correct. But why do IRS1 levels not correlate with AKT activity as judged by P-T308 levels? There is a significant difference in P-T308 levels between the different samples, which does not correlate with total IRS1 levels.

Fig 7A and 7B:
There is a problem with the WB. P-T194 is detected at app. 130kDa. Yet, the Ab against total MASTL detects only a band at 100Kda, but nothing at 130kDa. How is this possible?

Fig 7C:
In the kinase assay using recombinant mTORC1 and S6K1, GST-MASTL phosphorylated by mTORC1 runs higher than the one phosphorylated by S6K1. How is this possible?

Fig 7D:
It is not clear to this reviwer, why T194 suddenly does not any more play a role? Please explain. Fig. 7A and B address T194.

Fig 7F, G, H:
There is a break in the logic. S864A has a much weaker effect on Gwl activity than S861A (7F). Yet, S864 has a much more pronounced effect on the negative feedback mechanism (7G and H). How could this be? This does not make sense and questions the proposed mechanism.

We have just received a delayed additional review on your recent EMBO Journal submission, EMBOJ-2022-110833, from a fourth referee who had initially agreed to review the study, but subsequently gotten delayed and unresponsive. I am happy to say that this referee is also generally supportive of the study. Since I already sent you a final decision and revision invitation, I am not expecting you to additionally address the points of referee 4; but I am forwarding them to you (copied below) nevertheless, in the hope that you will find the included suggestions helpful for guiding your revision work.

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REFEREE 4

Malumbres EMBOJ-2022-110833

Here, the authors investigated a possible role for MASTL/Greatwall, a protein kinase that has been predominantly linked to the control of the G2/M transition, in mTOR pathway signaling to AKT. They started by

testing the effects of shRNA depletion of MASTL in MDA-MB-231, MCF7 and BT549 human breast cancer cells, which normally exhibit reduced mTORC1 signaling and phosphorylation of S6K1 and 4E-BP in response to nutrient starvation. Surprisingly, they found that lack of MASTL resulted in increased S6K1 and 4E-BP phosphorylation in glucose-starved cells. MASTL-depleted cells also exhibited increased phosphorylation of TSC2 T1462, a phosphorylation that inhibits TSC2 Rheb GAP activity. Next, using an inducible CRISPR knockout system, MASTL was efficiently deleted in MDA-MB-231cells, and consistently they found that TSC2 T1462, and AKT T308 and S473 phosphorylation was elevated in the knockout cells in the absence of glucose. In contrast to control cells, where the pT308 AKT signal dropped by 30 min, they found that glucose stimulation of MASTL KO cells led to sustained phosphorylation of AKT T308. Likewise, AKT pT308/pS473, S6K1 pT389 and TSC2 pT1462 levels induced in response to insulin stimulation, all showed sustained instead of transient phosphorylation in the MASTL KO cells. The delayed loss of mTORC1 signaling following insulin stimulation is known to be dependent on a negative feedback loop mediated by S6K, and, in contrast to control cells, knockdown of TSC2 in MDA-MB-231 MASTL KO cells failed to trigger the negative feedback loop. The loss of MASTL phenocopied the effects of rapamycin in overriding the feedback loop. Next, because of the known role of AKT in promoting glucose metabolism, they investigated the effects of MASTL depletion on cellular metabolism, and found that MASTL KO cells exhibited increased GLUT4 plasma membrane translocation, and also glucose transport and glycolysis, consistent with elevated AKT activity. In mouse tissues MASTL RNA levels were found to be expressed in pancreas, WAT, liver, and skeletal muscle and the levels of RNA were increased in liver upon ad libitum feeding. To test for a role of MASTL in glucose metabolism, they used a Mastlfl/fl mouse with a TAM-inducible Cre to elicit acute whole body Mastl deletion, and found the Mastl KO mice did not display any difference in insulin sensitivity, but did exhibit increased glucose clearance that was normalized by administration of the ETP46992 PI3K inhibitor, and elevated levels of pT308 AKT in skeletal muscle and liver, consistent with increased PI3K/AKT signaling being responsible for activating glucose uptake and metabolism in Mastl KO liver, WAT or skeletal muscle. One year old Mastl Δ/Δ mice fed ad libitum exhibited reduced glycemia compared to Mastl Δ/+ mice. Next, they tested whether a lack of MASTL phosphorylation of ENSA/ARPP19, the phospho-dependent inhibitors of the PP2A/B55 protein phosphatase complex, was responsible for these phenotypes though upregulation of PP2A-B55-mediated dephosphorylation. Combined knockdown of ENSA/ARPP19 in MDA-MB-231 cells increased pT308 AKT levels, whereas combined expression

of phosphomimic Ser to Asp mutants of ENSA S67D/ARPP19 S62D reduced pT308 AKT in MASTL KO MDA-MB-231 cells. Treatment of MDA-MB-231 cells with fostriecin or okadaic acid PP2A inhibitor decreased pT308 AKT partially restoring AKT inhibition in insulin treated in MAST-null cells. Next, they showed that the levels of phosphorylation of the IRS1 S616 and GRB10 S476 phosphosites, responsible for the feedback loop, were increased in MASTL-null cells. mTOR1/S6K mediate these feedback phosphorylations on IRS1 and GRB10, which lead to degradation of IRS and increased GRB10, respectively both of which reduce insulin receptor signaling. Knockdown of the B55a/B55d PP2A subunits increased phosphorylation of both sites, whereas MASTL KO reduced their phosphorylation, consistent with loss of PP2A/B55 inhibition. Knockdown of the B55 subunits also partially restored phosphorylation of the IRS1 and GRB2 sites in MASTL KO cells. Finally, they examined whether MASTL activity was regulated under these conditions, and showed that pT194 in the MASTL activation loop was increased during under feedback conditions, which was blocked by rapamycin or Torin inhibition of mTORC1. In vitro, both mTORC1 and S6K were shown to phosphorylate MASTL at T710, S716/T718, T722 and S878, and T710, S716/T718 and T722, respectively, although mTORC1 phosphorylations were stronger, particularly S878. S878 has a hydrophobic residue at +1, which would potentially make it a good mTORC1 site. In vitro a Mastl S864A mutant (equivalent to human MASTL S878) had slightly reduced ARPP19 phosphorylating activity, whereas an S861A mutant, at a key autophosphorylation site, had zero activity. Expression of mouse Mastl S864A or S861A failed to reduce AKT activity or GLUT4 membrane association in MASTL KO cells, suggesting that S864 is an important functional site, whose phosphorylation site could be important for mTORC1 regulation of MASTL activity and control of the negative feedback loop that attenuates insulin receptor signaling down the PI3K/AKT pathway by PP2A/B55.

Overall this is a nice story with convincing data obtained in both human cancer cell lines and mice that establishes an unexpected non-cell cycle role for MASTL/Greatwall in regulating the negative feedback pathway that damps down PI3K/AKT signaling downstream of insulin receptor activation, and indicates that the PP2A/B55 phosphatase complex has a key role in this pathway by dephosphorylating the inhibitory phosphorylation sites on IRS1 and GRB10. The finding that the MASTL/Greatwall kinase has a metabolic regulatory role in addition to its mitotic function is unexpected and important. The only weakness is the final step of the model, in which the authors implicate direct mTORC1-mediated phosphorylation and activation of MASTL as being needed to switch off the PP2A/B55 phosphatase via phosphorylation of ENSA/ARPP19. However, these studies are incomplete and further experiments are needed to establish this conclusion.

Points: 1. Figure 1B: Neither the text nor the legend indicates which cells were used in this panel - presumably MDA-MB-231 cells. The level of MASTL protein was greatly reduced by 72 hours, but it is not clear what fraction of the cells in the induced population were successfully deleted for MASTL. In EV1 the authors show that cell cycle distributions of parental and MASTL KO cells were similar at 72 hours, but from this analysis it is not clear whether there was an increase in mitotic cells in the MASTL KO population. How do the short term and long term proliferation profiles of the MASTL KO cells compare to those of the parental cells? Why was the level of pT389 S6K1 increased further with glucose stimulation in the MASTL KO cells, whereas the levels of pS473 AKT and pT1462 TSC2 were not.

2. Figure 2: Here too the identity of the MASTL KO cells needs to be included in the text and legend

3. Figure 3A: The authors ought to demonstrate how complete the MASTL knockdown was in these shRNA treated cells, ideally by staining for MASTL in parallel.

4. Figure 4G-I: It is surprising that whole body knockout of Mastl does not result in more serious mitotic defects and growth retardation? As far as I can, tell the authors previous studies with Mastllox/lox mice, where confined to studying MEFs to generate Mastl-null cells. Since Mastl Δ/Δ mice are embryonic lethal, this raises the question of how complete the Mastl knockout was in these mice. Possibly there is some stage during embryogenesis where Mastl is essential, and that Mastl is not essential in adult mice, but the authors should establish this. They need to include Mastl protein blots or IHC staining of different tissues of interest (or carry out quantitative PCR analysis for the mutant deleted allele) from Mastl Δ/Δ mice to demonstrate to what extent Mastl protein levels were reduced upon conditional KO in the long term or what fraction of cells in key tissues have in fact lost Mastl expression. Evidence for the extent of Mastl knockout and further discussion of the phenotypes of the Mastl Δ/Δ mice is essential.

5. Figure EV3D: The increase in pT308 and pS473 AKT levels in fasted one-year old Mastl Δ/Δ mice skeletal muscle was quite variable, and in many mice does not appear to have been that strong.

6. Figure 5: How much was ENSA/ARPP19 phosphorylation reduced in these MDA-MB-231 MASTL knockout cells?

7. Figure 7F-H: A number of additional experiments would be required to rigorously establish a role for mTORC1 phosphorylation of MASTL in regulation of the feedback loop. Here are some possibilities. The authors need to be more explicit (perhaps by adding a table) which MASLTL phosphosites they identified in cells, and under what conditions, and which sites they identified through in vitro phosphorylation and with which kinase. With regard to which sites might be directly phosphorylated by mTORC1, the majority of the well-documented mTORC1 phosphosites are Ser/Thr.Pro sites, although one or two have a Ser.Leu motif. Did the authors check the effects of a phosphomimic S878D MASTL mutation in the reconstitution system? The ideal way to study the role of S878 phosphorylation would be to make and use MASTL S878A and S878D knock-in mutations in MDA-MB-231 cells, which would enable analysis of the consequences of S878 phosphorylation for both insulin and glucose signaling. They should also use this system to analyze other phosphorylation events downstream of the insulin receptor, as they did in Figure 1. The fact that the exogenous GFP-Mastl proteins expressed in the isgMASTL MDA-MB-231 cells appear to have been hugely overexpressed is a cause for concern. In this regard, there are no Mastl blots in panel H. Does rapamycin or Torin treatment in cells inhibit S864/878 phosphorylation in vivo? Here pS864-specific antibodies would be useful. Through what mechanism would pS864/878 phosphorylation act as a priming event for autophosphorylation of S861/875 - the authors suggest a model in Figure EV4B but did not make mutations to test this. What are the possible functions of the other phosphorylation sites identified in this region of MASTL, two of which have Thr.Pro motifs - have any mutations been made?

## Answer to the Reviewers

## Referee #1:

The authors of this paper characterise a new signalling network that controls the activity of the PP2A/B55 phosphatase complex which regulates the dephosphorylation of Akt. The authors' data suggests that the MASTL protein kinase phosphorylates 2 proteins termed ENSA and ARPP19 that act as inhibitors of the PP2A/B55 complex. This stimulates the dephosphorylation of Akt thereby sustaining the mTORC1 and S6K1 dependent phosphorylation of IRS1. The authors also present some data that MASTL is directly phosphorylated and activated by mTORC1, and this reduces PP2A/B55 mediated dephosphorylation of IRS1 by the above pathway. They also show that loss of MASTL increases glucose uptake and glucose tolerance in the mouse model.

Overall, the data are strong and the results convincing, and this study would be of significant interest to researchers working on glucose tolerance, mitosis, and cancer PI3K/Akt signalling pathways as well as mTORC1 biology.

Below I outline some key points that I believe need attention in a revised version of this study.

> We thank the reviewer for the positive evaluation. We have addressed all the specific questions as discusses in the text below.

1. Can the authors add bar charts with statistics at the bottom of figs.1B, 1C and 1D? The bar chart in fig.1E does not correspond to the immunoblot shown above, that contains 2 time points of glucose stimulation. It is not clear what the time point corresponds to in the bar chart shown.

> We thank the reviewer for pointing out these problems. Indeed, the bar chart in Fig. 1E corresponds to 15 min Glc stimulation from several assays either using shRNA or isg*MASTL* (dox) and should be an independent panel referring to 1B and 1E (now 1C in the revised version). We apologize for this mistake. We have extended these data in the revised version by including new experiments and a new graph in figure 1D.
>
> Additionally, assays in BT-549 (Figure 1C) and MCF-7 (Figure 1D) have been repeated to reach n=3, and bar charts have been included in the revised version (Figures 1E and 1F, respectively).

2. The authors undertake lot of studies using MASTL knockout cells in figures 1 and 2. To confirm that the effects on Akt and other phosphorylation pathways that are examined are not a clonal or CRISPR off target effect, it would be important that the authors perform a rescue experiment, demonstrating the effects of re-expressing wild type and kinase inactive MASTL in MASTL deficient cells, to demonstrate that expression of wild type MASTL reduces Akt phosphorylation but not kinase inactive MASTL.

We performed a rescue experiment, as suggested by the reviewer, in which we re-expressed the wild-type version of mouse Mastl (intrinsically resistant to the sgRNA against human *MASTL*) and a kinase inactive mutant (Mastl S861A) in MASTL knock-out cells. This experiment appeared in Figure 7G in the original manuscript, and it shows that the wild-type version is able to rescue the effect of MASTL depletion by reducing the phosphorylation of AKT whereas the kinase inactive mutant not.

In addition, we have not only shown the effects of MASTL depletion on AKT phosphorylation through our CRISPR inducible system but also through RNAi in MDA-MB-231 cells (see figure 1C) and in other cell lines (see Figure 1E and F for BT-549 and MCF-7, or Figure EV1E for HepG2). In those assays we have used a shRNA against human MASTL whose specificity had already been validated in our previous manuscript (*Álvarez-Fernández et al., Cell Death Differ, 2018. PMID: 29229993*).

3. In fig.3, a control immunoblot showing that knockdown of the shRNA against MASTL reduces expression of the MASTL kinase should be shown in the main figure?

Agreed. A control immunoblot for Figure 3A, showing depletion of MASTL and increased AKT phosphorylation in MASTL-depleted cells upon insulin stimulation, was shown in Fig. EV2A in the original manuscript, and has now been included in the main Figure 3A. In addition, we have included new control immunoblots for MASTL depletion and AKT stimulation upon glucose re-addition (Figure EV2) in the revised version.

4. Similarly, in fig.4 immunoblotting evidence should be presented to show that MASTL-deficient cells lack the MASTL kinase in the main figure.

We reviewer is right but unfortunately antibodies do not recognize efficiently the mouse protein. We have thoroughly tested all available Mastl antibodies (including antibodies generated in our lab) in mouse tissues but, unfortunately, none of them work properly. Although they nicely recognize MASTL in human tissues, none of them give a reliable specific signal in mouse tissues, neither by immunoblotting or immunohistochemistry/immunofluorescence assays in paraffin or cryosections.

As an alternative we have now analyzed *Mastl* mRNA levels by qPCR with two specific probes against Mastl and we have been able to confirmed a significant reduction of MASTL mRNA both in liver and muscle tissues from those mice. This set of data is now included in the manuscript as Figure EV3F and EV3G.

5. The data shown in fig.3B most right-hand column does not look very persuasive. 2 of the 3 data points shown are in the same level as the control samples, with only one of the control data points being slightly lower. This does not look like the sort of experiment that would be easily reproduced independently.

Based on the description of the panel, we assume the reviewer refers to Figure 5C. The point of this experiment was to evaluate whether by inhibiting PP2A we were able to rescue the effect of MASTL depletion on AKT phosphorylation. Indeed, as pointed by the reviewer, there are no clear differences between MASTL knock-out cells (most right-hand column) and

MASTL-proficient control cells treated with okadaic acid. PP2A inhibition by okadaic acid therefore alleviates the increase in AKT phosphorylation caused by MASTL depletion, which agrees with the model proposed in the manuscript.

6. In figs.7A and 7b the authors study phosphorylation of MASTL at a residue termed Threonine194 that does not appear to be one of the key phosphorylation sites that mTORC1 or S6K1 phosphorylates. What is the relevance of studying this site? Why is it reduced by rapamycin if it is not phosphorylated by mTORC1 and S6K1? Is it a phosphorylation site that was identified in the mass spectrometry phospho-proteomic analysis?

We thank the reviewer for raising this important point. How MASTL activity is regulated is not yet fully understood, and most data come from regulation of MASTL during mitosis. Two residues have been proposed to be the activation loop sites, T194 and T741, critical for MASTL activation, together with the S875 autophosphorylation site in the extreme C-terminal (S861 in mouse Mastl). Unfortunately, there are no commercial antibodies against these critical sites and we have been unsuccessful trying to generate our own ones. The only exception is the antibody against the phospho-T194 used in this study (kindly provided by Dr. Hochegger, Sussex university). So, we decided to use that phospho-antibody as a surrogate of MASTL activation. As pointed by the reviewer, this site has not been identified as a potential mTOR site in our phospho-proteomic data or in previous studies. Indeed, T194 has been shown to be phosphorylated by CDK1/2 in mitosis. No information is available about possible kinases responsible for this phosphorylation in interphase. Interestingly, this phosphorylation has been proposed to be regulated by PP2A (*Rogers S, J Cell Sci, 2016. PMID: 26872783*), and these data suggest the possibility that sensitivity to mTOR inhibition might be due to the (MASTL-dependent) reactivation of PP2A.

7. The authors perform detailed phospho-proteomic analysis to map key phosphorylation sites that are phosphorylated by mTORC1 and S6K1. Has the primary data for this mass spec analysis been deposited in PRIDE database or another equivalent depository? Only a high-level summary of the mass spectrometry data is presented in the paper, and it is hard to evaluate the strength of this mass spec analysis. The mass spectrometry data should be better presented.

The mass-spectometry data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD032796. For peer reviewing purposes, the data set is available under the username: reviewer_pxd032796@ebi.ac.uk and password: ZUhRiLYA. This information is now included in the revised text of the manuscript.

In addition, we have also included a table in the revised version (table EV1) with more detailed data on the phosphoproteomic analysis to support Figures 7C and 7D. In this table, we show the normalized intensities for all phosphopeptides identified in the in vitro phosphorylation assay of recombinant GST-MASTL by mTORC1 and S6K1. Moreover, we have also included a table with the data on the phosphoproteomic analysis performed in vivo in G0 and G1 cells to support Figure EV4C (table EV2).

8. The authors mutate Ser861 in MASTL in experiments shown in Figure 7F-H, but this site does not appear to be a phosphorylation site according to the mass spectrometry data. The danger is that mutating a conserved Serine to an alanine can impact the activity of the kinase in a phosphorylation independent manner. The author's data does not necessarily mean that phosphorylation of this site is essential for regulating activity.

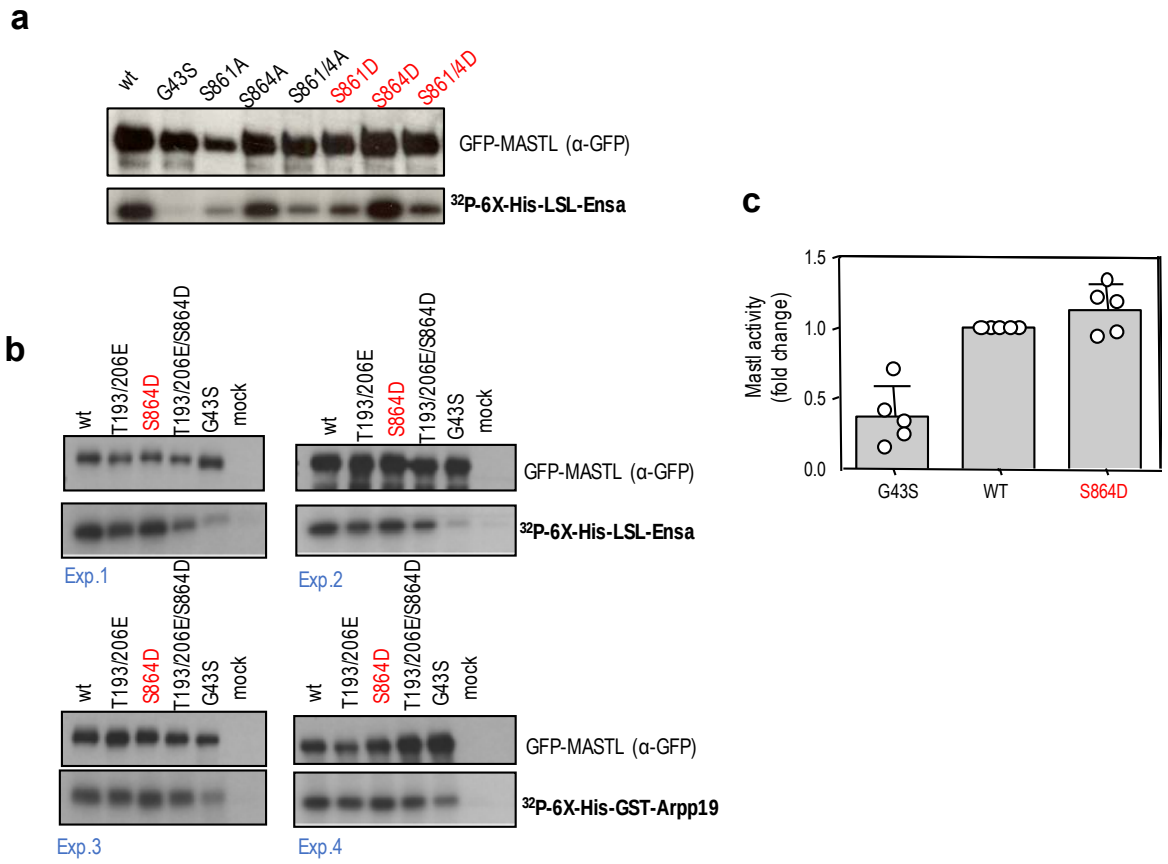
We agree with the reviewer. Our data do not prove that S861 is an essential phosphosite for MASTL activity. However, this has already been demonstrated by other laboratories. Phosphorylation of *Xenopus Mastl* S883 (the equivalent to S875 in human and S861 in mouse) was first reported in mitotic *Xenopus* extracts (*Vigneron et al., Mol Cell Biol, 2011, PMID: 21444715; Blake-Hodek et al., Mol Cell Biol, 2012 PMID:22354989*). In those studies, mutation of this site to alanine rendered MASTL inactive both in *Xenopus* and human versions but substitution to aspartic acid retains about 40-50% of activity, arguing that inactivity of the alanine mutant does not reflect structural requirements for serine in this position. In one of these earlier studies, this residue was demonstrated to be phosphorylated by Mastl itself, which has recently been confirmed in mammalian cells by our collaborators (*Hermida D. et al*., *Mol Cell Proteomics*, 2020. PMID: 31852836). This work shows that S875 is an autophosphorylation site essential for full kinase activation and with high occupancy during mitosis (more than 20% of protein). Interestingly, this study suggest that this site is also significantly phosphorylated in interphase.

The reason for mutating this important autophosphorylation site (S861 in mouse, S875 in human) in our study was to use it as an inactivating control mutation, as previously reported. It is important to note that the mTOR site reported in our study (S864 in mouse, 878 in human), is located in close proximity to that critical autophosphorylation site, and this opens the possibility that it might have a similar function and/or cooperate in possible protein interactions with the N-lobe and/or activation of the kinase. However, since mutation of S861 almost fully abolish the kinase activity of MASTL, combination with the mutation of the mTOR site S864 does not further leads to a significant reduction in kinase activity.

9. Have the authors attempted to mutate the 864/861 sites to aspartic or glutamic acid to mimic phosphorylation, either individually or together? This could provide more evidence that phosphorylation of these sites, activates the MASTL kinase if activation was observed

Yes, we have generated a phospho-mimicking mutation of the mTOR site (S864D) in order to further test the relevance of MASTL phosphorylation by mTORC1. Unfortunately, this mutant did not show any higher activity and it is similar to the wild-type version of MASTL (see panels **a-c** in the figure below). Since phosphorylation of this site alone would probably be insufficient to render an hyperactive MASTL kinase, we also decided to combine this mutant with the phospho-mimetic mutation in the autophosphorylation site of the C-terminus (S861D), but this combined mutant showed even less activity than the wild-type MASTL. In fact, the phospho-mimicking mutation of the autophosphorylation site (S861D) alone already showed a clear reduction in kinase activity compared to Mastl wt (panel **a** below). We also tried to combine the mTOR site phospho-mimetic mutant (S864D) with phospho-mimicking mutations in the activation loop (T193E/206E, T194/207 in human), but, unfortunately, none

of these combinations led to a clear increase in the kinase activity of MASTL (panel **b** below).



## Referee #2:

This is an interesting study by the Malumbres lab that presents a new link between cell cycle phosphatase control via MASTL and the mTor-AKT signalling cascade. The main message of the paper is that MASTL contributes to a negative feedback system that ensures a transient AKT activation following exposure to nutrients. The mechanisms of this signalling cascade are well worked out in the study and supported by experiments in human cell lines as well as in vivo studies in conditional MASTL knockout mice. The authors place MASTL downstream of mTor and present experiments that suggest that MASTL acts in this cascade via ENSA/ARPP19 dependent inhibition of PP2A-B55. Thus, MASTL activation following nutrient stimulation results in an increase in IRS1 and GRB10 phosphorylation and negative regulation of Insulin receptor signalling. There is a well-worked out link between TOR signalling and MASTL in budding and fission yeast but, to my knowledge, this study is the first to functionally link these two kinases in mammalian cells and implicate MASTL in metabolic control in mice.

The experiments in this study follow a logical progression, are mostly well executed and support the arguments of the authors. Overall, I believe this study represents a significant step forward in our understanding of MASTL and its functions outside of mitosis. I would recommend to publish this study in EMBOJ, after the following concerns have been addressed.

> We thank the reviewer for the detailed summary and positive comments on the relevance of the manuscript.

1) My major concern in the interpretation of the presented results lies in the differentiation between direct effect of MASTL depletion in interphase and potential indirect knock-on defects of aberrant mitotic progression, following loss of this essential mitotic kinase. The authors present data in MDA-MD-231 cells and show that at 72 hours depletion, MASTL is absent, but no cell cycle phenotypes are detected. This is shown in Figure EV1A for shRNA depletion. However, a previous study from the same lab (Alvarez-Fernandez et al. 2017) showed that these cells are sensitive to MASTL depletion.

I would suggest that the authors strengthen these data by performing a time-course analysis of MASTL depletion comparing depletion levels and cell cycle phenotypes both in the shRNA and the gRNA/Cas9 depleted cells. The FACS results in this experiment should also be quantified properly showing the standard deviation of three biological repeats.

> We fully agree with these comments and we thank the reviewer for raising this very relevant point. As indicated, we have previously shown that MASTL depletion impairs proliferation of MDA-MB-231 cells (*Álvarez-Fernández et al., Cell Death Differ, 2018 PMID: 29229993*). This was shown in long term colony formation assays (10-15 days after MASTL depletion) both using RNAi and CRISPR/Cas9 systems (Figure 1c). At earlier time points, we were also able to detect cell cycle defects (4n accumulation by FACS analysis as a consequence of mitotic

defects) but only in the sgRNA/Cas9 depleted cells, which is expected to be more efficient in inducing MASTL depletion than shRNA at the cell level, and having at least 4 days of doxycycline treatment (Fig.3 g-h and Fig. 4b in PMID: 29229993).

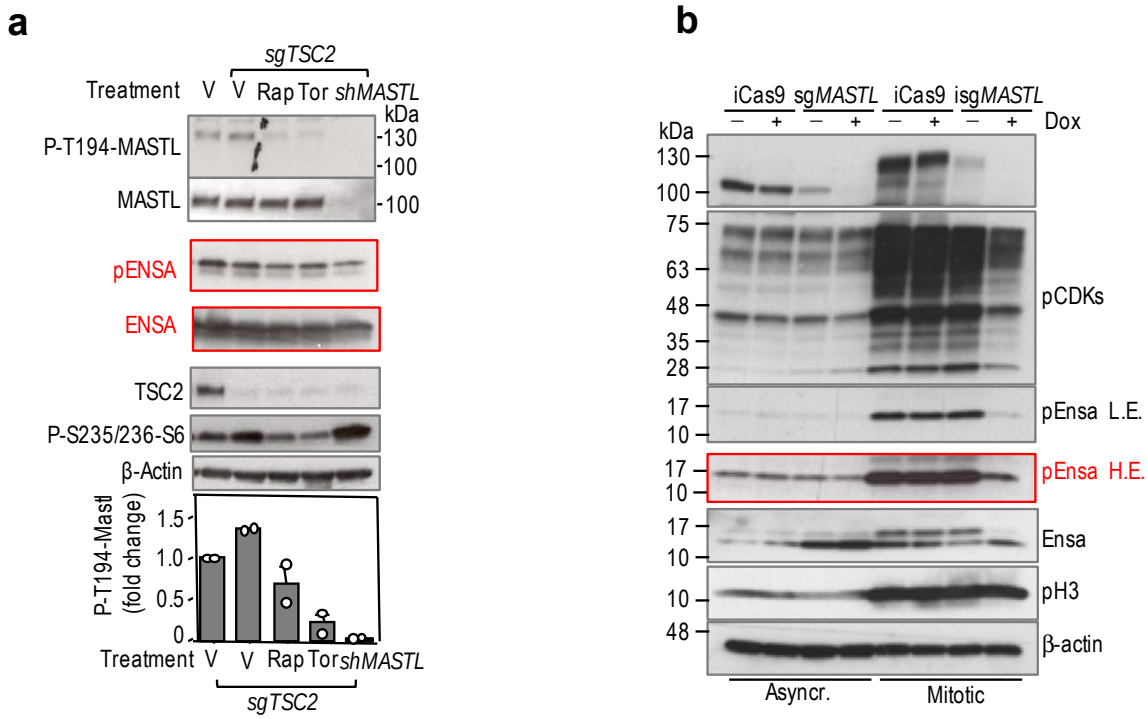
To strengthen these data and following the recommendation by the reviewer, we have now performed similar experiments in the current manuscript shortly after MASTL depletion; i.e. earlier than 72h either after shRNA infection, or doxycycline addition for the CRISPR/Cas9 system. As suggested by the reviewer, we have performed a time course analysis (48, 72 and 96h) in both systems, RNAi and CRISPR/Cas9. We have monitored the level of MASTL depletion by Western-blot and analyzed the cell cycle phenotype by FACS analysis (DNA content and pH3 staining to detect mitotic cells). Three independent experiments have been performed in both systems, and data are now included in the revised version of the manuscript as EV1A and EV1C for shRNA and isgRNA, respectively. Western-blot analysis and FACS plots are only shown for one representative experiment of each system and graphs reflects the average + SEM of the three replicates with the correspondent statistical analysis. In agreement with our previous published data, no cell cycle defects have been observed upon shRNA-mediated knockdown even at 96h, although a good level of MASTL depletion was already observed at 72h. In the CRISPR/Cas9 inducible system, an efficient depletion was already detected 48h upon doxycycline addition. No cell cycle differences were observed neither at 48 nor 72h, but an increased accumulation of 4n (mean 16.5% -dox vs. 25.6 % + dox) and over 4n cells (mean 0.7% -dox vs. 4.1 % + dox) was detected 96h after doxycycline addition, in agreement with our published results. This point has also been emphasized in the revised text.

2) Another important point of this study is the proposed direct activation of MASTL by mTOR phosphorylation of S878. The critical experiment here is the kinase activity measurement for MASTL. The in vitro experiments (MASTL pulldowns and ARPP19 immunoblots) are shown in EV4A. The observed changes in ARPP19 phosphorylation (an increase after TSC2 depletion and a decrease following Rapamycin treatment) are not convincing. This should be down at least three times and the significance of the differences analysed by a t-test. To confirm these data, the authors should also blot cell extracts with pS67 ENSA antibodies to test, if an increase in kinase activity can be directly measured.

We have repeated several times this assay and we found some variability in MASTL activity after TSC2 depletion, which might be explained by some technical issues since we are expressing MASTL and comparing its activity in two cell lines (control cells and stable sgTSC2 cells). What it is reproducible is the effect of mTOR inhibition on MASTL activity. In this case MASTL activity is measured in the absence or presence of mTOR inhibitors in the same background cells (sgTSC2), and we consistently observed a reduction in ARPP19 phosphorylation upon mTOR inhibition, which is statistically significant in the case of Torin but not Rapamycin, although it follows the same trend. Data from 4 independent experiments are now shown in the revised version as Figure EV4A, normalizing to control sgTSC2 cells.

We agree with the reviewer that a good confirmation might be obtained by using the phospho-ENSA (S67)/ARPP19 (S62) antibody in cell extracts. However, although we have

tried that antibody in several assays we cannot see any differences in signal, even in MASTL-depleted cells (see for instance panel a below corresponding to Figure 7B including ENSA and phospho-ENSA blots), suggesting its signal is not MASTL dependent in these conditions. Indeed, although in mitotic cell extracts this antibody shows an intense signal that gets strongly reduced upon MASTL depletion, it becomes much lower in interphase cells and, unfortunately, does not appear to be MASTL dependent (see panel b below corresponding to an adaptation of Figure 3e from our previous manuscript, Álvarez-Fernández et al. *Cell Death and Diff*, 2018; *PMID: 29229993*). In conclusion, we are not confident that this antibody is sensitive and/or specific enough to detect MASTL activity in non-mitotic cells.




Minor comments:

The text contains many small errors and imprecise descriptions. This should be corrected carefully before publication. Examples for this on page 5:

"Starvation of MDA-MB-231 cells for either growth factors or nutrients inhibited mTORC1 activity in control cells infected with a scrambled sequence"

Sequences were not transfected, this should be changed to shRNAs

"in the presence of specific small guide RNAs (sgRNAs)"

This should be "single guide RNA"

We thank the reviewer for pointing out these defects. These and other errors have now been corrected in the revised version of the text.

Figure 3

A - The image colours do not correspond with the colours reported in the figure legend. Also, "magenta" typo. The figure legend mentions a "histogram" and 800 cells scored per condition. The Figure does not show a histogram (but rather a swarm blot) and does not show data points for 800 cells. It is unclear what is being quantified and what statistical method was used to generate reported p-values.

Original images have been pseudocolored to magenta (GLUT4), cyan (DAPI) and yellow (Cell mask) to be color blindness friendly. In the original figure a representative experiment was shown in which we quantified the percentage of cells positive for GLUT4 at the plasma membrane in 16 fields in each condition summing up a total number of about 800 cells per condition. Each dot therefore represented the percentage of positive cells in one field.

We have now repeated this experiment to have three biological replicates and we have calculated the average of the percentage of GLUT4 positive cells in each experiment, normalized to the percentage of GLUT4 positive cells in the scramble condition. Therefore, the new figure in the revised version shows the mean ± SEM of the fold increase in GLUT4 localization at the plasma membrane from 3 experiments. Statistical significance has now been calculated with a 1-way ANOVA test, which reflects a significant increase in GLUT4 accumulation in MASTL-depleted cells upon insulin stimulation.

B - It should be made clear which data points originate from the same experiment (technical repeats). Statistical test should be only applied on independent results.

In the original figure all technical replicates from the 3 experiments were shown. This graph has now been substituted in the revised version by new plots showing the mean + SEM of the 3 independent experiments, and Student's t test has been applied to those 3 independent biological replicates.

Figure 4

A - The figure legend states that in all tissues, except pancreas, n = 3. However, only two data points are shown for eWAT and muscle tissues. Additionally, two datapoints reported for the eWAT tissue are conflicting.

The reviewer is right and we apologize for the mistake. We have now repeated this RT-qPCR using samples from the indicated tissues of 3 additional mice, except for pancreas, for which unfortunately RNA was degraded during the RNA extraction procedure. These new data are now presented in the revised version (new Fig. 4A), showing good expression of MASTL in all tissues analyzed compared with the most proliferative one (spleen), and less variability among individual mice than the original assay, also for eWAT.

Figure 5

B - Representative blot does not reflect the quantification. Ectopic expression of phosphomimetic ENSA-S67D and ARPP19-S62D in cells expressing MASTL appears to significantly increase AKT-T308 phosphorylation. In MASTL-depleted cells expression of ENSA-S67D and ARPP19-S62D reduces AKT-T308 phosphorylation.

> The reviewer is right. The blot showed in the original figure corresponds to the only replicate in which we detected an increase in AKT-T308 phosphorylation upon ectopic expression of phosphomimetic ENSA-S67D and ARPP19-S62D in cells expressing endogenous MASTL. We have now substituted that blot for a more representative one, and we have also revised the quantification, normalizing to control cells in each background (MASTL-expressing cells or MASTL KO cells). As clearly seen in the new plot, except for one experiment in one condition, in all cases ectopic expression of phosphomimetic ENSA /ARPP19 led to reduced AKT-308 phosphorylation.

Figure 6

D - Heterogeneity S6K1-T389 phosphorylation in MASTL-depleted tissues is not discussed.

> We think this is due to the high variability usually found in mouse tissues and the differences in epitope recovery during tissue protein extraction, especially for phosphoepitopes, such as P-T389-S6K1 in this case. We have acknowledged this variability in the revised version of the manuscript.

## Referee #3:

In the submitted manuscript, Sanz-Castillo investigate the mechanism that limits AKT activity. They show that Greatwall/MASTL suppresses PI3K-AKT activity via mTORC1- / S6K1-dependent phosphorylation of IRS1 and GRB10. Notably, ENSA/ARPP19, the downstream substrates of Greatwall/MASTL are involved in this process. Specifically, the authors can show that a phosphate-mimetic version of ENSA/ARPP19 rescues loss of Greatwall/MASTL. MASTL/Greatwall is directly phosphorylated by mTORC1 and S6K1. Thus, a mitotic-independent function of the Gwl-ENSA/ARPP-B55 axis modulates glucose response and negative feedback loops triggered by mTORC1-S6K1 activity. This is an interesting finding and I suggest publication, once the authors have addressed the following, major points:

We thank the reviewer for these positive comments.

These major points are key for the conclusions drawn by the authors and therefore must be rigorously addressed.

Figure 1:

- could the authors explain the effect of glucose re-addition in 1B?

In glucose-starved cells, a good correlation is detected between p-AKT, pTSC2 and p-S6K, and the level of those three phosphorylations is clearly increased in the absence of MASTL.

Glucose re-addition activates the feedback loop leading to reduced AKT phosphorylation and its downstream substrate TSC2 in the presence of active mTORC1 (high pS6K1), and therefore, dissociation of pS6K1 from pAKT/p-TSC2 takes place in control cells as expected (compare lanes 1 and 2).

- in Fig. 1E, glucose stimulation induces negative feedback, which leads to AKT inactivation. In 1E, MASTL depletion prevents AKT inactivation consistent with the idea that MASTL is involved in the negative feedback mechanism. However, in 1C and 1D, Akt gets partially inactivated by glucose even in the absence of MASTL, the difference to Ctrl KD is only minor. In 1B, there is no difference regarding P-T308-AKT at all between Ctrl and MASTL KD at all (although downstream targets like S6K1 show differences). Could the authors please clarify this discrepancy? It is an important data piece for the model.

As indicated above, the reduction in P-T308-AKT upon glucose stimulation and activation of the feedback loop is clearly detected in control cells. According to our model, that inactivation is much less pronounced in MASTL-depleted cells, in which higher phosphorylation of AKT-T308 is observed compared to control cells. We agree with the reviewer this is not so clear in the image in 1B likely due to low exposure conditions. Nevertheless, quantification of the raw data image rendered 2.5-fold increase in P-T308-AKT signal in MASTL-depleted cells compared to control in that experiment. In addition, we have now included the quantification of these differences in P-T308-AKT in MASTL-depleted cells compared to control ones from

at least 6 independent experiments (Figure 1D in the revised version), which leads to a statistically significant difference. Although the extent of such difference between control and MASTL-depleted cells is variable across different experiments, a higher signal in the absence of MASTL has been consistently detected in all experiments in conditions of glucose re-addition.

We agree with the reviewer that the effect in BT-549 and MCF-7 cell lines is lower compared to the one observed in MDA-MB-231 cells. This could be due to variable and less efficient knockdown in these cell lines. Also, please notice that we have only used shRNAs in those cells, but not a more complex CRISPR-mediated knockout system. Nevertheless, we have now repeated those assays to get 3 independent experiments, and quantifications are now included in the revised manuscript (Figures 1E and 1F for BT-549 and MCF-7, respectively). Data on MCF-7 cells revealed a consistent and significant increase in AKT phosphorylation in MASTL-depleted cells compared to control cells upon glucose stimulation. Same trend, although not statistically significant, was observed in BT-549 cells. These differences might be influenced by fact that this cell line harbours a deletion in the AKT-inactivating phosphatase PTEN that might affect its sensitivity to the feedback loop. It is also important to notice that variations in the status of the feedback loop, not being always on depending on the timing of glucose stimulation, might also explain some variability across experiments and cell lines.

- Fig. 1B: why does the level of P-T308-AKT not correlate with P-T389-S6K1, e.g., compare P-T308-AKT in lane 3 and 4 with the respective lanes of P-T389-S6K1

As indicated in the first point, the level of P-T-308-AKT does not correlate with P-T389-S6K1 in conditions of feedback activation also in control conditions (compare lane 1 and 2). On one hand, glucose activates mTOR (high pS6K1) but further activation of the feedback loop leads to reduced pAKT and pTSC2 to control and prevent an excessive and chronic activation of mTOR. In MASTL-depleted cells, a similar dissociation takes place but, due to the impairment in the proper establishment of the feedback loop, the reduction in pAKT and pTSC2 is much less observed.

Figure 2A + 2B:

TSC2 knockdown triggers activation of the feedback loop and AKT inhibition. Why did the authors stimulate TSC2 depleted cells with insulin? As shown in 2A, starvation + insulin by itself triggers negative feedback after 30min resulting in P-T308-ATK dephosphorylation. The leftmost lane of 2B would (according to the figure legend) correspond to the 30min timepoint of 2A (-Dox). Why is there a strong P-T308 signal in 2B, and no signal in 2A?

The reviewer is right and there is no need to stimulate with insulin when we trigger the feedback loop by depleting TSC2. These experiments have been done either in unstimulated cells or glucose-deprived cells in which there is strong P-T-308-AKT signal. We thank the reviewer for this observation, since there was a mistake in the figure legend that has now been corrected. We apologize for the misleading it has been caused.

Figure 4D:

Regarding glucose clearance in the presence/absence of PI3Ki in WT and MASTL KO cells. It is not really explained why PI3Ki has stronger effect in MASTL KO. If the effect goes via AKT there should be the same phenotype upon PI3Ki treatment.

In Figure 4D, treatment with a PI3K inhibitor rescues the phenotype of glucose tolerance found in MASTL KO mice. As pointed by the reviewer, and also referred in the text, MASTL KO mice are even more sensitive to the PI3Ki than WT mice. At this moment, we don't know the explanation for this but it might be due to the functional interaction of MASTL with other downstream targets of PI3K, such as SGK3; or with other PI3K-interacting signalling modules affecting glucose homeostasis such as LKB1, p38 MAPK or Myc, for instance. Alternatively, we cannot exclude different pharmacokinetics and tissue distribution of this compound between MASTL KO and MASTL WT mice.

An AKT inhibitor would probably be more appropriate to exclusively test the AKT-dependence. However, we decided to use a PI3K inhibitor since it is a potent and selective PI3Ki with good pharmacokinetic profile that was previously used in our institute to downregulate PI3K and also shown to lead to decreased AKT activity (Ortega-Molina et al., Cell Metabolism, 2015, PMID:25817535). Moreover, this inhibitor was previously used to inhibit the PI3K-AKT pathway in a glucose tolerance test (see figure below) similar to the one included in our manuscript, making it much easier to titrate and control its activity in these in vivo assays, therefore favouring a reduction in the number of mice to be used (3R reduction principle).

In summary, our data make us confident the effect of MASTL depletion on glucose tolerance is at least partially mediated by its effect on AKT signalling.

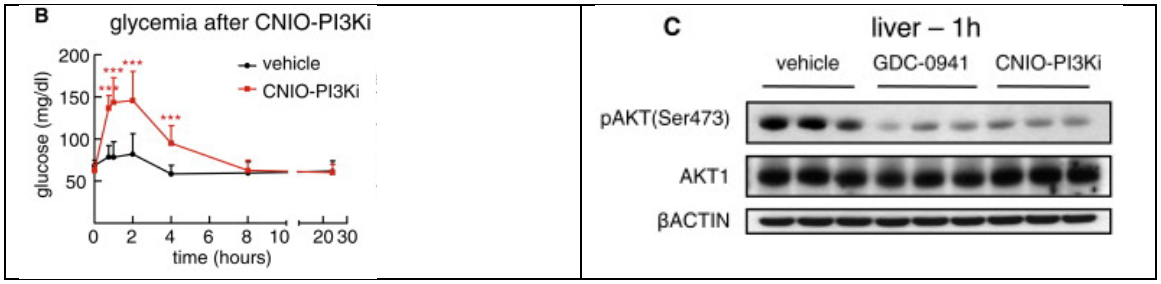



Figure from Ortega-Molina et al., Cell Metabolism, 2015

Figure 4E:

There is a discrepancy between 3B and 4E. In 4E, there is a significant difference in GLUT4PM localization under fasting conditions, but no difference under refeeding conditions. In Fig. 3B, it seems to be the opposite, no difference upon starvation but upon insulin stimulation. Please clarify.

We thank the reviewer for detecting this problem. We have checked the individual data and quantifications of Figure 4E. Glut4 signal was much higher in one of the control mice in refeeding conditions compared to the other two control mice, probably due to a problem in

the fiber orientation of the cut section. We have repeated that staining in a new tissue section and with the revised quantification a significant difference in Glut4 localization is also detected under refeeding (see new Figure 4E). Still, the difference is not as bigger as in fasting conditions, but this might be caused by the higher variability found in the refeeding condition, which depends on the *ad libitum* food intake of each mouse and cannot be so well controlled as the in vitro assays.

Moreover, we have also repeated the GLUT4 assays in vitro in cells to have 3 independent replicates. Quantification of the average ± SEM of those experiments is now showed in revised Figure 3A and EV2A, revealing a significant difference in GLUT4 localization both in glucose and insulin stimulation but also in glucose deprivation (EV2A), in agreement with our in vitro signalling data and the in vivo data. A similar trend is observed in serum deprivation (3A) although does not reach statistical significance. It is important to notice that in this condition of serum deprivation, glucose is present in the cell culture medium, whereas no nutrients at all are present in fasting conditions in the in vivo assays.

Figure 4F:

There is a discrepancy between 4F (glucose uptake) and 4E (GLUT4 PM localization). In EDL muscles, there is always an increase in glucose uptake in MASTL knockout cells (basal and insulin stimulated). GLUT4 PM localization is only affected under fasting conditions. In SOLEUS muscles it is even the opposite, i.e., no significant effect under fasting conditions, strong effect under insulin stimulation. Please explain.

As indicated above, revised data on Glut4 localization on muscle tissues also shows a significant difference under refeeding conditions (see new Figure 4E). It is also important to consider that the ex-vivo glucose uptake assays performed in Figure 4F are done under controlled conditions of insulin stimulation (100 nM 20 min), whereas we have not performed a specific insulin injection assay in vivo (Figure 4E) but a general refeeding condition, which implies *ad libitum* regular food intake for 2h after an overnight fasting.

In addition, the reason for not observing significant differences in basal conditions in SOLEUS might be due to the fact that this is a slow-twitch fiber with higher basal uptake of glucose due to higher expression of Glut4. On the other hand, the differences in MASTL-depleted cells upon insulin stimulation are more significant in this slow-twitch fiber (SOLEUS) compared with the less insulin-sensitive fast-twitch EDL.

Fig 5B:

The quantifications of P-T308-AKT (fold change) does not really correlate with the WB shown above. Please clarify.

The reviewer is right. The blot showed in the original figure corresponds to the only replicate in which we detected an increase in AKT-T308 phosphorylation upon ectopic expression of phosphomimetic ENSA-S67D and ARPP19-S62D in cells expressing MASTL. We have now substituted that blot for a more representative one, and we have also revised the quantification, normalizing to control cells in each background (MASTL-expressing cells or

MASTL KO cells). As clearly seen in the new plot, except for one experiment in one condition, in all cases ectopic expression of phosphomimetic ENSA /ARPP19 led to reduced AKT-308 phosphorylation.

Fig 5B:

ENSA/ARPP19 are the direct downstream substrates of MASTL. Phospho-mimetic ENSA/Arpp19 should act in a dominant manner. This implies that the presence or absence of MASTL should not play a role with respect to P-T308 phosphorylation of AKT, i.e., P-T308 levels should be the same in lanes 2 and 4. If anything, P-T308 levels should be lower in lane 2 because here in the presence of MASTL there is endogenous phosphorylated ENSA/ARPP19 in addition to the ectopic mimetic versions. Please, clarify.

Following the previous point, at this moment we do not know the reason by which reduction of P-T308 is more clearly detected in the absence of endogenous MASTL. One possibility might be that in the presence MASTL, endogenous phosphorylated ENSA/ARPP19 would compete with phosphomimetic ENSA/ARPP19 to bind PP2A-B55. And, if this were the case, according to the model of how ENSA/ARPP19 inhibits PP2A-B55 (by binding to the catalytic site as a very inefficient substrate), it might be very well possible that PP2A-B55 slowly auto-reactivates itself by dephosphorylating endogenous ENSA/ARPP19 and releasing its own inhibitor as already described, something that could not happen if only phosphomimetic mutants bind to PP2A-B55 instead (*Williams et al., Elife 2014, PMID:24618897; Labbe et al., Nat Comm 2021, PMID: 34117214*)

Fig 5C:

Along the same line: If PP2A is inhibited by OA / fostriecin, then why does it still make a difference if MASTL is present or not. This indicates that the mechanism is more complex than proposed by the authors. Importantly, these inhibitors are far from being PP2A-specific, i.e., the authors have to consider PP4 and PP6 inhibition.

The point of this experiment was to evaluate whether by inhibiting PP2A we were able to rescue the effect of MASTL depletion on AKT phosphorylation, based on the hypothesis it was mediated by the increased PP2A-B55 activity induced by deletion of MASTL. Although there is not a complete rescue, PP2A inhibition by okadaic acid (lane 5 vs 6) or fostriecin (lane 3 vs 4) alleviates the increase in AKT phosphorylation caused by MASTL depletion compared with control cells (lane 1 vs 2) in which the difference is much higher (see individual data points on bar chart), in agreement with our model. See also point 5 in comments to reviewer #1.

Importantly, these inhibitors are not PP2A-B55 specific and they also affect PP2A complexes with other B regulatory subunits not modulated by MASTL, or even other phosphatases such as PP4 or PP6, as indicated by the reviewer. The PP2A-B55 independent effects of those inhibitors might therefore contribute somehow to the outcome of this assay leading to a partial instead of a complete rescue. Data with phospho-mimetic ARPP19 and ENSA and

with siRNAs against ARPP19/ ENSA and B55 regulatory subunits in figures 5 and 6, respectively, support the involvement of this pathway downstream of MASTL in the regulation of AKT-mTORC1 signalling. Nevertheless, we cannot exclude the possibility of other signalling molecules beyond the ones here identified playing a role in MASTL phenotype.

Fig 6D:

The authors argue that total levels of IRS1 were higher in MASTL-null tissues compared to the control. This seems to be correct. But why do IRS1 levels not correlate with AKT activity as judged by P-T308 levels? There is a significant difference in P-T308 levels between the different samples, which does not correlate with total IRS1 levels.

Phospho-T-308-AKT signals were very variable between samples and showed no statistical difference ($p>0.05$) between control and Mastl KO tissues. As pointed by the reviewer, only few samples showed increased phosphorylation levels and correlated with the increased IRS1. We think this is due to the high variability usually found in mouse tissues and the differences in epitope recovery during tissue protein extraction specially for phosphoepitopes, such as P-T308-AKT.

Fig 7A and 7B:

There is a problem with the WB. P-T194 is detected at app. 130kDa. Yet, the Ab against total MASTL detects only a band at 100Kda, but nothing at 130kDa. How is this possible?

We think this is due to multiple phosphorylation sites, including autophosphorylation events, which take place once MASTL becomes active. This has actually been discussed in previous reports studying the regulation of Mastl kinase activity (*Vigneron et al., Mol Cell Biol, 2011, PMID: 21444715; Blake-Hodek et al., Mol Cell Biol, 2012 PMID:22354989*). The fact that MASTL total antibody recognizes only the lower band is indeed due to a preference for detection of non-phosphorylated forms of MASTL as we have now demonstrated (see Figure below). This is very clear in the case of the MASTL antibody raised in our laboratory (MASTL, clone 74C/B6, Abcam). This is a monoclonal antibody raised against recombinant GST-human MASTL full-length, probably not phosphorylated, so we suspect the epitope recognized by this antibody coincides with a phosphorylated region in the protein in mammalian cells. Importantly, pT94 signal disappears upon MASTL depletion (Figure 7B) confirming its specificity against MASTL protein.

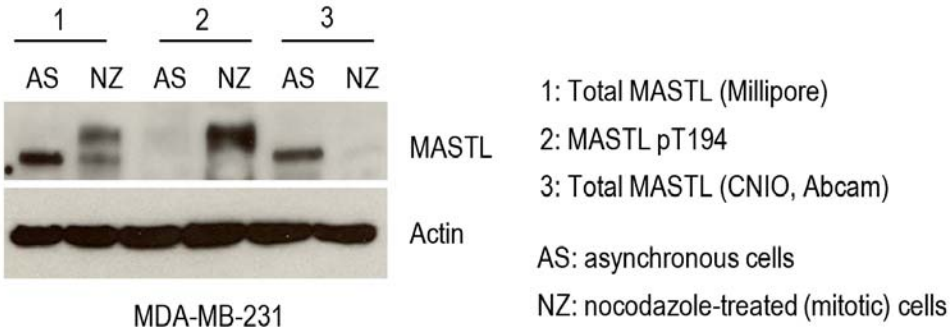



Fig 7C:

In the kinase assay using recombinant mTORC1 and S6K1, GST-MASTL phosphorylated by mTORC1 runs higher than the one phosphorylated by S6K1. How is this possible?

> Although similar number of phosphoresidues were identified in both conditions, in the case of phosphorylation by mTORC1 the intensity of pS878 phosphopeptide was two orders of magnitude higher than in control conditions or upon phosphorylation by S6K1 (see figure 7D and raw data in the new table EV1). This suggests that most GST-MASTL molecules are phosphorylated in this residue upon mTORC1 incubation, which might modify its charge/mass ratio triggering a reduction on its electrophoretic mobility.

Fig 7D:

It is not clear to this reviwer, why T194 suddenly does not any more play a role? Please explain. Fig. 7A and B address T194.

> We understand this might be a bit confusing. How MASTL activity is regulated is not yet fully understood but T194 has been proposed to be an activation loop site important for MASTL activation, together with the S875 autophosphorylation site in the extreme C-terminal (S861 in mouse Mastl). Unfortunately, there are no commercial antibodies against these critical sites and we have been unsuccessful trying to generate our own ones. The only exception is the phospho-T194 used in this study (kindly provided by Dr. Hochegger, Susex university).
>
> T194 has not been identified as a potential mTOR site in our phospho-proteomic data. Indeed, it has been shown to be phosphorylated by CDK1/2 in mitosis, but the kinase responsible for this phosphorylation in interphase is not known. We are therefore monitoring T194 phosphorylation in Fig. 7A and B is as a surrogate of MASTL activation. Once we found the potential activation of MASTL by mTOR based on pT194 signal (Figures 7A and B) and the in vitro kinase activity of MASTL (Fig. EV4A), we performed a phospho-proteomic analysis in recombinant GST-Mastl phosphorylated by mTOR in order to identify the potential mTOR sites in MASTL.

Fig 7F, G, H:

There is a break in the logic. S864A has a much weaker effect on Gwl activity than S861A (7F). Yet, S864 has a much more pronounced effect on the negative feedback mechanism (7G and H). How could this be? This does not make sense and questions the proposed mechanism.

The current model for MASTL activation suggests that MASTL requires first priming phosphorylation by other kinases followed by autophosphorylation on its C-terminal S875 (S861 in mouse) residue (*Hermida D. et al*., *Mol Cell Proteomics*, 2020. PMID: 31852836). In agreement with that, mutation of this site (S861 in mouse, S875 in humans) almost fully abolish the kinase activity of MASTL (kinase assay shown in Figure 7F), confirming that phosphorylation of S861/S875 seems to be the most critical and essential residue for MASTL activity.

Mutation of S864 (S878 in humans) alone significantly reduces the catalytic activity about 30-40%, suggesting it could work as a priming phosphorylation site for MASTL activation regulated by mTORC1 pathway. In agreement with that, functional assays showed in Figure 7G and 7H indicate that both mutants fail to rescue the effect induced by MASTL depletion on AKT phosphorylation and GLUT4 translocation. Whether S864A has a more pronounced effect on the feedback than S861A is difficult to conclude. Indeed, in panel 7G S864A seems to rescue a little bit the phosphorylation of AKT whereas S861A does not rescue at all and shows the same level in AKT phosphorylation as MASTL KO cells. In panel 7H, both mutants show similar behavior and none of them are able to rescue the phenotype of MASTL-depleted cells, suggesting that mutation in the mTOR site (with 30-40% less kinase activity) is sufficient to block the function of MASTL in these conditions.

## Referee #4

Here, the authors investigated a possible role for MASTL/Greatwall, a protein kinase that has been predominantly linked to the control of the G2/M transition, in mTOR pathway signaling to AKT. They started by testing the effects of shRNA depletion of MASTL in MDA-MB-231, MCF7 and BT549 human breast cancer cells, which normally exhibit reduced mTORC1 signaling and phosphorylation of S6K1 and 4E-BP in response to nutrient starvation. Surprisingly, they found that lack of MASTL resulted in increased S6K1 and 4E-BP phosphorylation in glucose-starved cells. MASTL-depleted cells also exhibited increased phosphorylation of TSC2 T1462, a phosphorylation that inhibits TSC2 Rheb GAP activity. Next, using an inducible CRISPR knockout system, MASTL was efficiently deleted in MDA-MB-231cells, and consistently they found that TSC2 T1462, and AKT T308 and S473 phosphorylation was elevated in the knockout cells in the absence of glucose. In contrast to control cells, where the pT308 AKT signal dropped by 30 min, they found that glucose stimulation of MASTL KO cells led to sustained phosphorylation of AKT T308. Likewise, AKT pT308/pS473, S6K1 pT389 and TSC2 pT1462 levels induced in response to insulin stimulation, all showed sustained instead of transient phosphorylation in the MASTL KO cells. The delayed loss of mTORC1 signaling following insulin stimulation is known to be dependent on a negative feedback loop mediated by S6K, and, in contrast to control cells, knockdown of TSC2 in MDA-MB-231 MASTL KO cells failed to trigger the negative feedback loop. The loss of MASTL phenocopied the effects of rapamycin in overriding the feedback loop. Next, because of the known role of AKT in promoting glucose metabolism, they investigated the effects of MASTL depletion on cellular metabolism, and found that MASTL KO cells exhibited increased GLUT4 plasma membrane translocation, and also glucose transport and glycolysis, consistent with elevated AKT activity. In mouse tissues MASTL RNA levels were found to be expressed in pancreas, WAT, liver, and skeletal muscle and the levels of RNA were increased in liver upon ad libitum feeding. To test for a role of MASTL in glucose metabolism, they used a Mastlfl/fl mouse with a TAM-inducible Cre to elicit acute whole body Mastl deletion, and found the Mastl KO mice did not display any difference in insulin sensitivity, but did exhibit increased glucose clearance that was normalized by administration of the ETP46992 PI3K inhibitor, and elevated levels of pT308 AKT in skeletal muscle and liver, consistent with increased PI3K/AKT signaling being responsible for activating glucose uptake and metabolism in Mastl KO liver, WAT or skeletal muscle. One year old Mastl Δ/Δ mice fed ad libitum exhibited reduced glycemia compared to Mastl Δ/+ mice. Next, they tested whether a lack of MASTL phosphorylation of ENSA/ARPP19, the phospho-dependent inhibitors of the PP2A/B55 protein phosphatase complex, was responsible for these phenotypes though upregulation of PP2A-B55-mediated dephosphorylation. Combined knockdown of ENSA/ARPP19 in MDA-MB-231 cells increased pT308 AKT levels, whereas combined expression of phosphomimic Ser to Asp mutants of ENSA S67D/ARPP19 S62D reduced pT308 AKT in MASTL KO MDA-MB-231 cells. Treatment of MDA-MB-231 cells with fostriecin or okadaic acid PP2A inhibitor decreased pT308 AKT partially restoring AKT inhibition in insulin treated in MAST-null

cells. Next, they showed that the levels of phosphorylation of the IRS1 S616 and GRB10 S476 phosphosites, responsible for the feedback loop, were increased in MASTL-null cells. mTOR1/S6K mediate these feedback phosphorylations on IRS1 and GRB10, which lead to degradation of IRS and increased GRB10, respectively both of which reduce insulin receptor signaling. Knockdown of the B55a/B55d PP2A subunits increased phosphorylation of both sites, whereas MASTL KO reduced their phosphorylation, consistent with loss of PP2A/B55 inhibition. Knockdown of the B55 subunits also partially restored phosphorylation of the IRS1 and GRB2 sites in MASTL KO cells. Finally, they examined whether MASTL activity was regulated under these conditions, and showed that pT194 in the MASTL activation loop was increased during under feedback conditions, which was blocked by rapamycin or Torin inhibition of mTORC1. In vitro, both mTORC1 and S6K were shown to phosphorylate MASTL at T710, S716/T718, T722 and S878, and T710, S716/T718 and T722, respectively, although mTORC1 phosphorylations were stronger, particularly S878. S878 has a hydrophobic residue at +1, which would potentially make it a good mTORC1 site. In vitro a Mastl S864A mutant (equivalent to human MASTL S878) had slightly reduced ARPP19 phosphorylating activity, whereas an S861A mutant, at a key autophosphorylation site, had zero activity. Expression of mouse Mastl S864A or S861A failed to reduce AKT activity or GLUT4 membrane association in MASTL KO cells, suggesting that S864 is an important functional site, whose phosphorylation site could be important for mTORC1 regulation of MASTL activity and control of the negative feedback loop that attenuates insulin receptor signaling down the PI3K/AKT pathway by PP2A/B55.

Overall this is a nice story with convincing data obtained in both human cancer cell lines and mice that establishes an unexpected non-cell cycle role for MASTL/Greatwall in regulating the negative feedback pathway that damps down PI3K/AKT signaling downstream of insulin receptor activation, and indicates that the PP2A/B55 phosphatase complex has a key role in this pathway by dephosphorylating the inhibitory phosphorylation sites on IRS1 and GRB10. The finding that the MASTL/Greatwall kinase has a metabolic regulatory role in addition to its mitotic function is unexpected and important. The only weakness is the final step of the model, in which the authors implicate direct mTORC1-mediated phosphorylation and activation of MASTL as being needed to switch off the PP2A/B55 phosphatase via phosphorylation of ENSA/ARPP19. However, these studies are incomplete and further experiments are needed to establish this conclusion.

> We thank very much the reviewer for this detailed summary of the manuscript and his/her positive evaluation of the relevance of the data generated in our work. We have addressed the remaining points as discussed below.

1. Figure 1B: Neither the text nor the legend indicates which cells were used in this panel - presumably MDA-MB-231 cells. The level of MASTL protein was greatly reduced by 72 hours, but it is not clear what fraction of the cells in the induced population were

successfully deleted for MASTL. In EV1 the authors show that cell cycle distributions of parental and MASTL KO cells were similar at 72 hours, but from this analysis it is not clear whether there was an increase in mitotic cells in the MASTL KO population. How do the short term and long term proliferation profiles of the MASTL KO cells compare to those of the parental cells?

> The reviewer is right and this assay was performed in MDA-MB-231 cells. This is now indicated both in the text and in the figure legend.
>
> The percentage of mitotic cells was determined by pH3 staining and no differences were found between control and MASTL KO cells. We have previously reported that MASTL depletion impairs proliferation of MDA-MB-231 cells (*Álvarez-Fernández et al., Cell Death Differ, 2018 PMID: 29229993*). This was shown in long term colony formation assays (10-15 days after MASTL depletion) both using RNAi and CRISPR/Cas9 systems (Fig. 1c of our previous manuscript). Being aware of this, and to avoid any interference with the mitotic function of MASTL, we have performed all experiments in the current manuscript shortly after MASTL depletion, that is no longer that 72h either after shRNA infection or doxycycline addition for the CRISPR/Cas9 system. We agree this is a very relevant point and as a suggestion of reviewer #2 we have now performed a more exhaustive cell cycle analysis in a time course manner (48, 72 and 96h) in both systems, RNAi and CRISPR/Cas9. We have monitored the level of MASTL depletion by Western-blot and analysed the cell cycle phenotype by FACS analysis (DNA content and pH3 staining to detect mitotic cells). These assays have been performed in triplicate in both systems, and data are now included in the revised version of the manuscript as EV1A and EV1C for shRNA and isgRNA, respectively. Western-blot analysis and FACS plots are shown from one representative experiment and graphs reflects the mean + SEM of the three replicates with the correspondent statistical analysis. In agreement with our previous published data, no cell cycle defects and no differences in the number of mitotic cells have been observed upon shRNA-mediated knockdown even at 96h, although a good level of MASTL depletion was already observed at 72h. In the CRISPR/Cas9 inducible system, an efficient depletion was already detected 48h upon doxycycline addition. No cell cycle differences were observed neither at 48 nor 72h, but an increased accumulation of 4n and over 4n cells was detected 96h after doxycycline addition, in agreement with our previous published results. This point has also been emphasized in the revised text.

Why was the level of pT389 S6K1 increased further with glucose stimulation in the MASTL KO cells, whereas the levels of pS473 AKT and pT1462 TSC2 were not.

> This also occurs in control cells (compare lane 1 and 2) as a consequence of the feedback loop. In glucose-starved cells, a good correlation is detected between p-AKT, pTSC2 and p-S6K, and the level of these three phosphorylations is clearly increased in the absence of MASTL. Glucose re-addition triggers the feedback loop leading to reduced AKT phosphorylation and its downstream substrate TSC2 in the presence of active mTORC1 (high pS6K1), and therefore, dissociation of pS6K1 from pAKT/p-TSC2 takes place in control cells as expected. On one hand, glucose activates mTOR (high pS6K1) but further activation

of the feedback loop leads to reduced pAKT and pTSC2 to control and prevent an excessive and chronic activation of mTOR. In MASTL-depleted cells, a similar dissociation takes place but, due to the impairment in the proper establishment of the feedback loop, the reduction in pAKT and pTSC2 is much less observed (lane 3 and 4)

2. Figure 2: Here too the identity of the MASTL KO cells needs to be included in the text and legend

Agrred. The cell line used, MDA-MB-231, is now indicated both in the text and legend.

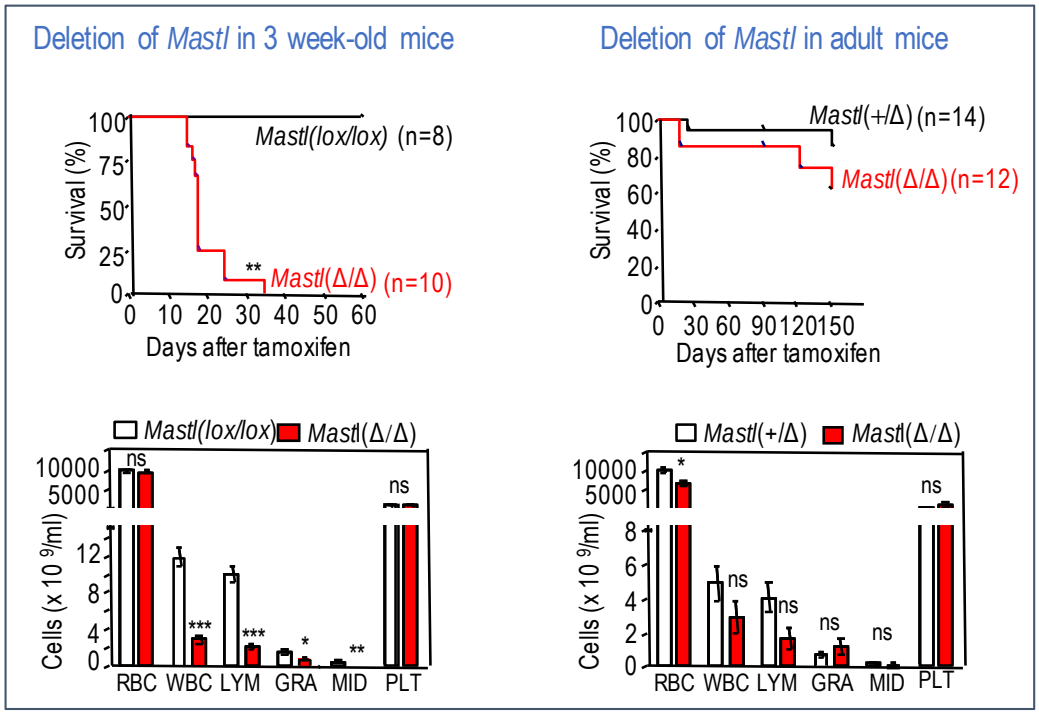
3. Figure 3A: The authors ought to demonstrate how complete the MASTL knockdown was in these shRNA treated cells, ideally by staining for MASTL in parallel.

The control immunoblot for Figure 3A, showing depletion of MASTL and increased AKT phosphorylation in MASTL-depleted cells upon insulin stimulation, was shown in Fig. EV2A in the original manuscript, and it has now been included in main Figure 3A. In addition, new control immunoblots for MASTL depletion and AKT stimulation upon glucose re-addition corresponding to Fig. EV2 have also been included in the revised version.

We have also tried to use several MASTL antibodies in immunofluorescence assays but, unfortunately, they do not work properly and we are not confident on the specificity of the detected signal.

4. Figure 4G-I: It is surprising that whole body knockout of Mastl does not result in more serious mitotic defects and growth retardation? As far as I can, tell the authors previous studies with Mastllox/lox mice, where confined to studying MEFs to generate Mastl-null cells. Since Mastl Δ/Δ mice are embryonic lethal, this raises the question of how complete the Mastl knockout was in these mice. Possibly there is some stage during embryogenesis where Mastl is essential, and that Mastl is not essential in adult mice, but the authors should establish this. They need to include Mastl protein blots or IHC staining of different tissues of interest (or carry out quantitative PCR analysis for the mutant deleted allele) from Mastl Δ/Δ mice to demonstrate to what extent Mastl protein levels were reduced upon conditional KO in the long term or what fraction of cells in key tissues have in fact lost Mastl expression. Evidence for the extent of Mastl knockout and further discussion of the phenotypes of the Mastl Δ/Δ mice is essential.

As indicated by the reviewer, deletion of *Mastl* leads to embryonic lethality (Álvarez-Fernández et al., *PNAS*, 2013, PMID: 24101512). Lethality also occurs in very young animals due to strong proliferative defects (see below). However, deletion of Mastl in adult mice did not compromise survival and did not lead to any obvious proliferative phenotype upon continuous tamoxifen treatment in diet for 6 months. As pointed by the reviewer, this suggest that Mastl is not essential in adult mice. This information is now cited and discussed in the text, and a survival curve in adult mice supporting these data is also included as EV3A.




For the assays reported in this manuscript, we have used adult mice (8-12 weeks plus 9 additional weeks on high-fat diet before applying tamoxifen). Moreover, we have performed acute tamoxifen treatment by intraperoneal injection just before the assays to ensure no interference with any potential proliferative defect. As already indicated in the text, no differences in body weight (Figure EV3B in the revised version) were observed in these conditions, neither alterations in highly proliferative tissues, such as intestine (Figure EV3C in the revised version).

To analyze the levels of Mastl protein expression in mouse tissues, we have thoroughly tested all available Mastl antibodies (including antibodies generated in our lab). Unfortunately, none of them work properly. Although they nicely recognize MASTL in human tissues, none of them give a reliable specific signal in mouse tissues, neither by immunoblotting or immunohistochemistry/immunofluorescence assays in paraffin or cryosections. As an alternative we have now analyzed Mastl mRNA levels by qPCR with two specific probes against Mastl and we have been able to confirmed a significant depletion of MASTL mRNA both in liver and muscle tissues from those mice. This set of data is now included in the manuscript as Figure EV3F and EV3G.

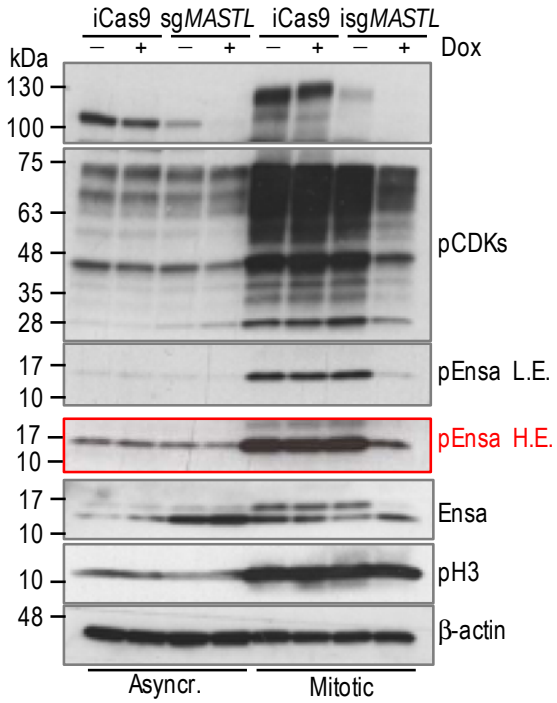
5. Figure EV3D: The increase in pT308 and pS473 AKT levels in fasted one-year old Mastl Δ/Δ mice skeletal muscle was quite variable, and in many mice does not appear to have been that strong.

We agree with the high variability usually detected in mice tissues. Nevertheless, quantification of both phospho-epitopes shows a significant increase in both phosphosites, which is more pronounced in case of pS473, in Mastl KO tissues compared to control ones.

Charts with those quantifications are now included in the new version of the figure (Figure EV3E in the revised version).

6. Figure 5: How much was ENSA/ARPP19 phosphorylation reduced in these MDA-MB-231 MASTL knockout cells?

We have tried to use the phosphor-ENSA (S67)/ ARPP19 (S62) antibody in several assays but we are not confident this antibody is sensitive and/or specific enough to detect MASTL activity in non-mitotic cells. Although in mitotic cell extracts this antibody shows an intense signal that gets strongly reduced upon MASTL depletion, it becomes much lower in interphase cells and, unfortunately, does not appear to be MASTL dependent (see high exposure (H.E.) blot added to Figure 3E from our previous manuscript, Álvarez-Fernández et al. *Cell Death and Diff*, 2018).




Adapted from Fig. 3E (Álvarez-Fernández et al., Cell Death Diff. 2018)

7. Figure 7F-H: A number of additional experiments would be required to rigorously establish a role for mTORC1 phosphorylation of MASTL in regulation of the feedback loop. Here are some possibilities. The authors need to be more explicit (perhaps by adding a table) which MASLTL phosphosites they identified in cells, and under what conditions, and which sites they identified through in vitro phosphorylation and with which kinase. With regard to which sites might be directly phosphorylated by mTORC1, the majority of the well-documented mTORC1 phosphosites are Ser/Thr.Pro sites, although one or two have a Ser.Leu motif. Did the authors check the effects of a phosphomimic S878D MASTL mutation in the reconstitution system? The ideal way to study the role of S878

phosphorylation would be to make and use MASTL S878A and S878D knock-in mutations in MDA-MB-231 cells, which would enable analysis of the consequences of S878 phosphorylation for both insulin and glucose signaling. They should also use this system to analyze other phosphorylation events downstream of the insulin receptor, as they did in Figure 1. The fact that the exogenous GFP-Mastl proteins expressed in the isgMASTL MDA-MB-231 cells appear to have been hugely overexpressed is a cause for concern. In this regard, there are no Mastl blots in panel H. Does rapamycin or Torin treatment in cells inhibit S864/878 phosphorylation in vivo? Here pS864-specific antibodies would be useful. Through what mechanism would pS864/878 phosphorylation act as a priming event for autophosphorylation of S861/875 - the authors suggest a model in Figure EV4B but did not make mutations to test this. What are the possible functions of the other phosphorylation sites identified in this region of MASTL, two of which have Thr.Pro motifs - have any mutations been made?

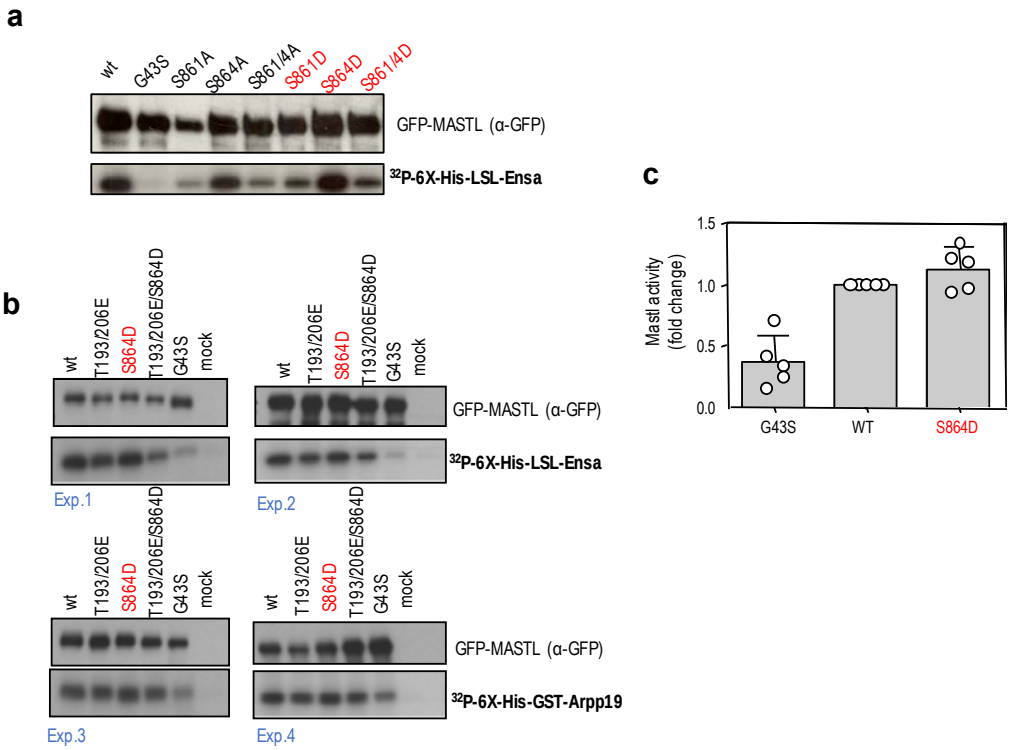
We thank the reviewer for these suggestions. We have included a new table in the revised version (table EV1) with more detailed data on the phosphoproteomic analysis to support Figures 7C and 7D. In this table, we show the normalized intensities for all phosphopeptides identified in the in vitro phosphorylation assay of recombinant GST-MASTL by mTORC1 and S6K. In this assay we identified 6 phosphorylation sites, of which four (T710, S716/T718, T722 and S878) were classified as potential mTORC1/S6K phosphosites based on their enrichment upon incubation with those kinases compared to control sample (GST-MASTL alone). Among those, we focused on S878 since it was exclusively phosphorylated by mTORC1 and to a much higher extent compared to the other identified sites (more than 100-fold). Moreover, S878 fits the mTORC1 consensus motif being a serine, which is preferred over threonine, and with a hydrophobic residue in position +1 (Khang *et al*., *Science*, 2013, PMID: 23888043 and Robitaille AM et al., *Science*, 2013, PMID:23429704). The other 3 sites are TP motifs that might also be phosphorylated by mTORC1, according to Robitaille AM *et al*. but were also found to be enriched upon phosphorylation by recombinant S6K1, and the intensities of those phosphopeptides were much lower compared to S878 (see table EV1 for details).

Moreover, three of those four phosphosites, T710 (T696 in mouse), T722 (T708 in mouse) and S878 (S864 in mouse) were also identified in vivo in mouse Mastl immunoprecipitates from G0 and G1 cells, although only S864 was induced upon serum stimulation (Figure EV4C and new table EV2). We have not performed mutations of those residues; however, phospho-mutants of the equivalent residues in *Xenopus* of T718 (T725 in *Xenopus*) and T722 (T729 in *Xenopus*) have already been reported with no significant effect on MASTL activity (*Blake-Hodek et al., Mol Cell Biol, 2012 PMID:22354989*). On the other hand, T710 has been identified as an autophosphorylation site enriched in mitosis (*Hermida D. et al*., *Mol Cell Proteomics*, 2020. PMID: 31852836). However, without properly testing those mutants in our functional assays, we cannot completely exclude their potential contribution to the regulation of MASTL in this context.

We agree with the reviewer it would be ideal to test the regulation of MASTL activity by mTORC1 and its sensitivity to mTOR inhibitors with phosphoantibodies against both the

S861/875 autophosphorylation site and the identified mTORC1 S864/S878 potential priming site but, unfortunately, they are not available at this moment.

We also agree with the reviewer that it would be ideal to also test phospho-mimicking mutations of the S864/878 site in rescue experiments. Indeed, we have generated that mutant (S864D in the mouse version) but, unfortunately, it did not show any higher activity but similar to the wild-type version of MASTL (see panels **a-c** in the figure below). Since phosphorylation of this site alone would probably be insufficient to hyperactive MASTL kinase, we also decided to combine this mutant with the phospho-mimetic mutation in the autophosphorylation site of the C-terminus (S861D in mouse) but this combined mutant showed even less activity than the wild-type MASTL. In fact, the phospho-mimicking mutation of the autophosphorylation site (S861D) alone already showed a clear reduction in kinase activity compared to Mastl wt (panel **a** below). We also tried to combine the mTOR site phospho-mimetic mutant (S864D) with phospho-mimicking mutations in the activation loop (T193E/206E, T194 /207 in human), but, unfortunately, none of these combinations led to a clear increase in the kinase activity of MASTL (panel **b** below).




How MASTL is regulated is not completely clear yet even in mitosis. The current model for MASTL activation suggests that MASTL requires first priming phosphorylation/s by other kinases in order to get autophosphorylated on its C-terminal S875 (S861 in mouse) residue (*Hermida D. et al.*, *Mol Cell Proteomics*, 2020. PMID: 31852836). We suggest here that phosphorylation of S864/878 by mTORC1 might be priming MASTL for autophosphorylation on S861/875 by facilitating its interaction with the N-terminal lobe. One option to test this

hypothesis would be to generate a combined mutant (S861D/S864A) to test whether phosphorylation in that site (S861) overcomes the requirement of the potential priming phosphorylation by mTORC1 in S878. However, the fact that the phospho-mimetic mutant in the autophosphorylation site (S861D) results in an inactive kinase precludes us from performing this assay to reach stronger conclusions.

Thank you for submitting your revised MASTL/PP2A-PI3K/Akt manuscript for our consideration. The original referees 1 and 3 have now assessed it once more, and are now fully supportive of publication. Before final acceptance, I would invite you to answer the last few specific comments of referee 3 through clarifications and possible reorganization of some presentations.

Referee #3:

The authors have addressed most of my points and the ms significantly benefits from the additional data. Before publication, I recommend that the authors address the following two remaining points:
- new Fig 1B - F
The figures and the respective quantifications are hard to interpret and this is mostly because the order of conditions in the bar graphs does not follow the order of lanes in the Western Blots.
In addition, this reviewer wonders if the biological significance of the feedback loop would not be easier to assess if all conditions would be normalized to a single condition, e.g. Ctrl KD/KO without glucose (as the authors did in the similar experiments of Fig.2). If for example there would be a 99% inactivation of Akt in the Ctrl KD and a 95% inactivation of Akt in the MASTL KD, then, according to the present way of quantification, this would mean that there is an impressive 5-fold difference in remaining Akt activity, although in reality it was almost completely inactivated in both cases.

# Answers to the Reviewers

## Referee #3:

The authors have addressed most of my points and the ms significantly benefits from the additional data. Before publication, I recommend that the authors address the following two remaining points:

- new Fig 1B - F

The figures and the respective quantifications are hard to interpret and this is mostly because the order of conditions in the bar graphs does not follow the order of lanes in the Western Blots.

In addition, this reviewer wonders if the biological significance of the feedback loop would not be easier to assess if all conditions would be normalized to a single condition, e.g. Ctrl KD/KO without glucose (as the authors did in the similar experiments of Fig.2). If for example there would be a 99% inactivation of Akt in the Ctrl KD and a 95% inactivation of Akt in the MASTL KD, then, according to the present way of quantification, this would mean that there is an impressive 5-fold difference in remaining Akt activity, although in reality it was almost completely inactivated in both cases.

We agree with the reviewer the way of representing the quantifications might be a bit confusing. We decided to normalize against control cells separately in glucose-deprived conditions and in glucose-stimulated cells to control for variability in the level of feedback activation among experiments. We have now modified the bar graphs in Figure 1 (panels D, E and F) normalizing to a single condition (control cells without glucose) and following the same order conditions as the lanes of the Western Blots. In case of Figure 1D we are only able to represent 3 independent experiments, which are those for which we had both conditions (cells without and with glucose in the same experiment) so we can normalize glucose-stimulated cells to glucose-deprived cells in the very same assay. Statistical analysis and text in figure legend have been modified accordingly.

Thank you for submitting your final revised manuscript for our consideration. I am pleased to inform you that we have now accepted it for publication in The EMBO Journal.

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**Abridged guidelines for 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.
- ideally, figure panels should include only measurements that are directly comparable to each other and obtained with the same assay.
- plots include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical
- if n<5, the individual data points from each experiment should be plotted. Any statistical test employed should be justified.
- Source Data should be included to report the data underlying figures according to the guidelines set out in the authorship guidelines on Data

**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.

**Please complete ALL of the questions below.**
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**Materials**

| Newly Created Materials | Information included in the manuscript? | In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section) |
|---|---|---|
| New materials and reagents need to be available; do any restrictions apply? | Yes | Materials and Methods |

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|---|---|---|
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**Design**

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| Experimental study design and statistics | Information included in the manuscript? | In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section) |
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| Include a statement about **sample size** estimate even if no statistical methods were used. | Yes | Material and Methods |
| Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. **randomization procedure**)? If yes, have they been described? | Yes | Material and Methods |
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| Describe **inclusion/exclusion criteria** if samples or animals were excluded from the analysis. Were the criteria pre-established?<br><br>If sample or data points were omitted from analysis, report if this was due to attrition or intentional exclusion and provide justification. | Not Applicable | |
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The MDAR framework recommends adoption of discipline-specific guidelines, established and endorsed through community initiatives. Journals have their own policy about requiring specific guidelines and recommendations to complement MDAR.

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## Data Availability

| Data availability | Information included in the manuscript? | In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section) |
|---|---|---|
| Have **primary datasets** been deposited according to the journal's guidelines (see 'Data Deposition' section) and the respective accession numbers provided in the Data Availability Section? | Yes | Data Availability Section |
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| Are **computational models** that are central and integral to a study available without restrictions in a machine-readable form? Were the relevant accession numbers or links provided? | Yes | Materials and Methods |
| If publicly available data were reused, provide the respective **data citations in the reference list**. | Yes | References |