EMBO reports

Macrophagic AMPKa1 orchestrates regenerative inflammation induced by glucocorticoids

Giorgio Caratti, Thibaut Desgeorges, Gaetan Juban, Ulrich Stifel, Aurélie Fessard, Mascha Koenen, Bozhena Caratti, Marine Theret, Carsten Skurk, Bénédicte Chazaud, Jan Tuckermann, and Rémi Mounier
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Corresponding author(s): Bénédicte Chazaud (benedicte.chazaud@inserm.fr) , Jan Tuckermann (jan.tuckermann@uni-ulm.de), Rémi Mounier (remi.mounier@univ-lyon1.fr)

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1st Editorial Decision **9th May 2022**

Dear Dr. Chazaud,

Thank you for transferring your manuscript to EMBO Reports, which was previously reviewed at The EMBO Journal.

Having read the manuscript and the referee reports, I would like to invite you to submit a revised manuscript to EMBO Reports as my colleague Daniel mentioned in his previous letter. In particular,

- Please strengthen the role of FOXO3 in AMPK mediated anti-inflammatory macrophage proliferation induced by GCs as per referee comments. Employing a macrophage specific FOXO3 knockout mouse line is not required for consideration here (referee #1, paragraph 6 of specific comments), but the in vitro findings need to be further supported as in referee reports.
- Elucidating the mechanism by which GCs induce AMPK phosphorylation is also not required, but please discuss possible mechanisms in light of earlier works (referee #1, paragraph 2 of specific comments).
- To address paragraph 3 of specific comments of referee #1, please acknowledge the shortcomings of the CTX experiment pointed out by referee #1 in the text and show some histological sections of regenerating muscles at different time points. Using an mdx mouse model is not required.

Please revise your manuscript with the understanding that the referee concerns (as in their reports) must be fully addressed and their suggestions taken on board. Please address all referee concerns in a complete point-by-point response. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

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You can submit the revision either as a Scientific Report or as a Research Article. For Scientific Reports, the revised manuscript can contain up to 5 main figures and 5 Expanded View figures. If the revision leads to a manuscript with more than 5 main figures it will be published as a Research Article. In this case the Results and Discussion section should be separate. If a Scientific Report is submitted, these sections have to be combined. This will help to shorten the manuscript text by eliminating some redundancy that is inevitable when discussing the same experiments twice. In either case, all materials and methods should be included in the main manuscript file

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1) a .docx formatted version of the manuscript text (including legends for main figures, EV figures and tables). Please make sure

that the changes are highlighted to be clearly visible.

2) individual production quality figure files as .eps, .tif, .jpg (one file per figure).

3) a .docx formatted letter INCLUDING the reviewers' reports and your detailed point-by-point responses to their comments. As part of the EMBO Press transparent editorial process, the point-by-point response is part of the Review Process File (RPF), which will be published alongside your paper. For more details on our Transparent Editorial Process, please visit our website: https://www.embopress.org/page/journal/14693178/authorguide#transparentprocess
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4) a complete author checklist, which you can download from our author guidelines (<http://embor.embopress.org/authorguide>). Please insert information in the checklist that is also reflected in the manuscript. The completed author checklist will also be part of the RPF.

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7) We would also encourage you to include the source data for figure panels that show essential data.

Numerical data should be provided as individual .xls or .csv files (including a tab describing the data). For blots or microscopy, uncropped images should be submitted (using a zip archive if multiple images need to be supplied for one panel). Additional information on source data and instruction on how to label the files are available <http://embor.embopress.org/authorguide#sourcedata>.

8) Our journal encourages inclusion of *data citations in the reference list* to directly cite datasets that were re-used and obtained from public databases. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records from which the data can be accessed. In the main text, data citations are formatted as follows: "Data ref: Smith et al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference. Further instructions are available at <http://embor.embopress.org/authorguide#datacitation>.

9) Please make sure to include a Data Availability Section before submitting your revision - if it is not applicable, make a statement that no data were deposited in a public database. Primary datasets (and computer code, where appropriate) produced in this study need to be deposited in an appropriate public database (see <http://embor.embopress.org/authorguide#dataavailability>).

Please remember to provide a reviewer password if the datasets are not yet public.

The accession numbers and database should be listed in a formal "Data Availability " section (placed after Materials & Method) that follows the model below. Please note that the Data Availability Section is restricted to new primary data that are part of this study.

Data availability

The datasets (and computer code) produced in this study are available in the following databases:

- RNA-Seq data: Gene Expression Omnibus GSE46843 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE46843)
- [data type]: [name of the resource] [accession number/identifier/doi] ([URL or identifiers.org/DATABASE:ACCESSION])

*** Note - All links should resolve to a page where the data can be accessed. ***

10) Regarding data quantification, please ensure to specify the name of the statistical test used to generate error bars and P values, the number (n) of independent experiments underlying each data point (not replicate measures of one sample), and the test used to calculate p-values in each figure legend. Discussion of statistical methodology can be reported in the materials and methods section, but figure legends should contain a basic description of n, P and the test applied.
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I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

Kind regards,

Deniz Senyilmaz Tiebe

Deniz Senyilmaz Tiebe, PhD
Editor
EMBO Reports

Referee #1:

In this study, Caratti et al. investigated the role of the AMP-activated protein kinase (AMPK) for glucocorticoid (GC)-mediated macrophage polarization towards a reparative phenotype, nicely demonstrating that AMPK is required for GC-induced macrophage polarization. The authors show that inactivation of AMPK did not blunt the canonical regulation of cytokines by GC but prevented phenotype transition, mostly via the well-known AMPK-substrate FOXO3. In vivo studies verified that AMPK is crucial for GC-stimulated resolution of inflammation in mouse models of acute lung injury and for skeletal muscle regeneration. The study convincingly demonstrates that GC-induced polarization of macrophages towards a reparative phenotype depends on AMPK and FOXO3, which is critical for resolution of inflammation and successful tissue repair.

The study is carefully done using appropriate techniques and controls. However, the gain of knowledge is moderate, since several previous studies already described a critical of AMPK for macrophage polarization (e.g. Sag et al., The Journal of Immunology, 2008, 181: 8633-8641) or reported that inhibition of AMPK enhances severity of acute lung injury (Park et al., Am J Physiol Lung Cell Mol Physiol 307: L735-L745, 2014 (not cited). Furthermore, it has already been demonstrated that AMK mediates GC-induced metabolic changes in some but not all cell types (Christ-Crain et al., FASEB J. 22, 1672-1683, 2008). Important mechanistic questions, i.e. how GCs activate AMPK in certain cell types (macrophages, hepatocytes) but repress AMPK in others (adipocytes, cardiomyocytes) were not addressed. It also remains unclear whether and how GR and the AMPK-target FOXO3 cooperate to regulate gene expression.

Specific comments

It remains completely unclear how GCs induce phosphorylation of AMPK at T172 and thereby activation in some cell types such as macrophages and liver cells and but not in others such as adipocytes, where GCs inhibit AMPK activity leading to increased lipogenesis and fat storage. In my opinion it is critical to unravel the mechanisms of GC-mediated activation of AMPK in macrophages.

The authors followed up a previous study, reporting that AMPK activation induces GR phosphorylation and activation via p38. The authors found that inactivation of AMPK diminished phosphorylation of GR at S211 but GR phosphorylation did not depend on p38 as reported before. How does AMPK lead to GR phosphorylation if not by p38? Is the AMPK-dependent phosphorylation of GR at S211 not relevant for canonical regulation of cytokines by GCs? Phosphorylation of GR at S211 has been shown to determine the magnitude of repression by GR. Is that not relevant for suppression of inflammatory cytokines?

The effects of dexamethasone treatment of acutely injured skeletal muscle are pretty weak, which makes me wonder whether CTX-induced acute muscle injury is an appropriate model to explore effects of GC-dependent AMPK activation on macrophage polarization. As shown in Fig. 2C muscle mass after regeneration is not affected by dexamethasone treatment and the changes in eMHC expression are moderate. The authors measured increased phagocytotic activity in vitro after dexamethasone treatment as an indicator of better resolution of

inflammation but this hardly seems to matter in vivo. It would be helpful to show some histological sections of regenerating muscles at different time points to validate differences in the resolution of inflammation after dexamethasone treatment (and its absence in AMPK mutants). Obviously, the study would gain a lot by analyzing a condition, in which GC-treatment is regularly used, i.e. mdx mice as a model for DMD. In contrast, the impact of dexamethasone treatment on acute lung injury is much more convincing.

The study shows that inactivation of AMPK prevents up-regulation of FOXO3-dependent genes upon GC-treatment. This finding corresponds to reduced up-regulation of AMPK-dependent genes after Foxo3-inactivation upon GC-treatment. However, the approach chosen to disentangle and display the different relationships is rather opaque. The authors should show a Venn diagram that depicts AMPK- and FOXO3-dependent genes (i.e. genes that fail to be upregulated in AMPK- and Foxo3-deficient macrophages upon GC-stimulation in comparison to WT cells) and overlaps the different groups. Such analysis should clearly indicate whether all or only a subset of AMPK-dependent genes requires FOXO3 and vice versa. The diagram shown in Fig. 4C does not serve such a purpose.

The mechanism by which AMPK activates FOXO3 was not analyzed in detail. Previous studies suggest that AMPK directly phosphorylates FOXO3 and increases transcriptional activity of FOXO3 without affecting the subcellular localization of FOXO3, which is the more common means to regulate activity of FOXO-proteins. The authors should analyze the phosphorylation status and the subcellular localization of FOXO3 in AMPK-mutant macrophages.

The role of FOXO3 in GC/AMPK-mediated macrophage polarization was only analyzed in vitro using Foxo3-mutant macrophages, indicating that AMPK-dependent genes have a strongly reduced response to GCs when Foxo3 is absent. The data would be more compelling when these findings are validated in vivo. Do mice in which Foxo3 was inactivated in macrophages show a similar phenotype (attenuated macrophage polarization and therefore attenuated regeneration upon GC treatment) as AMPK-mutants?

Based on differences in expression levels the authors assumed a common regulatory mechanism of AMPK and FOXO3 on GR actions. To explore this possibility ChIP-PCR experiments were performed to analyze loading of FOXO3 and GR on 4 genes, depending on the presence or absence of AMPK. FOXO3 and GR were recruited to the same genes and inactivation of AMPK reduced loading of both FOXO3 and GR. Based on these findings, the authors speculate that GR and FOXO3 may co-operate for DNA binding. There are several problems related to this experiment, which need to be addressed: (i) selection of only 4 genes for the analysis is not very telling. The authors should perform a genome-wide ChIPseq analysis for GR and FOXO3. (ii) AMPK is known to activate the GR, which was also investigated in this study (Fig. 1, diminished GR phosphorylation after AMPK inactivation). Thus, AMPK does not only affect FOXO3 but also the GR itself, meaning that no conclusion can be drawn about a potential cooperation of GR and FOXO3 for DNA binding, since both components are affected by AMPK. (iii) Expression of a constitutively active FOXO3 might bypass the effects of AMPK on FOXO3, which should allow valid conclusion about a potential cooperativity between GR and FOXO3.

The authors used an ATK inhibitor to indirectly activate FOXO3 in AMPK-deficient

macrophages. Restored GC-sensitivity was only assessed by measuring iNOS and CD206 expression. I do not understand why only NOS and CD206 expression was analyzed. It should be rather straightforward to do a RNAseq experiment, which will provide a much better view to what degree alternative activation of FOXO3 restores GC-sensitivity of AMPK-deficient macrophages. I also do not understand, why only an ATR-inhibitor was used, which causes several side effects. Expression of a constitutively active FOXO3 should be simple and is more specific.

The authors claim in the abstract that canonical cytokine regulation by GCs is not affected by the loss of AMPK. Although this most likely true, the analysis is somewhat sketchy. In Fig. 3E it is shown that dexamethasone fails to suppress CCL3 expression in AMPK-mutant macrophages. Isn't CCL3 an inflammatory cytokine? In contrast, supplemental Figure 3 shows that several cytokines are still normally suppressed by GCs even in the absence of AMPK but expression of GM-CSF increases upon LPS treatment after AMPK inactivation. What is the reason for that? Are ALL GC-suppressed inflammatory cytokines still suppressed when AMPK is absent?

The authors make some pretty strong statements, e.g. 'Whether and how GCs interfere with a metabolic sensor such as AMPK is completely unclear', which do not seem justified given the published literature.

Scale bars are missing in Fig. 1G, H; Fig. 3A.

Referee #2:

The manuscript by Caratti et al describes the role of AMPK in the restorative macrophage phenotype induced by glucorticoids. The authors use both post-injury muscle regeneration and acute lung injery to query the requirement for AMPK in macrophage activation and restoration. Overall, defining the role of AMPK in glucocorticoid activity is novel and interesting. This paper should be considered for publication if the following concerns and questions are addressed, and appropriate revisions made.

1) With the limited effect on P-ACC, additional AMPK targets should be evaluated with Dex-treatment to compare with 991 treatment.

2) In figure 2C, Dex treated was shown to decrease muscle mass in LysM-α1-/- and the conclusion was that Dex promotes muscle regeneration in a AMPKa1-dependent manner. However, this would imply that Dex has an effect in an AMPK-independent manner as well. A better description of these results is needed.

3) Is Foxo phosphorylation or translocation impacted by Dex-treatment?

4) Inhibition of FOXO3 signaling by MK2206 needs to be verified.

5) What percentage of FOXO3 dependent genes are also AMPK dependent? A more detailed comparison is needed.

6) What is the importance of Phosphorylation of GR? How is the phosphorylation of GR by AMPK integrated into the final model?

7) Do GR and Foxo3 directly interact? Are there any changes in interaction between GR and Foxo3 upon phosphorylation? Does P-GR change with Foxo3 inhibition?

8) Can direct AMPK activation mimic any effects of glucocorticoid treatment? Does AMPK activation promote macrophage restoration?

9) Figure 1D needs to be repeated as it appears there are blotting issues that may impact the quantification.

Minor Comments:

10) Nomenclature for AMPK-/- samples should be consistent (ie LysMα1 vs AMPK-/- vs LysM-α1-/-)

11) In Supplemental Figure 7, the order of the tracks should match the listed order in the figure legend.

Editor's comments

- Please strengthen the role of FOXO3 in AMPK mediated anti-inflammatory macrophage proliferation induced by GCs as per referee comments. Employing a macrophage specific FOXO3 knockout mouse line is not required for consideration here (referee #1, paragraph 6 of specific comments), but **the in vitro findings need to be further supported as in referee reports**.

We appreciate that you omitted the request for generating a new mouse line, such as LysM-Cre;Foxo3flox, which would have not been possible in a reasonable time frame. We did intensify our work concerning the activity of Foxo3 and the anti-inflammatory response in Foxo3 deficient macrophages by the following experiments:

1. *We determined the expression of inflammatory mediators in Foxo3a deficient primary macrophages (new Fig. 4H)*
2. *We determined nuclear localization of Foxo3 in AMPK knockout macrophages*
3. *We assessed the phosphorylation status of Foxo3 in AMPK mutant macrophages (New Figure 4F)*
4. *We measured Foxo3 activity in response to MK2206 (New Figure EV5E)*

- Elucidating the mechanism by which GCs induce AMPK phosphorylation is also not required, but please **discuss possible mechanisms in light of earlier works** (referee #1, paragraph 2 of specific comments).

According to the request we added the following section to the discussion, lines 679-94:
In our conditions, we found that GCs activate AMPK as efficiently as the potent and allosteric AMPK activator 991 in macrophages, indicating a previously unknown role of GCs in stimulating AMPK signaling. We found further that GR phosphorylation at Ser 211, a strong indicator of transcriptional activity (Wang et al, 2002), was found decreased. This was likely independent from p38 activation, but in agreement with elevated GR-S211 phosphorylation in the presence of phosphorylated AMPK in hepatocytes (Ratman et al., 2016). The activation of S211 phosphorylation is still poorly understood. Many different kinases were described to be involved in different cell types, such as JNKs, CDKs, Erks, Akt and GSK3 (Faus & Haendler, 2006). Recently, activity of protein phosphatase 1 alpha (PP1a) was associated with enhanced S211 phosphorylation in A549 cells, involving possibly GSK3-beta (Patt et al, 2020). To which other kinases and phosphatases are involved in GR-S211 phosphorylation dependent on AMPK is of interest to investigate in the future. However, we could not find in macrophages a decreased suppression of TNFα and other cytokines, nor a reduced induction of classical bona fide GR upregulated genes (canonical genes), such as Dusp1 and Gilz. We can only speculate that the residual Ser211 phosphorylation might be sufficient for the induction of these genes, while other GR target genes involved in phagocytosis are more vulnerable to the absence of AMPK1. Whether this can be directly linked to reduced Ser211 phosphorylation requires further investigations. Thus, cell type specific manners of GR-AMPK cross-talk exist. Here, we defined this cross-talk for macrophages and its implications for the anti-inflammatory actions of GCs.

- To address paragraph 3 of specific comments of referee #1, please acknowledge the shortcomings of the CTX experiment pointed out by referee #1 in the text and show some

INSTITUT NEUROMYOGENE
Université Claude Bernard Lyon 1 - CNRS UMR 5310 - INSERM U1217
8 Avenue Rockfeller - 69008 Lyon – France
Phone : (33) 4 26 68 82 46
remi.mounier@univ-lyon1.fr
http://www.musclestem.com
http://www.inmg.fr

histological sections of regenerating muscles at different time points. Using an mdx mouse model is not required.

The CTX model has been used by several laboratories as a standard model for investigating the resolution of inflammation in tissue repair. A difficult aspect of addressing the impact of GCs is their detrimental effects on myofiber homeostasis. We had to set up an experimental design to avoid GC impact on myofibers (described in Fig.EV1) that includes a single injection of GCs at the time of the resolution of inflammation. This of course limits the impact of GC action, if compared with several days of treatments.

To answer the reviewer's comment, we chose to analyze the resolution of inflammation by flow cytometry in vivo, which is more accurate than histological analyses (New Figure 2D, E). Indeed, at those early time points (days 2 and 4 post-injury), the cellularity of the muscle is very high and immunolabeling are hardly quantifiable. We then performed additional experiments demonstrating the impairment of polarization of macrophages in situ in LysMα1-/- upon GC treatment (Fig.2E, lines 515-18). Subsequently, later time points are presented on histological sections in Fig.2A to show the outcome on myofiber maturation. Thus, we cover in our analysis early and late time points of the inflammatory and healing response of the injury accordingly.

Referee #1

In this study, Caratti et al. investigated the role of the AMP-activated protein kinase (AMPK) for glucocorticoid (GC)-mediated macrophage polarization towards a reparative phenotype, nicely demonstrating that AMPK is required for GC-induced macrophage polarization. The authors show that inactivation of AMPK did not blunt the canonical regulation of cytokines by GC but prevented phenotype transition, mostly via the well-known AMPK-substrate FOXO3. In vivo studies verified that AMPK is crucial for GC-stimulated resolution of inflammation in mouse models of acute lung injury and for skeletal muscle regeneration. The study convincingly demonstrates that GC-induced polarization of macrophages towards a reparative phenotype depends on AMPK and FOXO3, which is critical for resolution of inflammation and successful tissue repair.

The study is carefully done using appropriate techniques and controls. However, the gain of knowledge is moderate, since several previous studies already described a critical of AMPK for macrophage polarization (e.g. Sag et al., The Journal of Immunology, 2008, 181: 8633-8641) or reported that inhibition of AMPK enhances severity of acute lung injury (Park et al., Am J Physiol Lung Cell Mol Physiol 307: L735-L745, 2014 (not cited). Furthermore, it has already been demonstrated that AMK mediates GC-induced metabolic changes in some but not all cell types (Christ-Crain et al., FASEB J. 22, 1672-1683, 2008). Important mechanistic questions, i.e. how GCs activate AMPK in certain cell types (macrophages, hepatocytes) but repress AMPK in others (adipocytes, cardiomyocytes) were not addressed. It also remains unclear whether and how GR and the AMPK-target FOXO3 cooperate to regulate gene expression.

We appreciate the evaluation of the reviewer about our study. To our knowledge, the role of AMPK in glucocorticoid responses in macrophages has not been investigated previously. Despite the previous findings that AMPK and GCs modulate inflammation in different paradigms a direct link has not been concluded in immune cells and goes beyond the previous knowledge of AMPK involvement in GC-induced in metabolic changes, e.g. in the liver. We agree that the question

why we have concomitant action of GR and AMPK in some cell types and opposite actions in other cell types is thrilling, but to address this, we would need to set up a complete new study that goes beyond the manuscript here, requiring many different mouse strains.
We addressed, as outlined above and below, the activity of Foxo3a in the presence and absence of AMPK.

Specific comments

§1 It remains completely unclear how GCs induce phosphorylation of AMPK at T172 and thereby activation in some cell types such as macrophages and liver cells and but not in others such as adipocytes, where GCs inhibit AMPK activity leading to increased lipogenesis and fat storage. In my opinion it is critical to unravel the mechanisms of GC-mediated activation of AMPK in macrophages.

Deciphering the molecular mechanisms leading to AMPK activation by GCs is of an utmost interest but a high challenge. In macrophages, our study show that GCs and AMPK signaling pathways cooperate in the regulation of a series of genes, mediated by Foxo3. Nevertheless, a part of GC action is also independent from AMPK signaling in macrophages. The new experiments in the revised version provide insights on the molecular mechanisms of AMPK action. They notably include the evidence that Foxo3 phosphorylation requires AMPK upon Dex treatment and that Foxo3 is required for the acquisition of an anti-inflammatory phenotype of macrophages in response to Dex (Fig.4F, Fig.4H). We also introduced a section about the complexity of these mechanisms in the discussion section (lines 679-94).

§2 The authors followed up a previous study, reporting that AMPK activation induces GR phosphorylation and activation via p38. The authors found that inactivation of AMPK diminished phosphorylation of GR at S211 but GR phosphorylation did not depend on p38 as reported before. How does AMPK lead to GR phosphorylation if not by p38? Is the AMPK-dependent phosphorylation of GR at S211 not relevant for canonical regulation of cytokines by GCs? Phosphorylation of GR at S211 has been shown to determine the magnitude of repression by GR. Is that not relevant for suppression of inflammatory cytokines?

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The CTX model has been used by several laboratories as a standard model for investigating the resolution of inflammation in tissue repair. While we agree that Mdx mice is a valuable model worthwhile to address as a disease model, this is more suitable for a follow up study, since we have to introduce this mouse strain and this is not feasible in the revision time. Here, we aimed to decipher the effect of GCs in a standard model of muscle injury. Indeed, a difficult aspect of addressing the impact of GCs is their detrimental effects on myofiber homeostasis. We had to set up an experimental design to avoid GC impact on myofibers (described in Fig.EV1) that includes a single injection of GCs at the time of the resolution of inflammation. This of course limits the impact of GC action, if compared with several days of treatments.

To answer the reviewer's comment, we chose to analyze the resolution of inflammation by flow cytometry in vivo, which is more accurate than histological analyses. Indeed, at those early time points (days 2 and 4 post-injury) (Fig. 2E), the cellularity of the muscle is very high and immunolabeling are hardly quantifiable. We then performed additional experiments demonstrating the impairment of polarization of macrophages in situ in LysMα1-/- upon GC treatment (Fig.2E, lines 515-18). Then, later time points are presented on histological sections in Fig.2A to show the outcome on myofiber maturation. Together with the demonstration that ex vivo phagocytosis of macrophages is not rescued by GCs in AMPK deficient macrophages (Fig.2D), our analysis covers early and late time points of the inflammatory and healing response of the injury accordingly.

§4 The study shows that inactivation of AMPK prevents up-regulation of FOXO3-dependent genes upon GC-treatment. This finding corresponds to reduced up-regulation of AMPK-dependent genes after Foxo3-inactivation upon GC-treatment. However, the approach chosen to disentangle and display the different relationships is rather opaque. The authors should show a

INSTITUT NEUROMYOGENE
Université Claude Bernard Lyon 1 - CNRS UMR 5310 - INSERM U1217
8 Avenue Rockfeller - 69008 Lyon – France
Phone : (33) 4 26 68 82 46
remi.mounier@univ-lyon1.fr
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Venn diagram that depicts AMPK- and FOXO3-dependent genes (i.e. genes that fail to be upregulated in AMPK- and Foxo3-deficient macrophages upon GC-stimulation in comparison to WT cells) and overlaps the different groups. Such analysis should clearly indicate whether all or only a subset of AMPK-dependent genes requires FOXO3 and vice versa. The diagram shown in Fig. 4C does not serve such a purpose.
We have included the Venn diagram in Fig.EV5B to allow the discrimination of AMPK dependent fraction of differential expressed genes in FOXO3 knockout macrophages.

§5 The mechanism by which AMPK activates FOXO3 was not analyzed in detail. Previous studies suggest that AMPK directly phosphorylates FOXO3 and increases transcriptional activity of FOXO3 without affecting the subcellular localization of FOXO3, which is the more common means to regulate activity of FOXO-proteins. The authors should analyze the phosphorylation status and the subcellular localization of FOXO3 in AMPK-mutant macrophages.
As requested by the reviewer, we performed those experiments. We observed that the localization of Foxo3 did not change in macrophages upon GC treatment in AMPK KO mice (Fig.EV5D). To evidence Foxo3 phosphorylation upon GC treatment, there is no suitable antibody for specific Foxo3 phosphorylation sites in mouse available. To overcome this obstacle, we pulled down all serine phosphorylated proteins and ran immunoblot against Foxo3. The results clearly indicate an increase of Foxo3 phosphorylation upon GC treatment, that does not occur in AMPK KO macrophages (Fig.4F). We are convinced that this last experiment demonstrates the need of AMPK for a GC dependent Foxo3 activation (lines 628-30).

§6 The role of FOXO3 in GC/AMPK-mediated macrophage polarization was only analyzed in vitro using Foxo3-mutant macrophages, indicating that AMPK-dependent genes have a strongly reduced response to GCs when Foxo3 is absent. The data would be more compelling when these findings are validated in vivo. Do mice in which Foxo3 was inactivated in macrophages show a similar phenotype (attenuated macrophage polarization and therefore attenuated regeneration upon GC treatment) as AMPK-mutants?
We agree that this is an important experiment. As the editor pointed out, generating a new mouse line such as LysM-Cre;Foxo3flox would take a too long time to be feasible in a revision process. Nevertheless, to answer the reviewer's comment, we performed analysis of Foxo3 BMDMs and showed that they hardly acquire the anti-inflammatory phenotype upon Dex treatment (Fig.4H, lines 633-34).

§7 Based on differences in expression levels the authors assumed a common regulatory mechanism of AMPK and FOXO3 on GR actions. To explore this possibility ChIP-PCR experiments were performed to analyze loading of FOXO3 and GR on 4 genes, depending on the presence or absence of AMPK. FOXO3 and GR were recruited to the same genes and inactivation of AMPK reduced loading of both FOXO3 and GR. Based on these findings, the authors speculate that GR and FOXO3 may co-operate for DNA binding. There are several problems related to this experiment, which need to be addressed: (i) selection of only 4 genes for the analysis is not very telling. The authors should perform a genome-wide ChIPseq analysis for GR and FOXO3. (ii) AMPK is known to activate the GR, which was also investigated in this study

INSTITUT NEUROMYOGENE
Université Claude Bernard Lyon 1 - CNRS UMR 5310 - INSERM U1217
8 Avenue Rockfeller - 69008 Lyon – France
Phone : (33) 4 26 68 82 46
remi.mounier@univ-lyon1.fr
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(Fig. 1, diminished GR phosphorylation after AMPK inactivation). Thus, AMPK does not only affect FOXO3 but also the GR itself, meaning that no conclusion can be drawn about a potential cooperation of GR and FOXO3 for DNA binding, since both components are affected by AMPK. (iii) Expression of a constitutively active FOXO3 might bypass the effects of AMPK on FOXO3, which should allow valid conclusion about a potential cooperativity between GR and FOXO3.

According to point (i) we do agree with the reviewer that the complexity of the molecular process between AMPK; GR and Foxo3 are not all unraveled by the experiments performed in our study. To assess how general Foxo3 and GR cooperation in the dependence of AMPK, the proposed ChIP Seq assays would be suitable. However, they would likely not provide more information about the mechanism itself on the cooperation between the two molecules. Given the effort to establish this double ChIP Seq experiment on a limited amount of available cells we did therefore not perform a ChIP Seq experiment for this study.

We addressed point (ii) by new experiments showing that Foxo3 phosphorylation is activated by GC treatment and is blunted in AMPK KO macrophages (Fig.4F), confirming the functional link between the 3 molecules.

Question (iii) was not addressable because, despite several attempts using 4 different approaches, BMDM (or THP1 cell line) we could not ensure transfection in these cell types during the revision period.

§8 The authors used an ATK inhibitor to indirectly activate FOXO3 in AMPK-deficient macrophages. Restored GC-sensitivity was only assessed by measuring iNOS and CD206 expression. I do not understand why only NOS and CD206 expression was analyzed. It should be rather straightforward to do a RNAseq experiment, which will provide a much better view to what degree alternative activation of FOXO3 restores GC-sensitivity of AMPK-deficient macrophages. I also do not understand, why only an ATR-inhibitor was used, which causes several side effects. Expression of a constitutively active FOXO3 should be simple and is more specific.

iNOS and CD206 are robust markers of the status of macrophages in vitro. As the reviewer can see, and as we showed previously (Mounier et al 2013 Cell Metab), the expression of inflammatory markers by macrophages, even in vitro, does not exhibit an on/off pattern. Indeed, at least 40% of the cells express all the markers in any condition. Moreover, we showed before that mRNA levels do not follow the protein level in BMDMs, as it is the case for several immune cells (that may sequester mRNAs for specific and rapid answer to stimuli) (Mounier et al 2013). We therefore think that immunolabeling for the markers is a better readout for determining the inflammatory status of macrophages. As mentioned below, despites several attempts, we did not succeed in transfecting macrophages, precluding the implementation of experiments using a CA-Foxo3 plasmid. Nevertheless, we showed that in the conditions tested using MK2206, the expression of two target genes of Foxo3 were upregulated, indicative of its activation (Fig.EV5E).

§9 The authors claim in the abstract that canonical cytokine regulation by GCs is not affected by the loss of AMPK. Although this most likely true, the analysis is somewhat sketchy. In Fig. 3E it is shown that dexamethasone fails to suppress CCL3 expression in AMPK-mutant macrophages. Isn't CCL3 an inflammatory cytokine? In contrast, supplemental Figure 3 shows that several cytokines are still normally suppressed by GCs even in the absence of AMPK but expression of

GM-CSF increases upon LPS treatment after AMPK inactivation. What is the reason for that? Are ALL GC-suppressed inflammatory cytokines still suppressed when AMPK is absent?
We thank the reviewer for raising this point and we now address in the discussion the question of the complexity of the regulation of the inflammatory response. Of course, the inflammatory response is a tightly regulated process, in terms of kinetics and amplitude and of nature, according to the immune cell and biological context considered. To achieve such a specific regulation, all inflammatory genes are not controlled similarly at the genomic levels, and require different transcription factors, or different combinations of transcription factors with accessory proteins, as well as different affinities for these protein complexes at their promoters or enhancers. Our study reveals a part of this complexity by showing a crosstalk between two signaling pathways, recruiting a third partner, Foxo3 for the regulation of some, but not all, inflammatory genes. Unraveling at the genomic level the distribution of genes dependent or not of this crosstalk is of course an exciting question but we do believe it is beyond the scope of the present study (discussion lines 679-94).

§10 The authors make some pretty strong statements, e.g. 'Whether and how GCs interfere with a metabolic sensor such as AMPK is completely unclear', which do not seem justified given the published literature.
We agree with the reviewer statement and dampened our statements (line 93).

Scale bars are missing in Fig. 1G, H; Fig. 3A.
We added the scale bars.

Referee #2

The manuscript by Caratti et al describes the role of AMPK in the restorative macrophage phenotype induced by glucorticoids. The authors use both post-injury muscle regeneration and acute lung injury to query the requirement for AMPK in macrophage activation and restoration. Overall, defining the role of AMPK in glucocorticoid activity is novel and interesting. This paper should be considered for publication if the following concerns and questions are addressed, and appropriate revisions made.

1) With the limited effect on P-ACC, additional AMPK targets should be evaluated with Dex-treatment to compare with 991 treatment.
After 991 treatment, the effects on Phospho-ACC were repeatedly observed in 6 biological independent experiments that all gave clear results. Of note, cells have been treated only 1 h at 1 µM before the WB analysis. This treatment was sufficient to have a robust increase of P-AMPK, but might be too short for phosphorylation of its targets in murine primary macrophages. Ahwazi et al., 2021 (Ahwazi et al. Biochem J. 2021 478:2977) have shown a more pronounced effect on 991 on P-ACC after treatment of cells (primary hepatocytes, C2C12 myotubes and U2OS) with a dose 10-fold higher (10 µM). Our explanation is reinforced since we found, in these conditions, a limited effect of 991 on an additional AMPK target, RAPTOR (data not shown).

INSTITUT NEUROMYOGENE
Université Claude Bernard Lyon 1 - CNRS UMR 5310 - INSERM U1217
8 Avenue Rockfeller - 69008 Lyon – France
Phone : (33) 4 26 68 82 46
remi.mounier@univ-lyon1.fr
http://www.musclestem.com
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2) In figure 2C, Dex treated was shown to decrease muscle mass in LysM-α1-/- and the conclusion was that Dex promotes muscle regeneration in a AMPKa1-dependent manner. However, this would imply that Dex has an effect in an AMPK-independent manner as well. A better description of these results is needed.
We apologize for the confusion and we rephrased the description of these results (lines 500-501).

3) Is Foxo phosphorylation or translocation impacted by Dex-treatment?
As requested by the reviewer, we performed those experiments. We observed that the localization of Foxo3 did not change in macrophages upon GC treatment in AMPK KO mice (Fig.EV5D). To show alterations of Foxo3 phosphorylation upon GC treatment, phospho-specific antibodies against Foxo3 for mouse were not available. We overcame this issue by pulling down all serine phosphorylated proteins and subsequently ran an immunoblot against Foxo3. The results clearly indicate an increase of Foxo3 phosphorylation upon GC treatment, that does not occur in AMPK KO macrophages (Fig.4F). This experiment demonstrates the need of AMPK for a GC dependent Foxo3 activation (lines 626-30).

4) Inhibition of FOXO3 signaling by MK2206 needs to be verified.
This experiment was performed by the demonstration that the expression of 2 targets genes of Foxo3 was upregulated in the presence of MK2206, indicative of its activation (Fig.EV5E).

5) What percentage of FOXO3 dependent genes are also AMPK dependent? A more detailed comparison is needed.
We added a Venn diagram of the results in Fig.EV5B to show the differentially expressed genes AMPK dependent genes in Foxo3 deficient cells (11.4%).

6) What is the importance of Phosphorylation of GR? How is the phosphorylation of GR by AMPK integrated into the final model?
Please see our answer to point n° 7 below.

7) Do GR and Foxo3 directly interact? Are there any changes in interaction between GR and Foxo3 upon phosphorylation? Does P-GR change with Foxo3 inhibition?
Despite we cannot exclude it, we believe it is very unlikely that Foxo3a inhibition affects GR phosphorylation. Due to the limited material from Foxo3a deficient macrophages available, we skipped this experiment in favor to the assessment of the anti-inflammatory phenotype of these cells. A direct interaction of GR and Foxo3 is possible, but needs establishment of ChIP on ChIP that was not feasible within the revision period available. We addressed however, as stated above, the phosphorylation of Foxo3 in dependence of AMPK (Fig.4F).
Given the complexity of the system, a section was added to the discussion, lines 679-94, as follows:
In our conditions, we found that GCs activate AMPK as efficiently as the potent and allosteric AMPK activator 991 in macrophages, indicating a previously unknown role of GCs in stimulating AMPK signaling. We found further that GR phosphorylation at Ser 211, a strong indicator of transcriptional activity (Wang et al, 2002), was found decreased. This was likely independent

INSTITUT NEUROMYOGENE
Université Claude Bernard Lyon 1 - CNRS UMR 5310 - INSERM U1217
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from p38 activation, but in agreement with elevated GR-S211 phosphorylation in the presence of phosphorylated AMPK in hepatocytes (Ratman et al., 2016). The activation of S211 phosphorylation is still poorly understood. Many different kinases were described to be involved in different cell types, such as JNKs, CDKs, Erks, Akt and GSK3 (Faus & Haendler, 2006). Recently, activity of protein phosphatase 1 alpha (PP1a) was associated with enhanced S211 phosphorylation in A549 cells, involving possibly GSK3-beta (Patt et al, 2020). To which other kinases and phosphatases are involved in GR-S211 phosphorylation dependent on AMPK is of interest to investigate in the future. However, we could not find in macrophages a decreased suppression of TNFα and other cytokines, nor a reduced induction of classical bona fide GR upregulated genes (canonical genes), such as Dusp1 and Gilz. We can only speculate that the residual Ser211 phosphorylation might be sufficient for the induction of these genes, while other GR target genes involved in phagocytosis are more vulnerable to the absence of AMPK1. Whether this can be directly linked to reduced Ser211 phosphorylation requires further investigations. Thus, cell type specific manners of GR-AMPK cross-talk exist. Here, we defined this cross-talk for macrophages and its implications for the anti-inflammatory actions of GCs.

8) Can direct AMPK activation mimic any effects of glucocorticoid treatment? Does AMPK activation promote macrophage restoration?
Indeed, in a previous study (Mounier et al., Cell Metab 2013), we have shown that AMPK activation is required in macrophages for the resolution of inflammation.

9) Figure 1D needs to be repeated as it appears there are blotting issues that may impact the quantification.
While we agree that the loading based on the Actin slightly differs, the picture is shown as an indication and the experiment was repeated in 3 biological independent experiments that all gave clear results when normalized to loading.

Minor Comments:

10) Nomenclature for AMPK-/- samples should be consistent (ie LysMα1 vs AMPK-/- vs LysM-α1-/-)
LysMα1 was modified for LysM-α1 and refers to the mice in which AMPα1K is depleted only in macrophages. AMPK$\alpha1^{-/-}$ refers to total KO animal or cells.

11) In Supplemental Figure 7, the order of the tracks should match the listed order in the figure legend.
This was modified accordingly.

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Dear Dr. Chazaud,

Thank you for the submission of your revised manuscript to our editorial offices. I have now received the reports from the two referees that I asked to re-evaluate your study, you will find below. As you will see, both referees think that the study is now suitable for publication in EMBO reports. Referee #1 has some remaining points and suggestions to improve the study I ask you to address in a final revised manuscript. Please also provide a final detailed p-b-p-response addressing all the remaining referee comments.

Moreover, I have these editorial requests I ask you to address:

- I would suggest to slightly shorten the title:
Macrophagic AMPKa1 orchestrates regenerative inflammation induced by glucocorticoids

- Please provide the abstract written in present tense throughout and with not more than 175 words.

- Please add up to 5 keywords to the title page below the abstract.

- Please remove the authors' ORCID IDs from the cover page of the manuscript text file. These will be automatically linked online to the author names.

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- Please make sure that all figure panels are called out separately and sequentially. Presently, there seems to be no callout for Fig. 3D. Please check.

- Please remove the referee tokens form the data availability section and make sure the datasets are public latest upon publication of the manuscript.

- Please name the two tables with primer information Table 1 and Table 2, put this as title and a legend, and also call these tables out accordingly in the methods section.

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- Thanks for providing the source data (SD). Please upload this as one pdf file (pptx files are not acceptable) per figure or as one folder with SD files for one figure ZIPed together (main and EV figures).

- Finally, please find attached a word file of the manuscript text (provided by our publisher) with changes we ask you to include in your final manuscript text. Please use the attached file as basis for further revisions and provide your final manuscript file with track changes, in order that we can see any modifications done.

In addition, I would need from you:
- a short, two-sentence summary of the manuscript (not more than 35 words).
- two to four short bullet points highlighting the key findings of your study (two lines each).
- a schematic summary figure (in jpeg or tiff format with the exact width of 550 pixels and a height of not more than 400 pixels) that can be used as a visual synopsis on our website.

I look forward to seeing the final revised version of your manuscript when it is ready. Please let me know if you have questions regarding the revision.

Please use this link to submit your revision: https://embor.msubmit.net/cgi-bin/main.plex

Achim Breiling
Senior Editor
EMBO Reports

Referee #1:

Caratti et al. have submitted a revised version of a manuscript that was previously reviewed for EMBO J. The authors investigated the role of the AMP-activated protein kinase (AMPK) for glucocorticoid (GC)-mediated macrophage polarization, demonstrating that AMPK is required for GC-induced macrophage polarization.

In my initial review, I made critical comments that the reasons for differences between cell types regarding GC-induced metabolic changes via AMK remain unsolved. The authors argue that addressing this problem will require disproportional efforts. I do not completely agree with the reasoning but I accept that such studies can be considered as outside of the scope of the current study.

Another critical point were the rather moderate effects of GC-dependent AMPK activation during acute muscle regeneration. I still believe that a model that involves sustained treatment with GCs would be better but I understand that the authors want to reserve such an approach for a follow-up study. The FACS analysis indeed revealed AMPK-dependent differences after GC injection in the abundance of immune cell subpopulations, but apparently this has no dramatic effects on muscle regeneration. Since the main focus is on the role of AMPK for GC-mediated macrophage polarization, a closer inspection of the morphology of regenerating muscles is probably dispensable. The newly introduced assessment of Lyve1+, FRb+, CD206+ cells by FACs is helpful and further strengthen the results. However, the authors should consider rephrasing the legend for Fig. 2, which reads: "Expression of the anti-inflammatory markers was assessed by flow cytometry in macrophages (CD64pos)". The authors assessed the abundance of marker-positive cells, which is something related but different.

I also asked to monitor subcellular localization of FOXO3 in AMPK-mutant macrophages. The authors performed additional immunofluorescence staining and observed no AMPK-dependent differences in the localization of FOXO3. All fine. However, the labelling of the panel in Fig. EV5 needs to be corrected: it should be Fig. EV5D and not Fig. EV5F. Furthermore, the authors complied to my request to analyze the phosphorylation state of FOXO3 in WT and AMPK-mutant cells after GC treatment. Since the authors claim that no specific antibodies against phosphorylation sites of FOXO3 that work in the mouse are available, pull-down assays for serin phosphorylation were done, which revealed reduced phosphorylation of FOXO3 upon AMPK depletion. These are nice results that strengthen the study. I do not have personal experience with phospho-specific FOAXO3 antibodies in the mouse but a quick search revealed several phospho-specific FOAXO3 antibodies by different vendors, which are described to work in the mouse (e.g. Phospho-FoxO3a (Ser253) Antibody #9466 from Cell Signaling).

Other comments were appropriately dealt with by doing additional RT-qPCR experiments, amending the discussion, or modifying the statements.

Referee #2:

The revisions are acceptable.

Editor's comments

Thank you for the submission of your revised manuscript to our editorial offices. I have now received the reports from the two referees that I asked to re-evaluate your study, you will find below. As you will see, both referees think that the study is now suitable for publication in EMBO reports. Referee #1 has some remaining points and suggestions to improve the study I ask you to address in a final revised manuscript. Please also provide a final detailed p-b-p-response addressing all the remaining referee comments.
See below for the pbp response of Referee#1's comments.

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Edit made.

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As requested, we merged results and discussion sections. The changes are shown in the tracking mode of word.

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We added these elements as a separate files in the submission system.

Referee #1:
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The reviewer is correct that phospho-FOXO3 antibodies do exist, however these are not for AMPK specific sites, but rather for AKT-dependent sites (S253) (https://doi.org/10.1074/jbc.274.24.17184). AMPK phosphorylates FOXO3 mainly at Ser413, Ser588, and Ser626 (https://doi.org/10.1074/jbc.M705325200) but no specific antibodies exist for these three sites with mouse immunogens. We felt using a non-specific phosphoserine antibody pulldown, followed by blotting for FOXO3 would both work in mouse cells, but also collect all potential AMPK-dependent phosphorylation sites in an unbiased way.

Other comments were appropriately dealt with by doing additional RT-qPCR experiments, amending the discussion, or modifying the statements.

INSTITUT NEUROMYOGENE
Université Claude Bernard Lyon 1 - CNRS UMR 5261 - INSERM U1315
8 Avenue Rockefeller - 69008 Lyon – France
Phone : (33) 4 26 68 82 46
remi.mounier@univ-lyon1.fr
http://www.musclestem.com
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Dr. Bénédicte Chazaud
Université Claude Bernard Lyon 1
Institut NeuroMyoGène
8 Avenue Rockfeller
Lyon 69008
France

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Reporting Checklist for Life Science Articles (updated January 2022)
This checklist is adapted from Materials Design Analysis Reporting (MDAR) Checklist for Authors. MDAR establishes a minimum set of requirements in transparent reporting in the life sciences (see Statement of Task: 10.31222/osf.io/9sm4x). Please follow the journal's guidelines in preparing your manuscript.
Please note that a copy of this checklist will be published alongside your article.

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 replicates.
- 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 Presentation.

2. Captions

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

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements.
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.
- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Please complete ALL of the questions below.
Select "Not Applicable" only when the requested information is not relevant for your study.

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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?	Not Applicable	

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For **antibodies** provide the following information: - Commercial antibodies: RRID (if possible) or supplier name, catalogue number and or/clone number - Non-commercial: RRID or citation	Yes	Materials and Methods

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Short novel DNA or RNA including primers, probes: provide the sequences.	Yes	Materials and Methods

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Cell lines: Provide species information, strain. Provide accession number in repository **OR** supplier name, catalog number, clone number, and/**OR** RRID.	Not Applicable	
Primary cultures: Provide species, strain, sex of origin, genetic modification status.	Yes	Materials and Methods
Report if the cell lines were recently **authenticated** (e.g., by STR profiling) and tested for mycoplasma contamination.	Not Applicable	

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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?	Not Applicable	
Include a statement about **blinding** even if no blinding was done.	Yes	Materials and Methods
Describe **inclusion/exclusion criteria** if samples or animals were excluded from the analysis. Were the criteria pre-established? If sample or data points were omitted from analysis, report if this was due to attrition or intentional exclusion and provide justification.	Not Applicable	
For every figure, are **statistical tests** justified as appropriate? Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. Is there an estimate of variation within each group of data? Is the variance similar between the groups that are being statistically compared?	Yes	Materials and Methods

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In the figure legends: state number of times the experiment was **replicated** in laboratory.	Yes	Figure legends
In the figure legends: define whether data describe **technical or biological replicates**.	Yes	Figure legends

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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)
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