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CARS senses cysteine deprivation to activate AMPK for cell survival

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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.)

1st Editorial Decision 23rd Mar 2021

Dear Ping,

Thank you again for the submission of your manuscript (EMBOJ-2021-108028) to The EMBO Journal and in addition providing us with a preliminary revision plan. As mentioned earlier, your study has been sent to three referees, and we have received reports from all of them, which I enclose below.

As you will see, the referees acknowledge the potential interest and novelty of your results in a timely context, although they also express major concerns. In more detail, the reviewers state considerable issues with the endogenous relevance of your findings at the level of CARS-CaMKK2-AMPK interaction and AMPK substrate activation. In addition, they point to limited insights into the relevance of this pathway in more cell-physiological settings. Finally, a number of weaknesses regarding robustness, data inconsistencies and missing control-rescue experiments are indicated, which compromise the impact of your results in the experts' view.

Given the interest stated and broader angle of your approach and findings, we are overall able to invite you to revise your manuscript experimentally to address the referees' comments, along the lines sketched in your outline.

Please feel free to contact me if you have any questions or need further input on the referee comments.

As you know, 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.

Thank you for the opportunity to consider your work for publication.
I look forward to your revision.

Kind regards,

Daniel

Daniel Klimmeck, PhD
Senior Editor
The EMBO Journal

Instructions for preparing your revised manuscript:

Please make sure you upload a letter of response to the referees' comments together with the revised manuscript.

Please also check that the title and abstract of the manuscript are brief, yet explicit, even to non-specialists.

When assembling figures, please refer to our figure preparation guideline in order to ensure proper formatting and readability in print as well as on screen:
https://bit.ly/EMBOPressFigurePreparationGuideline

IMPORTANT: When you send the revision we will require
- a point-by-point response to the referees' comments, with a detailed description of the changes made (as a word file).
- a word file of the manuscript text.
- individual production quality figure files (one file per figure)
- a complete author checklist, which you can download from our author guidelines (https://www.embopress.org/page/journal/14602075/authorguide).
- Expanded View files (replacing Supplementary Information)
Please see out instructions to authors
https://www.embopress.org/page/journal/14602075/authorguide#expandedview

Please remember: Digital image enhancement is acceptable practice, as long as it accurately represents the original data and conforms to community standards. If a figure has been subjected to significant electronic manipulation, this must be noted in the figure legend or in the 'Materials and Methods' section. The editors reserve the right to request original versions of figures and the original images that were used to assemble the figure.

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The revision must be submitted online within 90 days; please click on the link below to submit the revision online before 21st Jun 2021.

Link Not Available

Referee #1:

AMP-activated protein kinase (AMPK) is a crucial sensor of cellular energy status that is activated by increased AMP:ATP (or ADP:ATP) ratios. Upon activation, AMPK inhibits anabolism and promotes catabolism to restore energy homeostasis. A growing body of evidence supports the idea that AMPK activity is also influenced by diverse metabolites including glycogen and lipid intermediates. However, the ability of AMPK to respond to changes in amino acid availability is poorly understood. In the current study, Yuan and colleagues provide evidence that cysteine depletion triggers AMPK activation in a manner dependent on a physical association between AMPK, cysteinyl-tRNA synthetase (CARS) and calcium/calmodulin-dependent protein kinase kinase 2 (CaMKK2). The authors also demonstrate that AMPK activation contributes, in a subset of cell lines, to cell survival following cysteine depletion.

This is a very novel and elegant study with, for the most part, robust data provided to support the conclusions. The authors have been very successful in contextualizing their study and the significance of their findings and, as a result, this study should be of interest to a broad audience spanning cell signalling, cell metabolism and cancer biology.

In order to support the conclusions, the authors should address the following major concerns.

The authors rely on AMPK Thr172 phosphorylation status to monitor AMPK activity. However, AMPK is susceptible to numerous post-translational modifications that can impact activity. The authors should therefore monitor phosphorylation of the AMPK substrate ACC, which will provide a more robust indication of AMPK activity. At the very least, it would be important to show p-ACC blots in Fig 1B/C/D/F/G.

All of the immunoprecipitation experiments that support an interaction between CARS/CaMKK2/AMPK (Fig 1H, 2B/E, 3F) rely on overexpression of tagged proteins. Where possible, the authors should perform endogenous IP experiments.

Minor concerns that should be addressed are outlined below.

It is not clear from the methods section if dialyzed serum is used for amino acid deprivation studies, in particular Fig 1A. The authors refer to a previous paper that mentions "FBS used is not dialyzed" (Methods Enzymol 587:465, 2017). Dialyzed serum must be used to ensure that there is no contribution of amino acids from the serum.

The authors should take care to talk about cystine-deficient media/cystine deprivation, as opposed to cysteine-deficient media/cysteine deprivation.

p-AMPK levels are so much higher in the second panel of Fig 1A, which makes it difficult to compare the amino acid dropouts. At the very least, a different exposure should be shown. Ideally, the sample derived following cystine deprivation should be included in both panels.

Given that the N- and C-terminal fragments of AMPK gamma2 are roughly the same size, the authors should check if the arrows in Fig 3C are highlighting the correct bands.

The authors should take care to mention how many biological replicates were performed for each experiment, in particular where statistical analysis is performed.

Referee #2:

This manuscript by Yuan and colleagues implements a variety of molecular and cellular models to understand links between amino acid metabolism and AMP-activated protein kinase (AMPK) phosphorylation at T172. The authors elucidate a role for cysteine content in modulating the phosphorylation status of AMPK that is dependent on Calcium/Calmodulin Dependent Protein Kinase Kinase 2 (CaMKK2). Further experiments expand this cysteine sensing AMPK/CaMKK2 axis to include interactions between cysteinyl-tRNA synthetase (CARS), CaMKK2, and the γ2 subunit of AMPK (AMPKγ2). The isolation of this pathway to AMPKγ2 is dependent on the N-terminal portion of AMPKγ2, which is known to be a definitive breakpoint in homology between this AMPKγ isoform

and AMPKγ1 or AMPKγ3. Finally, the authors assess the capacity for cysteine deprivation in combination with CaMKK2 inhibition as a means to limit cell viability and stimulate cell death across a panel of cancer cell lines.

The data presented definitively indicate the capacity for cysteine deprivation to increase AMPK phosphorylation in specific cell lines. The necessity and sufficiency for CaMKK2 and CARS for this effect in cells expressing AMPKγ2 are demonstrated through a series of complex and well controlled studies. This is a critical step forward in understanding the direct impacts of different metabolites on AMPK phosphorylation. The authors demonstrate a clear focus on delineating the mechanistic underpinnings of this set of interactions and signals.

However, there are gaps in understanding how this pathway is impacting downstream signals or outputs of AMPK signaling. Additionally, the relevance of this pathway to normal physiology or even pathological settings in vivo remains to be seen. The authors also fail to address the critical role of cysteine in glutathione metabolism and cellular redox status, which may impact a number of metabolic processes including those regulated by AMPK.

Reviewer major comments

1. The manuscript implements a considerable array of molecular and cellular models to delineate the mechanism underlying cysteine deprivation dependent AMPK phosphorylation. The authors often refer to this increase in phosphorylation of AMPKα at T172 as increased activity. While this phosphorylation is known to impart increased activation potential of AMPK it is critical to demonstrate that activation is actually enhanced. Demonstrating phosphorylation of downstream substrates of AMPK (ACC, ULK1, and Raptor)) using widely used commercially available antibodies by western blot under cysteine deprived conditions would sufficiently demonstrate AMPK activation.

2. Additionally, demonstrating alterations in these downstream targets under various cellular conditions that effect this novel pathway (e.g., CARS over expression, CaMKK2 shRNA knockdown, and expression of n-terminal AMPKγ2) would greatly enhance the impact of the manuscript and put it in the context of well-described effectors studied by many labs.

3. The studies presented do an excellent job of delineating a novel mechanism for cysteine dependent modulation of AMPK phosphorylation. The authors also acknowledge the relationship between AMPK and cellular redox status. Despite cysteine being a critical component of cellular redox metabolism, through direct effects and its contributions to glutathione metabolism, there are no references to the effects that cysteine deprivation has on the redox status of the cells, the production of reactive oxygen species, glutathione content, or glutathione redox status. The authors provide references to the potential benefits of limiting cysteine availability in vivo to various cancers (Cramer et al. 2016; Poursaitidis et al. 2017). Both of these references cite effects on reactive oxygen species production and redox handling as critical drivers of cell death in their respective models. Understanding the exact mechanisms underlying cysteine deprivation-mediated initiation of the CARS-CaMKK2-AMPK cascade is likely beyond the scope of this manuscript. However, given the known impacts of cysteine deprivation on ROS dependent cell death and the modulation of cell death by the CARS-CaMKK2-AMPK in this manuscript it would benefit the manuscript to demonstrate the effects of their cysteine deprivation model on redox status and glutathione metabolism. Authors should measure GSH:GSSG ratios in cysteine deprived cells and determining the impacts of various interventions (e.g. CARS over expression, CaMKK2 shRNA

knockdown, and expression of n-terminal AMPKγ2) on this set of measurements, as this would greatly improve the manuscript and add clarity about any potential effect of ROS.

Reviewer minor comments

1. The authors discuss the impacts of the AMPK isoform expression on functional redundancy and expression localization specificity. Indeed, critical work has been done to delineate tissue expression profiles of AMPKγ subunits across multiple organisms (Mahlapuu et al., Am J Physiol Endocrinol Metab, 2004) and the functional implications of differences in AMPKγ subunit homology (Willows et al., Biochem J, 2017). The authors describe their in silico comparisons of AMPKγ subunits, but do not acknowledge or cite that this has been previously done. This observation should be considered within the context of previous work on AMPKγ subunits with supporting citations.

2. The authors provide significant evidence as to the involvement of AMPKγ2, specifically, in this novel pathway and the protein domains involved in AMPKγ2-CARS interactions. The authors also demonstrate that cell lines lacking AMPKγ2 do not have cysteine deprivation-dependent activation of AMPK and are susceptible to cell death induced by lack of cysteine. The authors do not address how the reliance of this pathway on AMPKγ2 could be more or less relevant to specific types of cancer. Discussing which cell types and/or cancers would be most impacted by the AMPKγ2-dependence of the observed phenotypes would help to establish the scope of potential therapeutic applications.

3. The authors utilize a number of abbreviations and acronyms for proteins that are not fully defined including AMPK and CaMKK2. Providing full names of proteins and enzymes upon initial introduction would be helpful for the reader as not all readers may be familiar with certain proteins and their naming conventions.

4. The authors refer to AMPK as a "molecule" in the abstract and introduction. Referring to AMPK as a kinase or enzyme would be more appropriate due to its heterotrimeric nature referenced by the authors.

5. The section heading "Cystine deprivation activates AMPK through CAMKK2" uses cystine, which is the oxidized dimeric form of cysteine. The title and most of the manuscript reference cysteine. This should be made consistent.

6. Cystine deprivation activates AMPK through CAMKK2, Paragraph 1: "isoleucine or leucine deprivation also marginally activated AMPK". Comment: Using the term marginally for a treatment that significantly increased AMPK phosphorylation ~4-fold is misleading. Simply remove the word marginally.

7. CARS is critical for CaMKK2-mediated AMPK activation under cysteine deprivation conditions, Paragraph 1: "...we speculated that they sensor is likely an upstream factor..." Comment: Simply correct "they" to "the".

8. CARS senses cysteine starvation to activate AMPK by binding to AMPKγ2, Paragraph 1: "On the other hand, the CARS protein structure analysis based on the online website UniProt (http://www.ebi.ac.uk/interpro/), we constructed vectors expressing three truncated fragments of the CARS protein..." Comment: This sentence is confusing and should be edited for clarification."

9. Figure 1A: Comment: The labeling of bars in the densitometry histogram is inconsistent (only some bars are labeled) and inaccurate ("Hle" appears to be a combination of histidine and isoleucine abbreviations). The authors should clarify the labeling of this graph.

Referee #3:

The manuscript by Yuan and colleagues reports on an interesting phenomenon whereby cysteine starvation leads to activation of AMPK-gamma2 containing complexes mediated by CAMKK2. The authors suggest that this may have important implications in some cancer cells that are resistant to cysteine deprivation, although this remains highly speculative. The model the authors present is straightforward but there are some obvious questions that arise that have not been addressed in the current manuscript. Dealing with these issues would strengthen greatly the impact of the study.

Major Points:
1. In general, most of the data shown are from single blots, with no attempt at quantification. It would be important to include some evidence of replication and quantification throughout the paper. In addition (and importantly) blots are often shown without reference to molecular mass standards, and/or evidence of antibody validation.

2. An exception to the lack of quantification is shown in Fig. 1A, but this immediately presents a problem of its own. In the top panel of the blot, the pAMPK is very low in the control (Nor), but clearly increased in the minus cys condition. However, in the lower panel, the pAMPK signal in the control is relatively high (obviously higher than in the control lane above). A rough estimate by eye would suggest that the pAMPK:total AMPK values for control would well below 1 for the top panel, and above 1 for the bottom panel (so at least an order of magnitude difference). As similar problem is evident for some of the amino acids. This is a worry. How were the values in the bar graph calculated? Any attempt at normalising the data between the two blots would introduce significant concerns.

3. Was any attempt made to determine binding between endogenous proteins (rather than using over-expressed proteins)? As the authors point out, AMPK is a heterotrimeric complex. It is important to show that in the over-expression studies (where usually it appears only a single AMPK subunit is transfected) whether the other two subunits are present. Related to this point, in the experiment shown in Fig. 3A, results showing interaction when alpha, beta and gamma subunits are co-transfected should be included. In vivo, AMPK would be expected to be present as the heterotrimeric complex, so recapitulating this in the experimental model is important.

4. The model for CAMKK2 dependence is intriguing, but the authors don't monitor whether cysteine starvation alters calcium levels in the cell. This is an important aspect of the mechanism (either way - if calcium is not increased by cysteine starvation how is CAMKK2 activated?) and so should be included. Related to this, the study by Dalle Pezze (Nat Comms 2016) that the authors cite reports that re-addition of amino acids activates AMPK (implying that amino acid starvation inhibits AMPK) via CAMKK2. A more nuanced discussion on this point is required here.

5. The finding that activation is restricted to the gamma2 AMPK complex is very interesting. In most cell types, gamma2 AMPK is a minor component (gamma1 complexes accounting for most of the AMPK present in the cell). This raises some complex mechanistic issues. In Fig. 2D, knockdown of

CARS reduces basal AMPK phosphorylation (suggesting total AMPK activity is affected - including gamma1-containing complexes). In Fig. 3D/E, knockdown of gamma2 has little effect on basal AMPK phosphorylation (suggesting that total AMPK activity is not affected - which is what would be expected if gamma2 is a minor component of total AMPK). It is difficult to reconcile these different findings. Monitoring phosphorylation of endogenous downstream AMPK targets (e.g. pACC, pULK1) would provide useful information related to this issue. Adding this data for both cysteine starvation, CARS knockdown and CARS over-expression would be very helpful and informative.

6. Related to point 5, the suggestion that activation of AMPK (other than gamma2-containing complexes) by AICAR can rescue the growth phenotype following cysteine starvation is interesting and perhaps counter-intuitive. AICAR is known to have AMPK-independent effects, so it would be good to complement this study with more selective (and potent) direct AMPK activators (2nd generation activators such as the Pfizer and Merck compounds).

Point-by-point response to the comments

Summary of Editor's comments:

Thank you again for the submission of your manuscript (EMBOJ-2021-108028) to The EMBO Journal. Your study has been sent to three reviewers for evaluation. As you will see, the referees acknowledge the potential interest of your findings, although they also express major concerns. In more detail, the reviewers state substantial issues with the endogenous relevance of your findings at the level of CARS-CaMKK2-AMPK interaction and AMPK substrate activation. In addition, the referees point to number of weaknesses regarding robustness, data inconsistencies and missing control-rescue experiments, which compromise the impact of your results in their view.

These are important points in our view, and given the substantial criticisms raised, we find it difficult to commit to going further with this manuscript The EMBO Journal. Before making the final decision, I would offer you the chance to read the reports and to let us know about your view on the critique and how the concerns raised by the referees could be addressed within the time-frame of a revision. It would therefore be helpful if you could already at this point provide me with a preliminary point-by-point response on what data could be included in the revised manuscript. In this way, we can better agree on the exact experimental requirements for the revision. I will then re-consult with the referees to determine if such a revision would address their concerns. I would like to stress that I need strong endorsement from the referees in order to fully commit to a revised manuscript.

Please feel free to contact me with any questions related to this matter. By conducting this exchange at the current stage I hope to avoid inviting a revision with a high risk of being rejected by the referees following extensive experimental efforts on your side.

Response: We are very grateful to our editor for your kind decision to encourage us to proceed with this intensive revision. We also thank the referees for the very constructive and insightful comments and suggestions that have helped us with the preparation of a revised manuscript. In the past three months, we have performed additional new experiments and have addressed all the concerns and comments raised by our referees. Here we resubmit a substantially improved manuscript along with our point-by-point responses.

For the referee's convenience, we have appended in this file all the revised figures, which we labeled as **Figure R1** to **Figure R14**.

EMBOJ-2021-108028-Referee reports

Referee #1: (Remarks to the Author):

AMP-activated protein kinase (AMPK) is a crucial sensor of cellular energy status that is activated by increased AMP:ATP (or ADP:ATP) ratios. Upon activation, AMPK inhibits anabolism and promotes catabolism to restore energy homeostasis. A growing body of evidence supports the idea that AMPK activity is also influenced by diverse metabolites including glycogen and lipid intermediates. However, the ability of AMPK to respond to changes in amino acid availability is poorly understood. In the current study, Yuan and colleagues provide evidence that cysteine depletion triggers AMPK activation in a manner dependent on a physical association between AMPK, cysteinyl-tRNA synthetase (CARS) and calcium/calmodulin-dependent protein kinase kinase 2 (CaMKK2). The authors also demonstrate that AMPK activation contributes, in a subset of cell lines, to cell survival following cysteine depletion.

This is a very novel and elegant study with, for the most part, robust data provided to support the conclusions. The authors have been very successful in contextualizing their study and the significance of their findings and, as a result, this study should be of interest to a broad audience spanning cell signalling, cell metabolism and cancer biology.

Response: We are grateful for the reviewer's positive comments that well summarized the major findings and significance of our study. We also appreciate his/her constructive comments and suggestions for this manuscript.

In order to support the conclusions, the authors should address the following major concerns.The authors rely on AMPK Thr172 phosphorylation status to monitor AMPK activity. However, AMPK is susceptible to numerous post-translational modifications that can impact activity. The authors should therefore monitor phosphorylation of the AMPK substrate ACC, which will provide a more robust indication of AMPK activity. At the very least, it would be important to show p-ACC blots in Fig 1B/C/D/F/G.

Response: We fully agree with the reviewer's suggestion. Considering that AMPK activates and regulates different substrates under different stresses (Zong et al., 2019), monitoring the AMPK substrate ACC and other well-known substrates such as Raptor will provide a more robust indication of AMPK activity. Following this suggestion, we detected the phosphorylation levels of ACC and Raptor and found that they are consistent with phosphorylation levels of AMPK shown in Fig 1B/C/D/F/G, 2D/F, 3D/E in the original manuscript (**Figures R1-R6**), these results indeed provide a more robust indication of AMPK activation. We have included Figures R1-6 as **Figures 1B-D, 1F, 1G, 2E, 2H, 3D-E** in the revised manuscript, respectively.

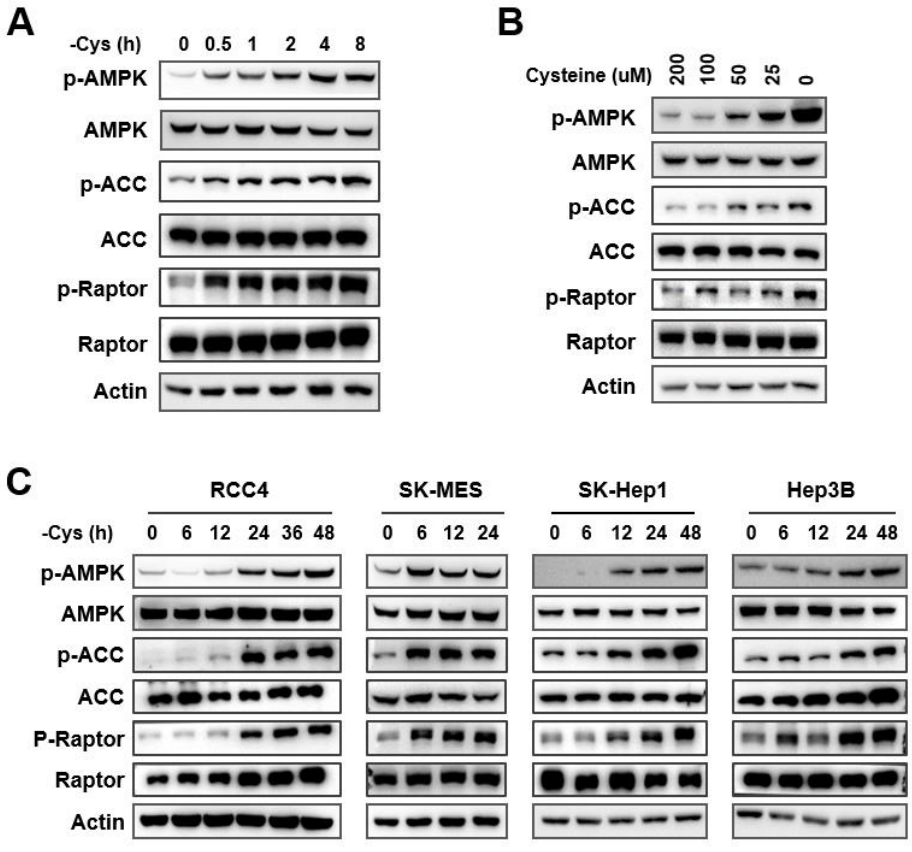

Figure R1. Cystine deprivation activates AMPK and its substrates. (**A-C**) 293T cells were treated with cystine-deficient medium for 0, 0.5, 1, 2, 4, or 8 hours (**A**) or treated with medium containing 200 μM, 100 μM, 50 μM, 25 μM or 0 μM cystine for 8 hours (**B**); RCC4, SK-MES, SK-hep-1 or Hep3B cells were treated with cystine-deficient medium for indicated time (**C**). Protein levels of p-AMPK, p-ACC, p-Raptor and total AMPK, ACC, Raptor were measured by WB, and actin served as the loading control. Please see also **Figures 1B-D** in the revised manuscript.

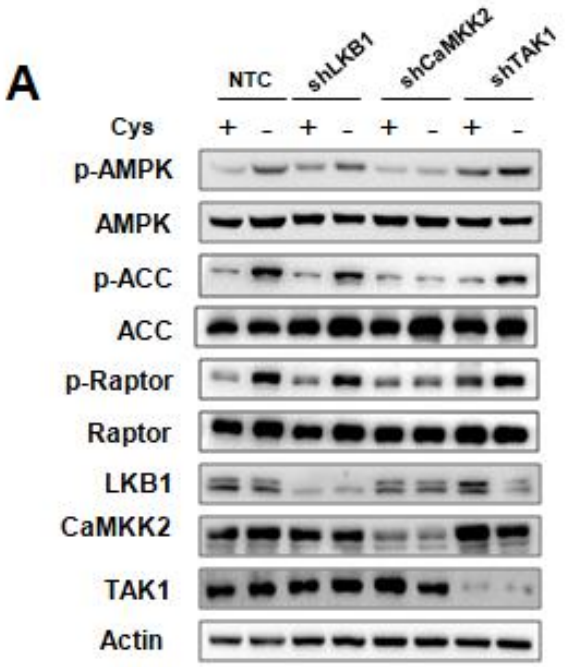

Figure R2. Cystine deprivation activates AMPK through CaMKK2. (**A**) WB analysis of p-AMPK, p-ACC, p-Raptor and total AMPK, ACC, Raptor protein expression in 293T cells transfected with shRNAs targeting LKB1, CaMKK2, TAK1 or non-targeting control (NTC) that were treated with cystine-deficient medium for 8 hours. Actin served as the loading control. Please see also **Figure 1F** in the revised manuscript.

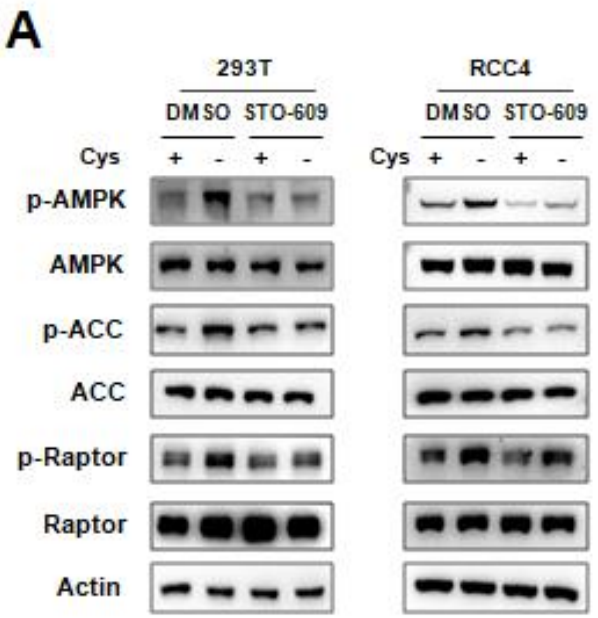

Figure R3. Cystine deprivation-induced AMPK activation was suppressed by CaMKK2 inhibitor STO-609. (A) WB analysis of p-AMPK, p-ACC, p-Raptor and total AMPK, ACC, Raptor protein expression in 293T cells and RCC4 cells treated with 1 μg/ml STO-609 or DMSO for 8 hours during cystine deprivation. Actin served as the loading control. Please see also **Figure 1G** in the revised manuscript.

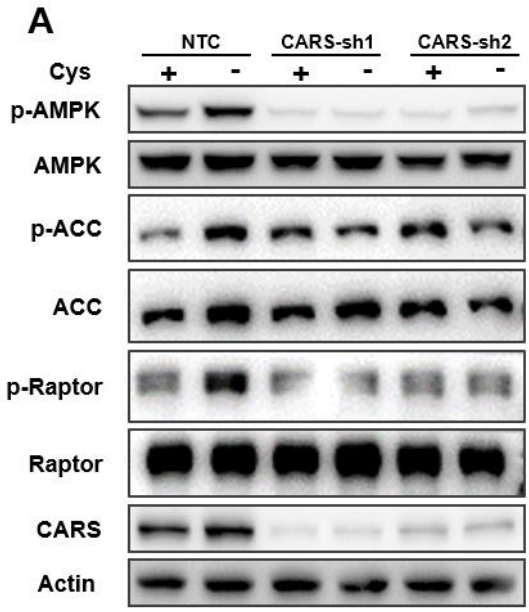

Figure R4. Cystine deprivation-induced AMPK activation was suppressed by CARS knockdown. (**A**) WB analysis of p-AMPK, p-ACC, p-Raptor and total AMPK, ACC, Raptor protein expression in 293T cells transfected with shRNAs targeting CARS that were further treated with cystine-deficient medium for 8 hours. Actin served as the loading control. Please see also **Figure 2E** in the revised manuscript.

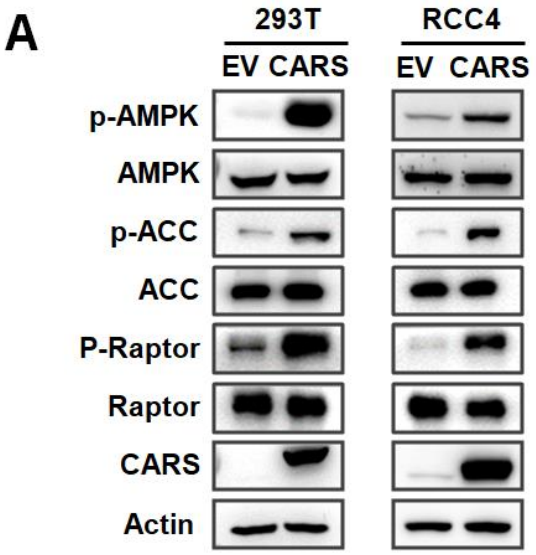

Figure R5. CARS overexpression promotes the activation of AMPK and its substrates. (**A**) WB analysis of p-AMPK, p-ACC, p-Raptor and total AMPK, ACC, Raptor, CARS protein expression in 293T cells and RCC4 cells overexpressing Flag-CARS. Actin served as the loading control. Please see also **Figure 2H** in the revised manuscript.

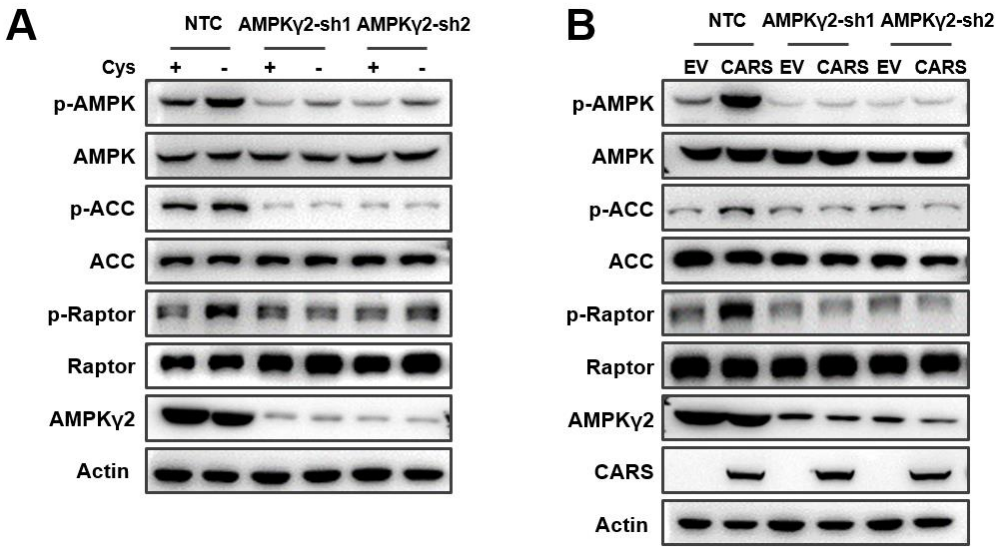

Figure R6. Cystine deprivation- or CARS overexpression-induced AMPK activation was suppressed by AMPKγ2 knockdown. (A-B) WB analysis of p-AMPK, p-ACC, p-Raptor and total AMPK, ACC, Raptor protein expression in 293T cells transfected with siRNAs targeting AMPKγ2 that were further treated with cystine-deficient medium for 8 hours (**A**) or transfected with Flag-CARS for 48 hours (**B**). Actin served as the loading control. Please see also **Figure 3D-3E** in the revised manuscript.

All of the immunoprecipitation experiments that support an interaction between CARS/CaMKK2/AMPK (Fig 1H, 2B/E, 3F) rely on overexpression of tagged proteins. Where possible, the authors should perform endogenous IP experiments.

Response: We appreciate the reviewer's suggestion. Accordingly, we performed additional experiments using dialyzed serum to provide the endogenous IP result of Fig 1H, 2B/E, 3F. Concerned with the potential issues related to antibody specificity for endogenous IP, we detected and validated the interaction between endogenous AMPKα vs endogenous CaMKK2 (**Figure R7A, R7C**), endogenous CaMKK2 vs endogenous CARS (**Figure R7B**), and AMPKγ2 with HA tag vs endogenous CARS (**Figure R7D**) under indicated conditions. We have now included Figure R7A-D as **Figures 1I, 2C/G, 3G** in the revised manuscript, respectively.

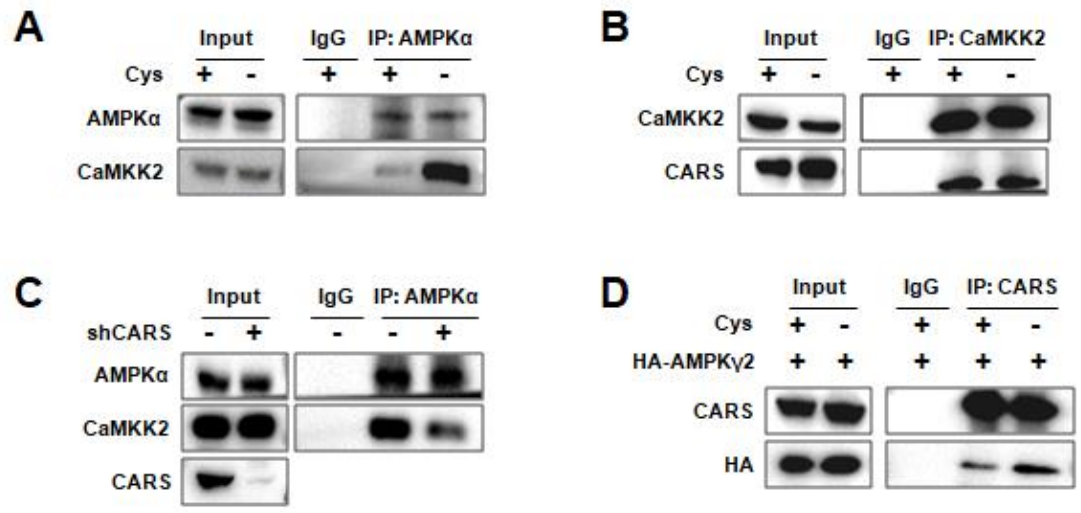

Figure R7: Detection of the binding activity of AMPKα VS CaMKK2, CaMKK2 VS CARS, and HA-AMPKγ2 VS CARS under the indicated conditions. (**A**) 293T cells cultured with cystine-deficient medium or complete medium for 8 hours were harvested and subjected to immunoprecipitation with anti-AMPKα, followed by WB analysis with anti-AMPKα and anti-CaMKK2. (**B**) 293T cells cultured with cystine-deficient medium or complete medium for 8 hours were harvested and subjected to immunoprecipitation with anti-CaMKK2, followed by WB analysis with anti- CaMKK2 and anti-CARS. (**C**) 293T cells were transfected with shRNAs targeting CARS or NTC, then they were cultured with cystine-deficient medium for 8 hours. Cell lysates were immunoprecipitated with anti-AMPKα and subjected to WB analysis with anti-AMPKα and anti-CaMKK2. (**D**) 293T cells were transfected with HA-AMPKγ2 for 48 hours and then cultured with cystine-deficient medium or complete medium for 8 hours. Cell lysates were immunoprecipitated with anti-CARS, followed by WB analysis with anti-HA and anti-CARS. The medium used in Figure R7 is prepared with dialyzed serum. Please see also **Figures 1I, 2C/G, 3G** in the revised manuscript.

Minor concerns that should be addressed are outlined below.

It is not clear from the methods section if dialyzed serum is used for amino acid deprivation studies, in particular Fig 1A. The authors refer to a previous paper that mentions "FBS used is not dialyzed" (Methods Enzymol 587:465, 2017). Dialyzed serum must be used to ensure that there is no contribution of amino acids from the serum.

Response: This is a good point. Following this suggestion, we have carried out some key experiments with dialyzed serum, including Figures 1A, 1H, 2B, 2E, 3F in the original manuscript. Results shown that cystine deprivation with dialyzed serum markedly activated AMPK, as indicated by its phosphorylation and the

phosphorylation of ACC and Raptor proteins in 293T cells, although isoleucine or leucine deprivation also activated AMPK (**Figure R8A**), which has been included in the revised manuscript as Figure 1A. Further experiments shown that cystine deprivation activated AMPK and its substrates including ACC and Raptor, in both medium with dialyzed serum or non-dialyzed serum (**Figure R8B**). Additionally, we performed endogenous IP results with dialyzed serum (**Figure R7**), which led to similar conclusions as Figures 1H, 2B/E, 3F in the original manuscript.

Thank the reviewer for pointing this important issue. Accordingly, we also revised the description of the serum in the section of "Materials and Methods" part in the revised manuscript.

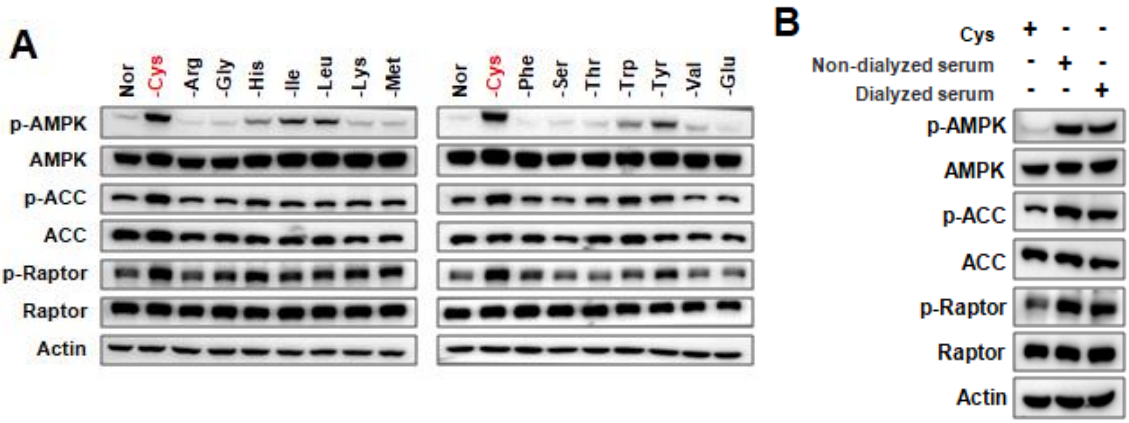

Figure R8. Cystine deprivation markedly activates AMPK and its substrates. (A) 293T cells were cultured in complete medium supplemented with dialyzed serum for 24 hours which was then replaced with medium in which one amino acid was eliminated and cultured for 8 hours. Then, WB was performed to measure p-AMPK, p-ACC, p-Raptor and total AMPK, ACC, Raptor protein expression. Actin served as the loading control. Please see also **Figure 1A** in the revised manuscript. (**B**) 293T cells were cultured in complete medium supplemented with dialyzed serum or non-dialyzed serum for 24 hours which was then replaced with medium in which cystine was eliminated and cultured for 8 hours. WB was performed to measure p-AMPK, p-ACC, p-Raptor and total AMPK, ACC, Raptor, CARS protein expression. Actin served as the loading control.

The authors should take care to talk about cystine-deficient media/cystine deprivation, as opposed to cysteine-deficient media/cysteine deprivation.

Response: We thank the reviewer for pointing this out. Following this suggestion, to keep consistent, we replaced "cysteine-deficient media/cysteine deprivation" with

"cystine-deficient media/cystine deprivation" throughout this manuscript during the revision.

p-AMPK levels are so much higher in the second panel of Fig 1A, which makes it difficult to compare the amino acid dropouts. At the very least, a different exposure should be shown. Ideally, the sample derived following cystine deprivation should be included in both panels.

Response: We thank our reviewer for this suggestion. We repeated Fig 1A in the original manuscript with dialyzed serum and provided western blotting images with p-AMPK, p-ACC, p-Raptor and total AMPK, ACC, Raptor, and included cystine deprivation conditions in both panels (**Figure R8A**). Again, the results confrimed our obsevation that cystine deprivation activated AMPK. Additionally, we deleted the densitometry histogram and provided the values of p-AMPK/AMPK, p-ACC/ACC, p-Raptor/Raptor ratios to indicate AMPK activation.

Given that the N- and C-terminal fragments of AMPK gamma2 are roughly the same size, the authors should check if the arrows in Fig 3C are highlighting the correct bands.

Response: Yes, our reviewer is right. We have revised this part to make sure that the arrows point to the correct positions in the revised manuscript.

The authors should take care to mention how many biological replicates were performed for each experiment, in particular where statistical analysis is performed.

Response: As a matter of fact, we have provided the information of biological replicates in the "**Statistical Analysis**" of the methods section on Page 16 in the original manuscript. To make this clear to the reader, we also emphasized this point in the Figure legends of Figure 4B, D, G and Figure EV 4A, B, D during the revision.

Referee #2: (Remarks to the Author):

This manuscript by Yuan and colleagues implements a variety of molecular and cellular models to understand links between amino acid metabolism and AMP-activated protein kinase (AMPK) phosphorylation at T172. The authors elucidate a role for cysteine content in modulating the phosphorylation status of AMPK that is dependent on Calcium/Calmodulin Dependent Protein Kinase Kinase 2

(CaMKK2). Further experiments expand this cysteine sensing AMPK/CaMKK2 axis to include interactions between cysteinyl-tRNA synthetase (CARS), CaMKK2, and the γ2 subunit of AMPK (AMPKγ2). The isolation of this pathway to AMPKγ2 is dependent on the N-terminal portion of AMPKγ2, which is known to be a definitive breakpoint in homology between this AMPKγ isoform and AMPKγ1 or AMPKγ3. Finally, the authors assess the capacity for cysteine deprivation in combination with CaMKK2 inhibition as a means to limit cell viability and stimulate cell death across a panel of cancer cell lines.

The data presented definitively indicate the capacity for cysteine deprivation to increase AMPK phosphorylation in specific cell lines. The necessity and sufficiency for CaMKK2 and CARS for this effect in cells expressing AMPKγ2 are demonstrated through a series of complex and well controlled studies. This is a critical step forward in understanding the direct impacts of different metabolites on AMPK phosphorylation. The authors demonstrate a clear focus on delineating the mechanistic underpinnings of this set of interactions and signals.

However, there are gaps in understanding how this pathway is impacting downstream signals or outputs of AMPK signaling. Additionally, the relevance of this pathway to normal physiology or even pathological settings in vivo remains to be seen. The authors also fail to address the critical role of cysteine in glutathione metabolism and cellular redox status, which may impact a number of metabolic processes including those regulated by AMPK.

Response: We appreciate the reviewer for the positive comments and insightful summary of our exciting discovery. Indeed, we elucidate the novel role of cystine in the regulation of CaMKK2-dependent activation of AMPK. We also appreciate the reviewer for his/her valuable concerns and suggestions, which have certainly helped us strengthen this study. As these important concerns are further detailed in his/her major comments, we will address them one by one below.

Reviewer major comments

1. The manuscript implements a considerable array of molecular and cellular models to delineate the mechanism underlying cysteine deprivation dependent AMPK

phosphorylation. The authors often refer to this increase in phosphorylation of AMPKα at T172 as increased activity. While this phosphorylation is known to impart increased activation potential of AMPK it is critical to demonstrate that activation is actually enhanced. Demonstrating phosphorylation of downstream substrates of AMPK (ACC, ULK1, and Raptor)) using widely used commercially available antibodies by western blot under cysteine deprived conditions would sufficiently demonstrate AMPK activation.

Response: We fully agree with our reviewer on this point. Following this suggestion, we detected the phosphorylation levels of ACC and Raptor and found that they are consistent with the phosphorylation levels of AMPK shown in Fig. 1B/C/D/F/G in the original manuscript (**Figures R1-3**). These results indeed provide a more robust indication of AMPK activation and we have included these data as **Figure 1B-D, 1F, 1G** in the revised manuscript, respectively. We have addressed the similar important concerns by the reviewer # 1, here we present the results again for your convenience.

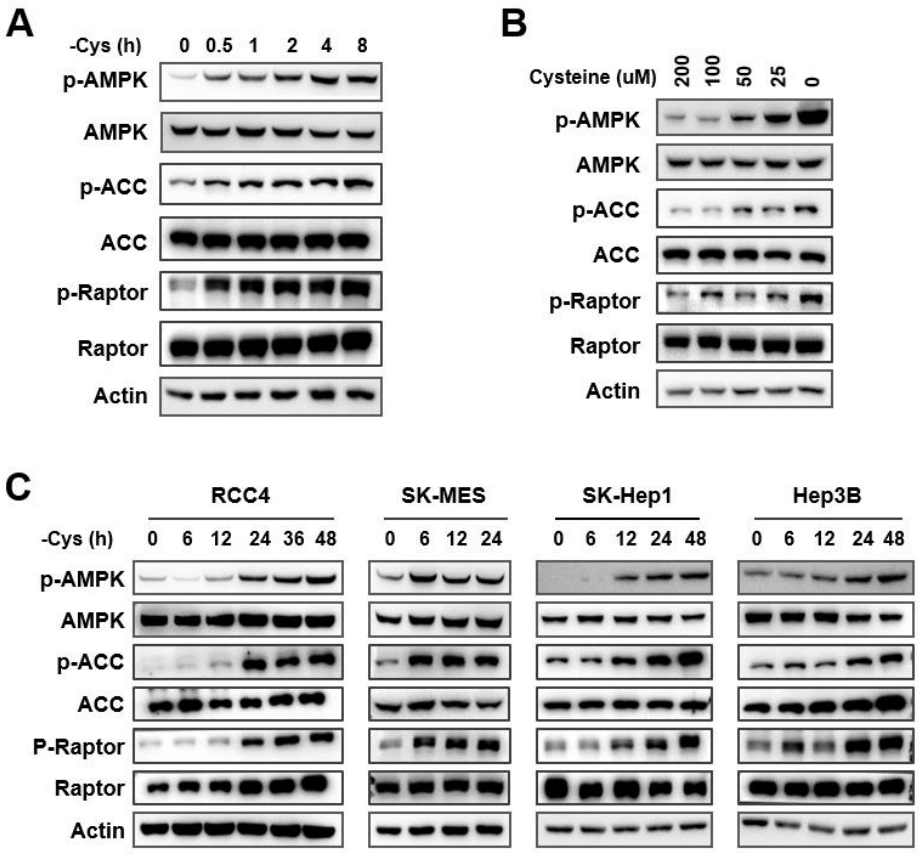

Figure R1. Cystine deprivation activates AMPK and its substrates. (**A-C**) 293T cells were treated with cystine-deficient medium for 0, 0.5, 1, 2, 4, or 8 hours (**A**) or treated with medium containing 200 μM, 100 μM, 50 μM, 25 μM or 0 μM cystine for 8 hours (**B**); RCC4, SK-MES,

SK-hep-1 or Hep3B cells were treated with cystine-deficient medium for indicated time (**C**). Protein levels of p-AMPK, p-ACC, p-Raptor and total AMPK, ACC, Raptor were measured by WB, and actin served as the loading control. Please see also **Figures 1B-D** in the revised manuscript.

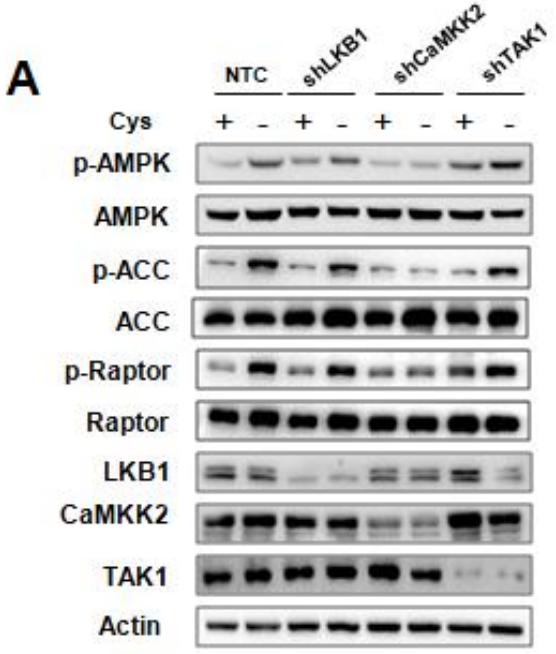

Figure R2. Cystine deprivation activates AMPK through CaMKK2. (**A**) WB analysis of p-AMPK, p-ACC, p-Raptor and total AMPK, ACC, Raptor protein expression in 293T cells transfected with shRNAs targeting LKB1, CaMKK2, TAK1 or non-targeting control (NTC) that were treated with cystine-deficient medium for 8 hours. Actin served as the loading control. Please see also **Figure 1F** in the revised manuscript.

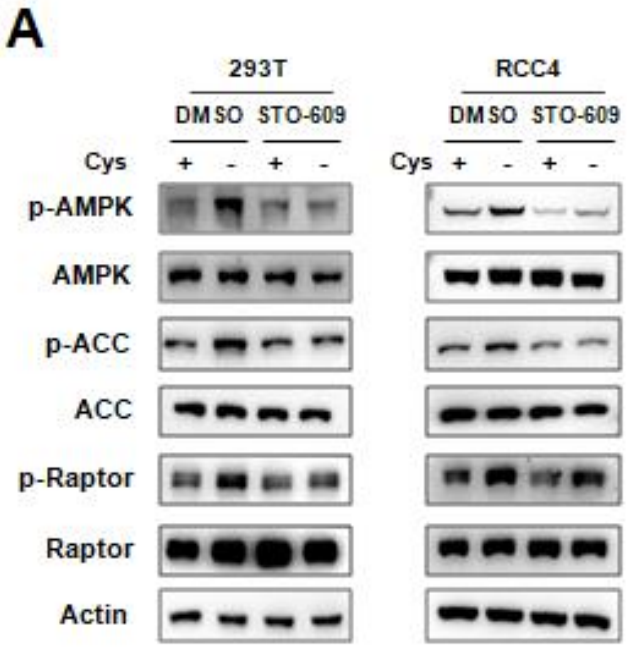

Figure R3. Cystine deprivation-induced AMPK activation was suppressed by CaMKK2 inhibitor STO-609. (A) WB analysis of p-AMPK, p-ACC, p-Raptor and total AMPK, ACC,

Raptor protein expression in 293T cells and RCC4 cells treated with 1 μg/ml STO-609 or DMSO for 8 hours during cystine deprivation. Actin served as the loading control. Please see also **Figure 1G** in the revised manuscript.

2. Additionally, demonstrating alterations in these downstream targets under various cellular conditions that effect this novel pathway (e.g., CARS over expression, CaMKK2 shRNA knockdown, and expression of n-terminal AMPKγ2) would greatly enhance the impact of the manuscript and put it in the context of well-described effectors studied by many labs.

Response: Thanks for this suggestion. We examined the phosphorylation levels of ACC and Raptor under various cellular conditions that may affect this novel pathway. Specifically, we found that overexpression of CARS promotes the phosphorylation of AMPK substrates such as ACC and Raptor (**Figure R5**), but knockdown of CaMKK2 shown the reverse trends (**Figure R2**). We have included these data in the revised manuscript (**Figures 2H, 1F**).

Additionally, overexpression of the wild-type AMPKγ2, but not N-terminus, or C-terminus of AMPKγ2 activates the phosphorylation of AMPK and its substrates (**Figure R9**). These results indicate that, although N-terminus of AMPKγ2 binds to CARS, only the full length AMPKγ2 has the ability to activate AMPK.

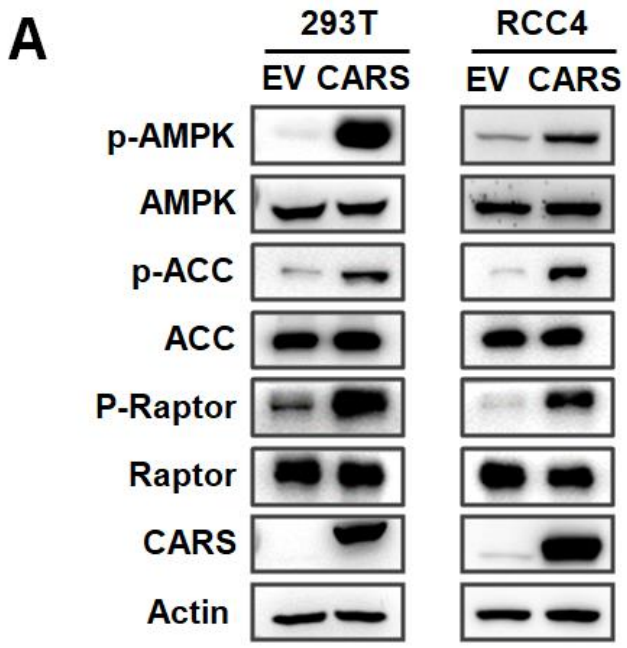

Figure R5. CARS overexpression promotes the activation of AMPK and its substrates. (**A**) WB analysis of p-AMPK, p-ACC, p-Raptor and total AMPK, ACC, Raptor, CARS protein expression in 293T cells and RCC4 cells overexpressing Flag-CARS. Actin served as the loading control. Please see also **Figure 2H** in the revised manuscript.

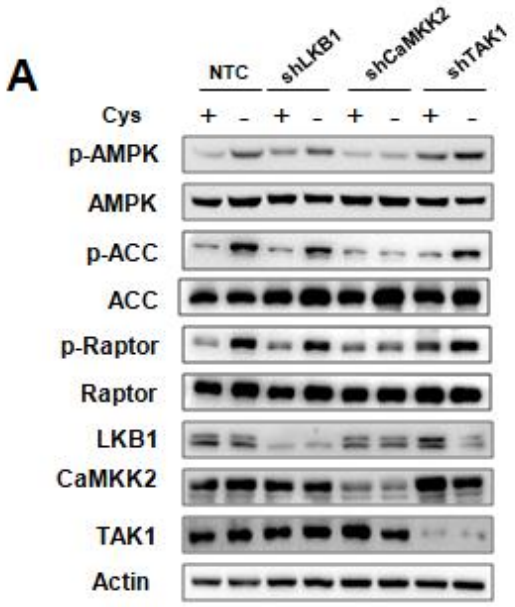

Figure R2. Cystine deprivation activates AMPK through CaMKK2. (**A**) WB analysis of p-AMPK, p-ACC, p-Raptor and total AMPK, ACC, Raptor protein expression in 293T cells transfected with shRNAs targeting LKB1, CaMKK2, TAK1 or non-targeting control (NTC) that were treated with cystine-deficient medium for 8 hours. Actin served as the loading control. Please see also **Figure 1F** in the revised manuscript.

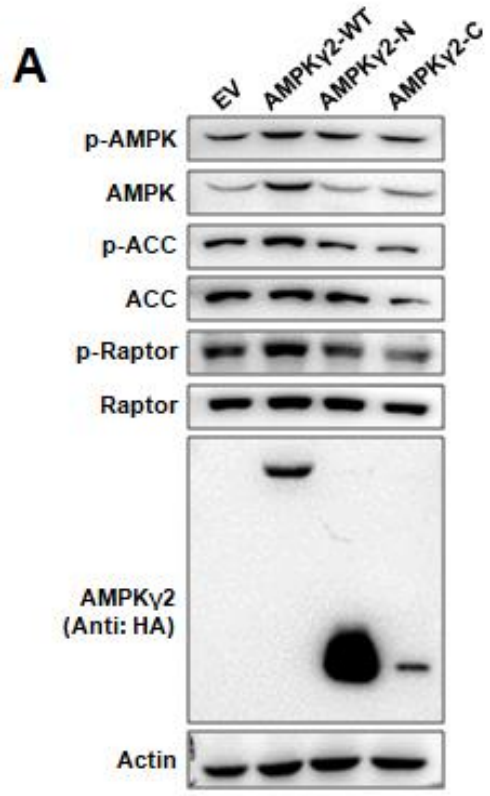

Figure R9. Overexpression of the wild-type AMPKγ2, but not N-terminus, or C-terminus of AMPKγ2 activates AMPK and its substrates. (**A**) WB analysis of p-AMPK, p-ACC, p-Raptor and total AMPK, ACC, Raptor protein expression in 293T cells overexpressing wild-type AMPKγ2, N-terminus, or C-terminus of AMPKγ2. Actin served as the loading control.

3. The studies presented do an excellent job of delineating a novel mechanism for cysteine dependent modulation of AMPK phosphorylation. The authors also acknowledge the relationship between AMPK and cellular redox status. Despite cysteine being a critical component of cellular redox metabolism, through direct effects and its contributions to glutathione metabolism, there are no references to the effects that cysteine deprivation has on the redox status of the cells, the production of reactive oxygen species, glutathione content, or glutathione redox status. The authors provide references to the potential benefits of limiting cysteine availability in vivo to various cancers (Cramer et al. 2016; Poursaitidis et al. 2017). Both of these references cite effects on reactive oxygen species production and redox handling as critical drivers of cell death in their respective models. Understanding the exact mechanisms underlying cysteine deprivation-mediated initiation of the CARS-CaMKK2-AMPK cascade is likely beyond the scope of this manuscript. However, given the known impacts of cysteine deprivation on ROS dependent cell death and the modulation of cell death by the CARS-CaMKK2-AMPK in this manuscript it would benefit the manuscript to demonstrate the effects of their cysteine deprivation model on redox status and glutathione metabolism. Authors should measure GSH:GSSG ratios in cysteine deprived cells and determining the impacts of various interventions (e.g. CARS over expression, CaMKK2 shRNA knockdown, and expression of n-terminal AMPKγ2) on this set of measurements, as this would greatly improve the manuscript and add clarity about any potential effect of ROS.

Response: We thank our reviewer for his/her positive comments and concerns on our novel findings. We agree with the reviewer that measure GSH:GSSG ratios is important to demonstrate the effects of cystine deprivation on redox status and glutathione metabolism. Following the reviewer's suggestion, we knocked down of CARS, CaMKK2, or AMPKγ2 in cells treated with or without cystine-deficient medium, and then measure GSH:GSSG ratios, as well as ROS levels. Results shown that cystine deprivation dramatically suppressed GSH:GSSG ratios, but knockdown of CARS, CaMKK2, or AMPKγ2 has litter effect on GSH:GSSG ratio and ROS level under cystine deprivation conditions (**Figure R10**). In addition, little changes of ROS

levels between normal and cystine deprivation conditions were observed, probably due to the short treatment time, which is consistent with the previous report (Wu et al., 2021). These results suggest that cysteine deprivation-activated CARS-CaMKK2-AMPK cascade in our study is independent of cellular redox status.

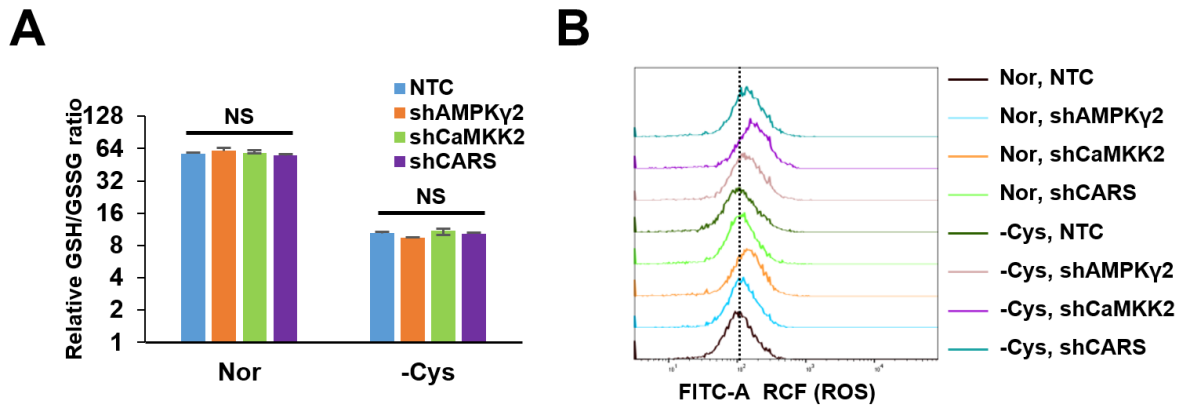

Figure R10. Knockdown of CARS, CaMKK2, or AMPKγ2 has marginal effect on GSH:GSSG ratio and ROS level under cystine deprivation conditions. (A, B) The GSH:GSSG ratio (**A**) and ROS level (**B**) were performed in 293T cells transfected with shRNAs targeting NTC, CARS, CaMKK2 or AMPKγ2 and further treated with cystine-deficient medium for 8 hours. GSH:GSSG ratio were measured using kit (Cat # G263) purchased from Dojindo following the manufacturer's instruction. For ROS detection, cells were stained with 5-(and-6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (Invitrogen; Cat # C400) and analyzed by flow cytometers following the manufacturer's instruction.

Reviewer minor comments

1. The authors discuss the impacts of the AMPK isoform expression on functional redundancy and expression localization specificity. Indeed, critical work has been done to delineate tissue expression profiles of AMPKγ subunits across multiple organisms (Mahlapuu et al., Am J PhysiolEndocrinolMetab, 2004) and the functional implications of differences in AMPKγ subunit homology (Willows et al., Biochem J, 2017). The authors describe their in silico comparisons of AMPKγ subunits, but do not acknowledge or cite that this has been previously done. This observation should be considered within the context of previous work on AMPKγ subunits with supporting citations.

Response: We thank our reviewer for pointing this out. We have now discussed the expression profiles of AMPKγ subunit and the different functional implications in AMPKγ subunit homology within the context of previous related work and have cited these important papers on page 7 in the revised manuscript (Mahlapuu et al., 2004;

Willows et al., 2017) during the revision.

2. The authors provide significant evidence as to the involvement of AMPKγ2, specifically, in this novel pathway and the protein domains involved in AMPKγ2-CARS interactions. The authors also demonstrate that cell lines lacking AMPKγ2 do not have cysteine deprivation-dependent activation of AMPK and are susceptible to cell death induced by lack of cysteine. The authors do not address how the reliance of this pathway on AMPKγ2 could be more or less relevant to specific types of cancer. Discussing which cell types and/or cancers would be most impacted by the AMPKγ2-dependence of the observed phenotypes would help to establish the scope of potential therapeutic applications.

Response: It's a good point. We thus referred to databases such as GEPIA to perform more analysis and studied the correlation between the cancer types and AMPKγ2 expressions. The results shown that AMPKγ2 is upregulated in kidney chromophobe (KICH), acute myeloid leukemia (LAML), pancreatic adenocarcinoma (PAAD), and thymoma (THYM); but downregulated in bladder urothelial carcinoma (BLCA), skin cutaneous melanoma (SKCM), and uterine carcinosarcoma (UCS) (**Figure R11**). Therefor, we further discussed this important issue of AMPKγ2-dependent potential therapeutic applications such as precision therapy on page 12 in the "Discussion" section in the revised manuscript.

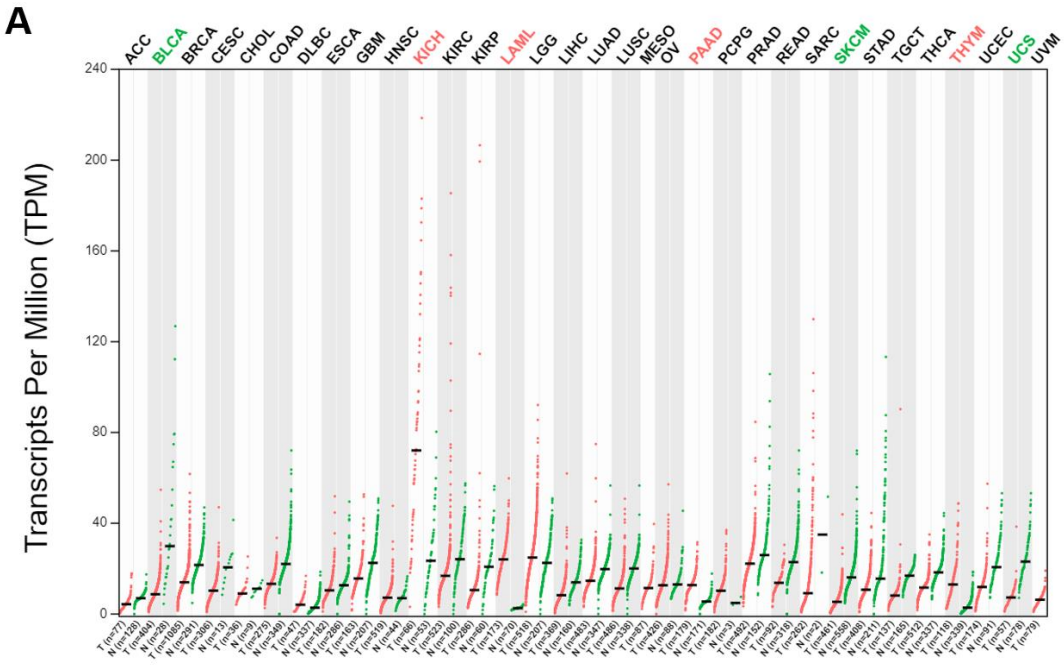

Figure R11. mRNA profile of PRKAG2 (AMPKγ2) in different tumors (Data from GEPIA). (A) The gene expression profile of AMPKγ2 across all tumor samples and paired normal tissues (Dot plot). Each dots represent expression of samples. Red: upregulated; Green: downregulated.

Tumor abbreviations: ACC: Adrenocortical carcinoma; BLCA: Bladder Urothelial Carcinoma; BRCA: Breast invasive carcinoma; CESC: Cervical squamous cell carcinoma and endocervical adenocarcinoma; CHOL: Cholangio carcinoma; COAD: Colon adenocarcinoma; DLBC: Lymphoid Neoplasm Diffuse Large B-cell Lymphoma; ESCA: Esophageal carcinoma; GBM: Glioblastoma multiforme; HNSC: Head and Neck squamous cell carcinoma; KICH: Kidney Chromophobe; KIRC: Kidney renal clear cell carcinoma; KIRP: Kidney renal papillary cell carcinoma; LAML: Acute Myeloid Leukemia; LGG: Brain Lower Grade Glioma; LIHC: Liver hepatocellular carcinoma; LUAD: Lung adenocarcinoma; LUSC: Lung squamous cell carcinoma; MESO: Mesothelioma; OV: Ovarian serous cystadenocarcinoma; PAAD: Pancreatic adenocarcinoma; PCPG: Pheochromocytoma and Paraganglioma; PRAD: Prostate adenocarcinoma; READ: Rectum adenocarcinoma; SARC: Sarcoma; SKCM: Skin Cutaneous Melanoma; STAD: Stomach adenocarcinoma; TGCT: Testicular Germ Cell Tumors; THCA: Thyroid carcinoma; THYM: Thymoma; UCEC: Uterine Corpus Endometrial Carcinoma; UCS: Uterine Carcinosarcoma; UVM: Uveal Melanoma.

3. The authors utilize a number of abbreviations and acronyms for proteins that are not fully defined including AMPK and CaMKK2. Providing full names of proteins and enzymes upon initial introduction would be helpful for the reader as not all readers may be familiar with certain proteins and their naming conventions.

Response: We thank the reviewer for pointing this out. We have now provided full names of AMPK and CaMKK2 in the “Abstract” section upon initial introduction in the revised manuscript.

4. The authors refer to AMPK as a "molecule" in the abstract and introduction. Referring to AMPK as a kinase or enzyme would be more appropriate due to its heterotrimeric nature referenced by the authors.

Response: We have made corresponding revisions in the revised manuscript.

5. The section heading "Cystine deprivation activates AMPK through CAMKK2" uses cystine, which is the oxidized dimeric form of cysteine. The title and most of the manuscript reference cysteine. This should be made consistent.

Response: We thank the reviewer for pointing this out. Following the similar suggestion of reviewer 1, we have replaced “cysteine-deficient media/cysteine deprivation” with “cystine-deficient media/cystine deprivation” throughout the manuscript during the revision.

6. Cystine deprivation activates AMPK through CAMKK2, Paragraph 1: "isoleucine or leucine deprivation also marginally activated AMPK". Comment: Using the term marginally for a treatment that significantly increased AMPK phosphorylation ~4-fold is misleading. Simply remove the word marginally.

Response: Thanks for this suggestion. We have made corresponding changes in the revised manuscript.

7. CARS is critical for CaMKK2-mediated AMPK activation under cysteine deprivation conditions, Paragraph 1: "...we speculated that they sensor is likely an upstream factor..." Comment: Simply correct "they" to "the".

Response: Thank you for pointing this out. We have corrected it in the revised manuscript.

8. CARS senses cysteine starvation to activate AMPK by binding to AMPKγ2, Paragraph 1: "On the other hand, the CARS protein structure analysis based on the online website UniProt (http://www.ebi.ac.uk/interpro/), we constructed vectors expressing three truncated fragments of the CARS protein..." Comment: This sentence is confusing and should be edited for clarification."

Response: We apologize for the confusion. We have now corrected this sentence as below: On the other hand, based on the CARS protein structure analysis via the online website UniProt (http://www.ebi.ac.uk/interpro/), we constructed three vectors expressing the N-terminus (N), middle fragment (M) and C-terminus (C) of the CARS proteins, respectively, in which the N-terminus fragment contains cysteine binding and catalytic domains, and the middle fragment contains tRNA binding domain. We have made corresponding change on page 7-8 in the revised manuscript.

9. Figure 1A: Comment: The labeling of bars in the densitometry histogram is inconsistent (only some bars are labeled) and inaccurate appears to be a combination of histidine and isoleucine abbreviations). The authors should clarify the labeling of this graph.

Response: We thanked the reviewer for pointing this out. In the revised manuscript, we have deleted this densitometry histogram and provided the values of p-AMPK/AMPK, p-ACC/ACC, p-Raptor/Raptor ratios to indicate AMPK activation in Figure 1A along with the blots in other Figures.

Thank you for all your constructive and insightful comments and suggestions.

Referee #3: (Remarks to the Author):

The manuscript by Yuan and colleagues reports on an interesting phenomenon whereby cysteine starvation leads to activation of AMPK-gamma2 containing complexes mediated by CAMKK2. The authors suggest that this may have important implications in some cancer cells that are resistant to cysteine deprivation, although this remains highly speculative. The model the authors present is straightforward but there are some obvious questions that arise that have not been addressed in the current manuscript. Dealing with these issues would strengthen greatly the impact of the study.

Response: We thank the reviewer for your encouraging and constructive comments. During the revision, we have addressed all your concerns and suggestions to strengthen the impact of this study.

Major Points:

1. In general, most of the data shown are from single blots, with no attempt at quantification. It would be important to include some evidence of replication and quantification throughout the paper. In addition (and importantly) blots are often shown without reference to molecular mass standards, and/or evidence of antibody validation.

Response: Following the reviewer's suggestion, we provided quantitative results of protein signaling and added the molecular mass standards (kDa) throughout this manuscript. Our antibodies are generally verified by qRT-PCR and WB using the cells expressing specific tartgeting shRNAs. Additionally, we also verified our antibodies through Research Resource Identifiers (RRIDs) (https://scicrunch.org/resources) and provided this information in the revised manuscript. For more details, please refere to **Table R1** with RRIDs information. Please see also **Supplementary Table 2** in the revised manuscript.

Table R1. Information of antibodies used in this study		
Reagent or Resource	**Source**	**Identifier**
Anti-p-AMPK antibody	CST	Cat# 2535; RRID:AB_331250
Anti-AMPK antibody	CST	Cat# 2532; RRID:AB_330331
Anti-AMPKγ2 antibody	CST	Cat# 2536s; RRID:AB_2170335
Anti-CARS antibody	Bethyl	Cat# A302-409A; RRID:AB_1907292
Anti-CaMKK2 antibody	Proteintech	Cat# 11549-1-AP; RRID:AB_2259441
Anti-LKB1 antibody	Proteintech	Cat# 10746-1-AP; RRID:AB_2271311
Anti-TAK1 antibody	Proteintech	Cat# 12330-2-AP; RRID:AB_2140101
Anti-Actin antibody	Proteintech	Cat# 66009-1-Ig; RRID:AB_2687938
Anti-AHCYantibody	Proteintech	Cat# 66019-1-Ig; RRID:AB_11044194
Anti-GCN2 antibody	Sangon Biotechnology	Cat# D121890;
Anti-SIRT3 antibody	CST	Cat# 2627; RRID:AB_2188622
Anti-p-Raptor-S792 antibody	CST	Cat# 2083, RRID:AB_2249475
Anti-Raptor antibody	Proteintech	Cat# 20984-1-AP; RRID:AB_11182390
Anti-p-ACC-S79 antibody	CST	Cat# 3661, RRID:AB_330337
Anti-ACC antibody	Proteintech	Cat# 67373-1-Ig; RRID:AB_2882621
Anti-Flag antibody	Sigma-Aldrich	Cat# F1804; RRID:AB_262044
Anti-His-Tag antibody	Sangon Biotechnology	Cat# D110002
Anti-GST-Tag antibody	Proteintech	Cat# 10000-0-AP; RRID:AB_11042316
Anti-HA-HRP antibody	Proteintech	Cat# 2999; RRID:AB_1264166
Goat Anti-Rabbit IgG (H + L)-HRP	Bio-Rad	Cat# 1706515; RRID:AB_11125142
Goat Anti-Mouse IgG (H + L)-HRP	Bio-Rad	Cat# 1706516; RRID:AB_11125547

2. An exception to the lack of quantification is shown in Fig. 1A, but this immediately presents a problem of its own. In the top panel of the blot, the pAMPK is very low in the control (Nor), but clearly increased in the minus cys condition. However, in the lower panel, the pAMPK signal in the control is relatively high (obviously higher than in the control lane above). A rough estimate by eye would suggest that the pAMPK:total AMPK values for control would well below 1 for the top panel, and above 1 for the bottom panel (so at least an order of magnitude difference). As similar

problem is evident for some of the amino acids. This is a worry. How were the values in the bar graph calculated? Any attempt at normalising the data between the two blots would introduce significant concerns.

Response: We apologize for not making this clear. Following the suggestions of our three reviewers, we repeated Fig 1A with dialyzed serum and provide western blotting images with phosphorylation levels of AMPK, ACC and Raptor to indicate AMPK activation, including cystine deprivation conditions in both panels (**Figure R8A**), which has been included in the revised manuscript as Figure 1A. In this way, the effect of deficiency of individual amino acid can be compared with cystine deficiency group and the normal control group. Further experiments shown that cystine deprivation activated AMPK and its substrates including ACC and Raptor, in both medium with dialyzed serum or non-dialyzed serum (**Figure R8B**). We have addressed the similar important concerns raised by the reviewer # 1, here we present the results again for your convenience.

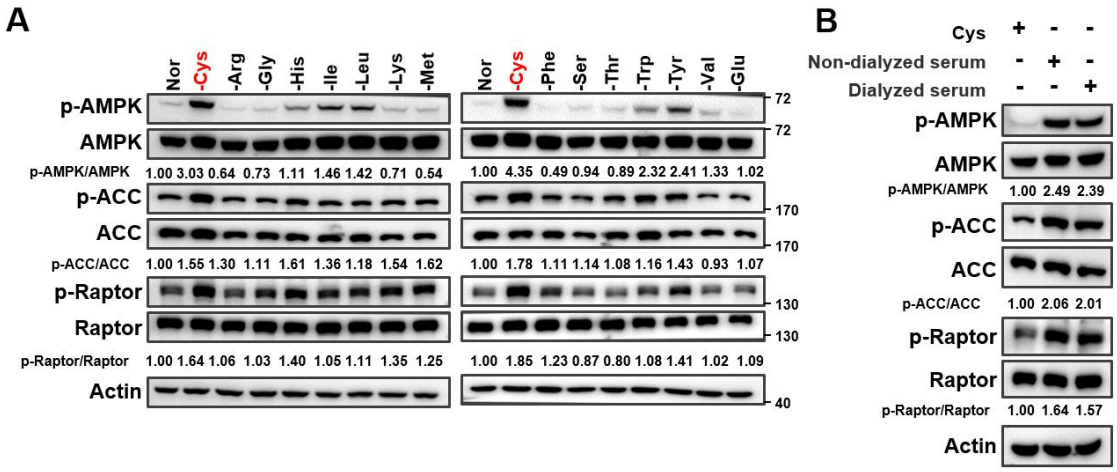

Figure R8. Cystine deprivation markedly activates AMPK and its substrates. (A) 293T cells were cultured in complete medium made of dialyzed serum for 24 hours which was then replaced with medium in which one amino acid was eliminated and cultured for 8 hours. Then, WB was used to measure p-AMPK, p-ACC, p-Raptor and total AMPK, ACC, Raptor protein expression. Actin served as the loading control. Please see also **Figure 1A** in the revised manuscript. (**B**) 293T cells were cultured in complete medium made of dialyzed serum or non-dialyzed serum for 24 hours which was then replaced with medium in which cystine was eliminated and cultured for 8 hours. WB was used to measure p-AMPK, p-ACC, p-Raptor and total AMPK, ACC, Raptor, CARS protein expression. Actin served as the loading control.

3. Was any attempt made to determine binding between endogenous proteins (rather

than using over-expressed proteins)? As the authors point out, AMPK is a heterotrimeric complex. It is important to show that in the over-expression studies (where usually it appears only a single AMPK subunit is transfected) whether the other two subunits are present. Related to this point, in the experiment shown in Fig. 3A, results showing interaction when alpha, beta and gamma subunits are co-transfected should be included. In vivo, AMPK would be expected to be present as the heterotrimeric complex, so recapitulating this in the experimental model is important.

Response: We thank the reviewer for this suggestion. Following the similar suggestions of reviewer 1 and reviewer 3, we performed additional Co-IP experiments to study the interaction between endogenous proteins. Concerned with the potential issues related to antibody specificity for endogenous IP, we detected and validated the interaction between endogenous AMPKα vs endogenous CaMKK2 (**Figure R7A, R7C**), endogenous CaMKK2 vs endogenous CARS (**Figure R7B**), and AMPKγ2 with HA tag vs endogenous CARS (**Figure R7D**) under indicated conditions. We have now included Figure R7A-D as **Figures 1I, 2C/G, 3G** in the revised manuscript, respectively.

In order to evaluate the interaction of CARS and AMPK heterotrimeric complex/subunits under the conditions of Fig 3A in the original manuscript, we co-transfecteded alpha, beta and gamma subunits in the cells and found that CARS could interact with the AMPK complex (**Figure R12**). Through analysis of the results of Figure R12, and Figure 3A in our original manuscript, we think CARS could interact with the AMPK complex via AMPKγ2.

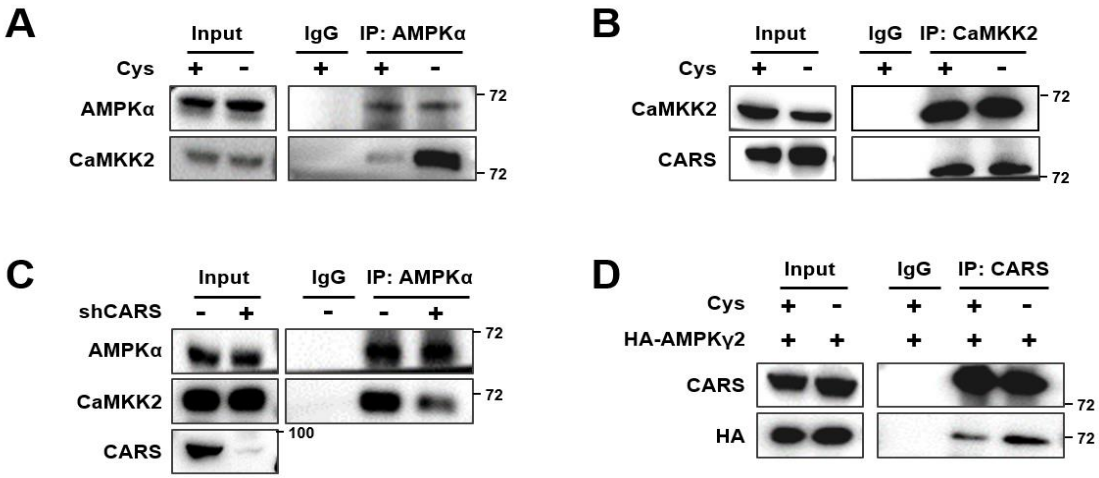

Figure R7: Detection of the binding activity of AMPKα VS CaMKK2, CaMKK2 VS CARS, and HA-AMPKγ2 VS CARS under the indicated conditions. (**A**) 293T cells cultured with cystine-deficient medium or complete medium for 8 hours were harvested and subjected to immunoprecipitation with anti-AMPKα, followed by WB analysis with anti-AMPKα and anti-CaMKK2. (**B**) 293T cells cultured with cystine-deficient medium or complete medium for 8 hours were harvested and subjected to immunoprecipitation with anti-CaMKK2, followed by WB analysis with anti- CaMKK2 and anti-CARS. (**C**) 293T cells were transfected with shRNAs targeting CARS or NTC, then they were cultured with cystine-deficient medium for 8 hours. Cell lysates were immunoprecipitated with anti-AMPKα and subjected to WB analysis with anti-AMPKα and anti-CaMKK2. (**D**) 293T cells were transfected with HA-AMPKγ2 for 48 hours and then cultured with cystine-deficient medium or complete medium for 8 hours. Cell lysates were immunoprecipitated with anti-CARS, followed by WB analysis with anti-HA and anti-CARS. The medium used in Figure R7 is prepared with dialyzed serum. Please see also **Figures 1I, 2C/G, 3G** in the revised manuscript.

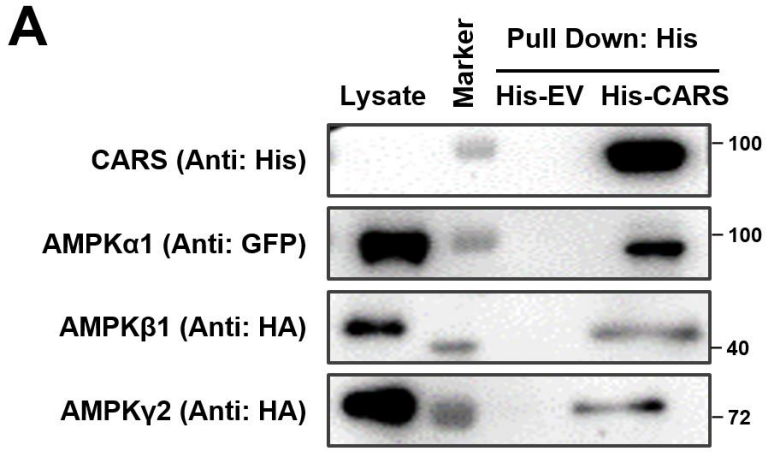

Figure R12: Interation of AMPKα, AMPKβ, or AMPKγ with CARS. (**A**) His pull down analysis using recombinant His-CARS proteins and cell lysates from 293T cells co-expressing AMPKα1, AMPKβ1 and AMPKγ2. After incubation for 4 hours with pull-down buffer (PDB) (150 mM NaCl, 50 mM Tris (pH 7.5), 0.1% NP-40, 5 mM DTT), His beads were pelleted and washed with PDB buffer followed by elution of proteins and western blot analysis with anti-His, anti-GFP, and anti-HA antibodies.

4. The model for CAMKK2 dependence is intriguing, but the authors don't monitor whether cysteine starvation alters calcium levels in the cell. This is an important aspect of the mechanism (either way - if calcium is not increased by cysteine starvation how is CAMKK2 activated?) and so should be included. Related to this, the study by DallePezze (Nat Comms 2016) that the authors cite reports that re-addition of amino acids activates AMPK (implying that amino acid starvation inhibits AMPK) via CAMKK2. A more nuanced discussion on this point is required here.

Response: We thank the reviewer for the insightful suggestion. Accordingly, we have

performed additional experiments and found that, although amino acid deprivation promoted accumulation of intracellular calcium levels (Ghislat et al., 2012), ther is no significant changes of intracellular calcium levels under cystine starvation conditions compared with normal conditions (**Figure R13**). We thus believe that the activation of CaMKK2 in our study is mainly resulted from the interaction of CaMKK2 and AMPKα. We have now further discussed this important issue of the relationship between amino acid, CaMKK2, and AMPK on page 10 in the revised manuscript.

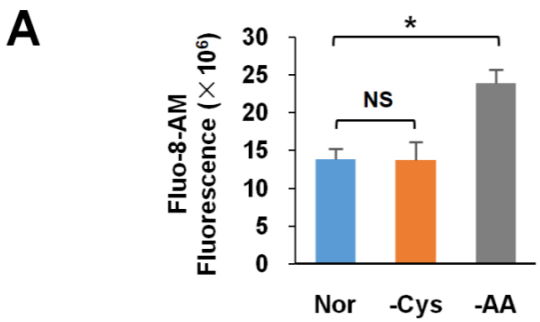

Figure R13. Intracellular calcium levels were not affected under cystine deprivation conditions. 293T cells were treated with complete medium, cystine-deficient medium, or amino acids-deficient medium for 8 hours, followed by incubation with the fluorescent calcium binding dye Fluo-8 AM. After 1 hour of incubation, $Ca2^{+}$ concentration was monitored by HCS imaging system (PerkinElmer, USA) and quantified by Harmony software.

5. The finding that activation is restricted to the gamma2 AMPK complex is very interesting. In most cell types, gamma2 AMPK is a minor component (gamma1 complexes accounting for most of the AMPK present in the cell). This raises some complex mechanistic issues. In Fig. 2D, knockdown of CARS reduces basal AMPK phosphorylation (suggesting total AMPK activity is affected - including gamma1-containing complexes). In Fig. 3D/E, knockdown of gamma2 has little effect on basal AMPK phosphorylation (suggesting that total AMPK activity is not affected - which is what would be expected if gamma2 is a minor component of total AMPK). It is difficult to reconcile these different findings. Monitoring phosphorylation of endogenous downstream AMPK targets (e.g. pACC, pULK1) would provide useful information related to this issue. Adding this data for both cysteine starvation, CARS knockdown and CARS over-expression would be very helpful and informative.

Response: We thank the reviewer for pointing this out. In Fig. 3D/E, knockdown of AMPKγ2 indeed had little effect on basal AMPK phosphorylation in our original manuscript. We think it might be due to the lower knockdown eficiency of AMPKγ2

shRNAs. During the revision, we knocked down AMPKγ2 more efficiently by siRNA and observed that cystine deprivation- or CARS overexpression-induced AMPK phosphorylation was attenuated by AMPKγ2 knockdown (**Figure R6**). Moreover, we also detected phosphorylation levels of ACC and Raptor to indicate AMPK activity in the original Figure 1B/C (cystine starvation) (**Figure R1**), 2D/F (CARS knockdown and CARS over-expression) (**Figures R4-5**), 3D/E (AMPKγ2 knockdown) (**Figure R6**). We have included Figures R1, R4, R5, R6 as **Figures 1B-C, 2E, 2H, 3D-E** in the revised manuscript, respectively. As we have answered the similar important questions by the reviewer # 1, here we present the results again for your convenience.

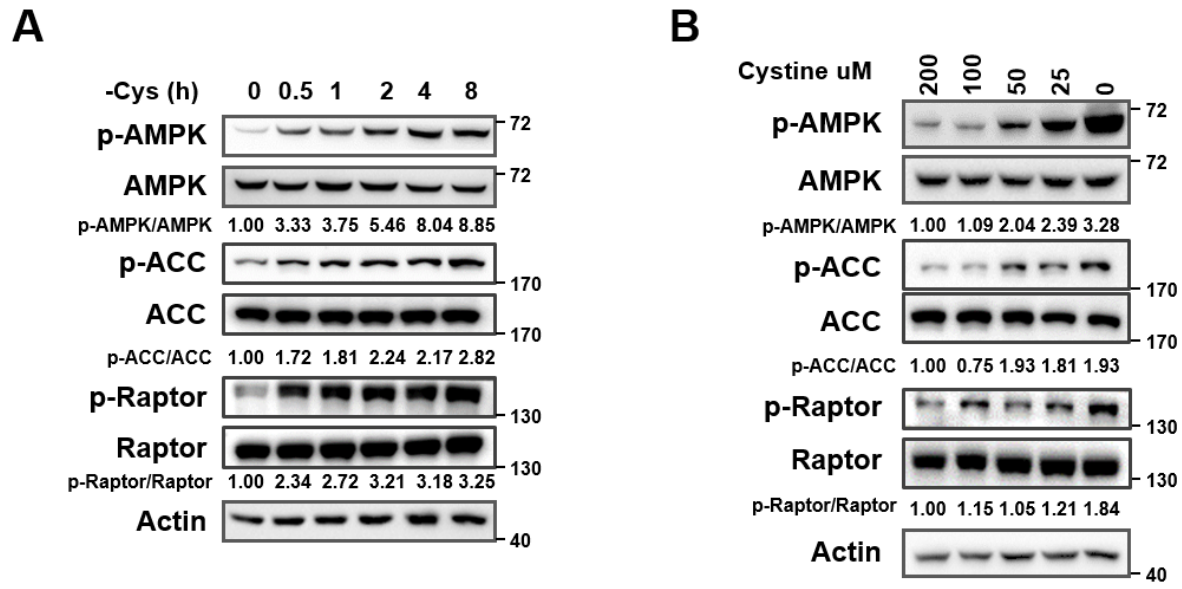

Figure R1. Cystine deprivation activates AMPK and its substrates. (**A-B**) 293T cells were treated with cystine-deficient medium for 0, 0.5, 1, 2, 4, or 8 hours (**A**) or treated with medium containing 200 μM, 100 μM, 50 μM, 25 μM or 0 μM cystine for 8 hours (**B**);. Protein levels of p-AMPK, p-ACC, p-Raptor and total AMPK, ACC, Raptor were measured by WB, and actin served as the loading control. Please see also **Figures 1B-C** in the revised manuscript.

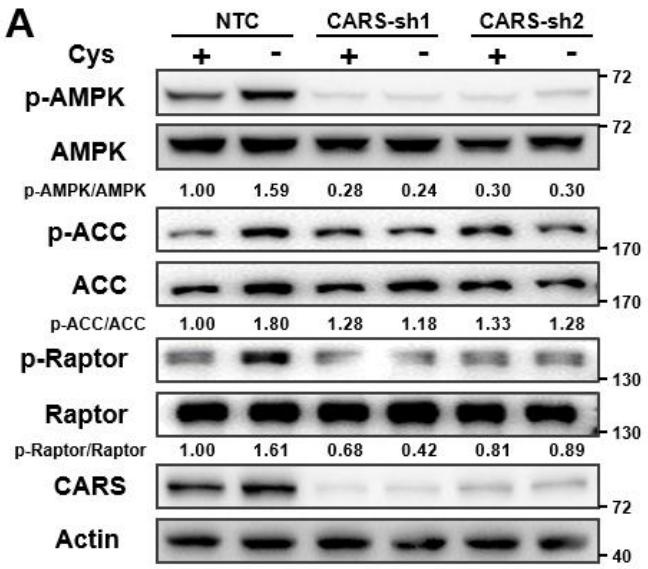

Figure R4. Cystine deprivation-induced AMPK activation was suppressed by CARS knockdown. (**A**) WB analysis of p-AMPK, p-ACC, p-Raptor and total AMPK, ACC, Raptor protein expression in 293T cells transfected with shRNAs targeting CARS that were further treated with cystine-deficient medium for 8 hours. Actin served as the loading control. Please see also **Figure 2E** in the revised manuscript.

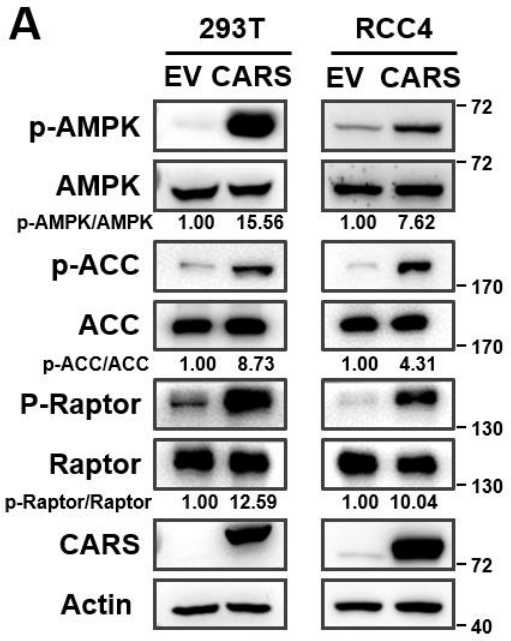

Figure R5. CARS overexpression promotes the activation of AMPK and its substrates. (**A**) WB analysis of p-AMPK, p-ACC, p-Raptor and total AMPK, ACC, Raptor, CARS protein expression in 293T cells and RCC4 cells overexpressing Flag-CARS. Actin served as the loading control. Please see also **Figure 2H** in the revised manuscript.

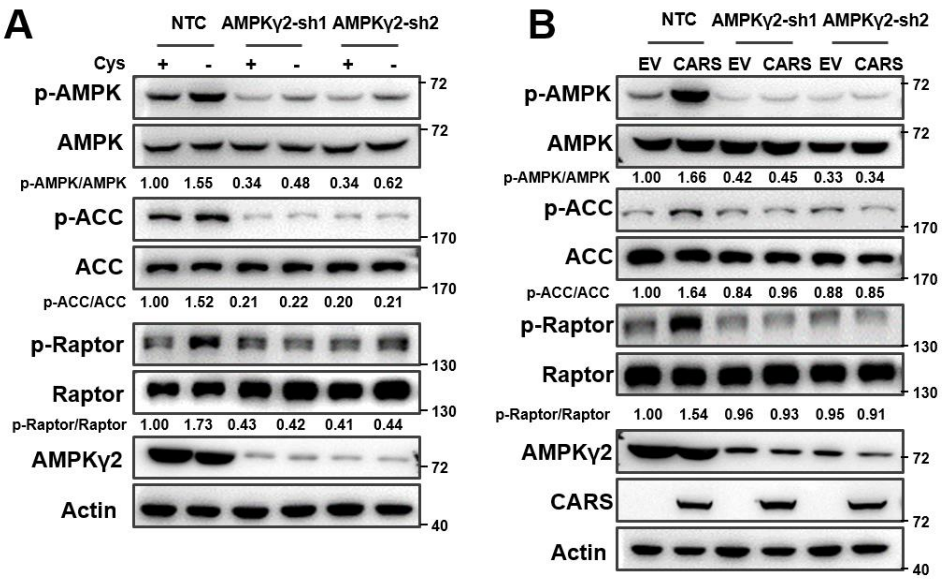

Figure R6. Cystine deprivation- or CARS overexpression-induced AMPK activation was suppressed by AMPKγ2 knockdown. (A-B) WB analysis of p-AMPK, p-ACC, p-Raptor and total AMPK, ACC, Raptor protein expression in 293T cells transfected with siRNAs targeting AMPKγ2 that were further treated with cystine-deficient medium for 8 hours (**A**) or transfected with Flag-CARS for 48 hours (**B**). Actin served as the loading control. Please see also **Figure 3D-3E** in the revised manuscript.

6. Related to point 5, the suggestion that activation of AMPK (other than gamma2-containing complexes) by AICAR can rescue the growth phenotype following cysteine starvation is interesting and perhaps counter-intuitive. AICAR is known to have AMPK-independent effects, so it would be good to complement this study with more selective (and potent) direct AMPK activators (2nd generation activators such as the Pfizer and Merck compounds).

Response: We agree with the reviewer's comments. There were reports of AMPK-independent effects of AICAR (Boss et al., 2016; Kirchner et al., 2018). Following this suggestion, we used the second-generation of AMPK activator PT1 and GSK (Jensen et al., 2015; Sujobert et al., 2015) to repeat the experiments of Figure 4C/D in the original manuscript. Results shown that knockdown of CARS, CaMKK2 or AMPKγ2 markedly induced cell death under cystine deprivation conditions, which was attenuated by addition of the AMPK activator PT1, and GSK (**Figure R14A**). Similar results were observed when cell death was detected by flow cytometry (**Figure R14B**). We have included these data in the revised manuscript

(**Figures EV4C-D**).

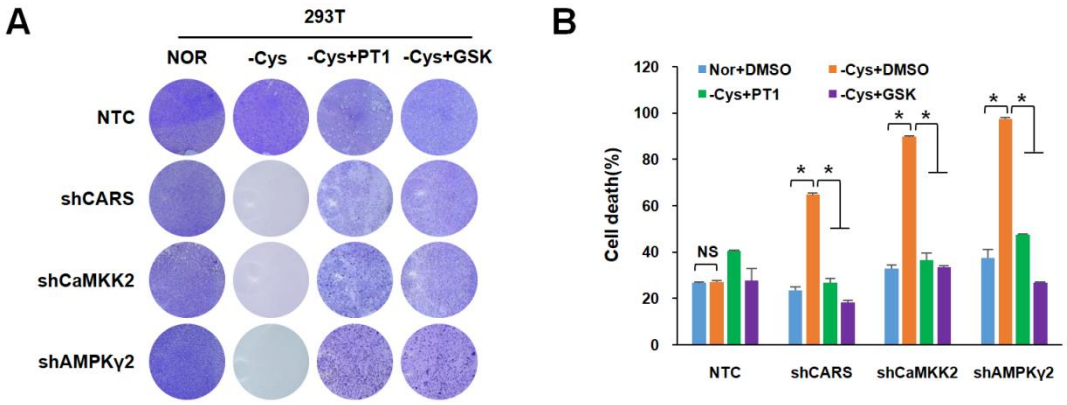

Figure R14. Knockdown of CARS, CaMKK2, or AMPKγ2-induced cell death was attenuated by addition of AMPK activator PT1 or GSK under cystine deprivation conditions. (**A, B**) The crystal violet assay (**A**) and apoptosis rate assay (**B**) were performed in 293T cells transfected with shRNAs targeting NTC, CARS, CaMKK2 or AMPKγ2 and further treated with cystine-deficient medium for 24 hours with or without 100 μM PT1, or 30 μM GSK. NS, not significant; *P < 0.05, compared with the indicated groups. Please see also **Figures EV4C-D** in the revised manuscript.

Thank you for all your insightful comments and suggestions that have helped us improve this study substantailly.

Reference

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1st Revision - Editorial Decision 23rd Jul 2021

Dear Ping,

Thank you for submitting your revised manuscript (EMBOJ-2021-108028R) to The EMBO Journal. Your amended study was sent back to the reviewers for re-evaluation, and we have received comments from two of them, which I enclose below. Please note that we have editorially assessed your response to referee #2 and found the issues raised by him/her to be satisfactorily addressed. As you will see, the other referees stated that their critique have been comprehensively considered and resolved and they are now broadly in favour of publication, pending minor revision.

Thus, we are pleased to inform you that your manuscript has been accepted in principle for publication in The EMBO Journal.

Please consider the remaining minor issues stated by the referees carefully by adding complementary data or introducing caveats in the text where appropriate, We also need you to address a number of points related to formatting and data representation at re-submission as detailed below.

Please contact me at any time if you have additional questions related to below points.

As you might remember from previous experience, every paper at the EMBO Journal now includes a 'Synopsis', displayed on the html and freely accessible to all readers. The synopsis includes a 'model' figure as well as 2-5 one-short-sentence bullet points that summarize the article. I would appreciate if you could provide this figure and the bullet points.

Thank you for giving us the chance to consider your manuscript for The EMBO Journal. I look forward to your final revision.

Again, please contact me at any time if you need any help or have further questions.

Kind regards,

Daniel

Daniel Klimmeck PhD
Senior Editor
The EMBO Journal

Formatting changes required for the revised version of the manuscript:

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>> Provide main figures and EV figures as individual, high-resolution .tiff files.

>> Dataset EV legends: EV tables should be uploaded separately with their legends/titles removed from the manuscript and zipped to each file.

>> Recheck callouts and their correct order in the main text for Figures EV1C,D ; EV2F ; EV3A and EV4A,B.

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Referee #1:

It is now widely recognised that changes in extracellular nutrient availability influence numerous biological processes. However, there is a surprising paucity of knowledge regarding the relationship between changes in amino acid availability and regulation of the critical energy/nutrient-sensing enzyme AMPK. This excellent study provides compelling mechanistic insight into the regulation of AMPK activity following cysteine deprivation and demonstrates that AMPK activation can contribute to cell survival following cysteine deprivation.

The authors have thoroughly addressed both the major and minor concerns raised in my initial review and I support publication of this article. The only other suggestion I have relates to the following comment on Page 4 - "The results showed that, of the 15 amino acids tested, cystine deprivation markedly activated AMPK, as indicated by its phosphorylation, in both 293T cells and RCC4 cells, although isoleucine or leucine deprivation also activated AMPK (Figs 1A and EV1A). Thus, we focused on cystine deprivation and AMPK activation." Quantification of phospho/total protein ratios (AMPK, ACC and Raptor) in Fig 1A suggests that Trp and Tyr deprivation might also impact AMPK activity. It is therefore not entirely accurate to suggest that cysteine, isoleucine and leucine are the only amino acids that impact AMPK and the statement should be rephrased to better reflect the data. This is a minor point as, without question, the most significant activation of AMPK is induced upon cystine deprivation.

Referee #3:

The authors have made considerable changes to their original submission and as a result, the manuscript is improved. There still remain a few significant concerns that weaken the study. If the

authors could address these it would remove any lingering issues I have with the manuscript.

1. Although the authors have added some evidence of quantification of the blots, many of the figures still report single blots, so there is no evidence of reproducibility. Have the blots been replicated using independent samples, or are they single studies? This should be made clear throughout the manuscript. If the blots are from single studies this weakens the conclusions. For instance, in Figure R9 in the rebuttal response the data is not very convincing and quantification and evidence of reproducibility would be required to substantiate the authors claims.

2. The apparent preference for CARS/CAMKK2 binding to gamma2 is intriguing but still requires some additional controls to be conclusive. In Figure R7 panels A and B, CARS/CAMKK2 association with endogenous AMPK following immunoprecipitation should be monitored in cells lacking gamma2 (using siRNA depletion). This would be a more convincing experiment to show specific interaction with gamma2 than deleting AMPK alpha or over-expressing gamma2. In Fig. R7 panel B, the band detected in the IP with CARS appear to migrate differently from the CARS blot in the input lane. An additional IP from cells depleted with CARS would help resolve this issue.

3. AMPK activation rescuing cell growth (Figure R14) still seems counter-intuitive to me. It would be helpful to include a control treatment with AMPK alpha depletion to determine whether knockdown of total AMPK attenuates cell growth rescue. PT1 is not a good compound to use in this case as it's mechanism of action is disputed (see Jensen et al PMID: 25695398). At any rate it is not a second generation AMPK activator binding via the ADaM site.

The authors performed the requested editorial changes.

2nd Revision - Editorial Decision 4th Aug 2021

Dear Ping,

Thank you for submitting the revised version of your manuscript. I have now evaluated your amended manuscript and concluded that the remaining minor concerns have been sufficiently addressed.

Thus, I am pleased to inform you that your manuscript has been accepted for publication in the EMBO Journal.

Please note that it is EMBO Journal policy for the transcript of the editorial process (containing referee reports and your response letter) to be published as an online supplement to each paper. I would thus like to ask for your consent on keeping the additional referee figures included in this file.

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If you have any questions, please do not hesitate to call or email the Editorial Office.

Thank you again for this contribution to The EMBO Journal and congratulations on a successful publication! Please consider us again in the future for your most exciting work.

Kind regards,

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The data shown in figures should satisfy the following conditions:

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- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
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- a statement of how many times the experiment shown was independently replicated in the laboratory.
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Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

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

B- Statistics and general methods

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1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	No Pre-determination of sample sizes was done. Instead, at least three independent experiments were performed to support the statements.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	N/A
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No samples were excluded from the anlysis.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	N/A
For animal studies, include a statement about randomization even if no randomization was used.	N/A
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	The investigators were blinded to allocation during experiments and outcome assessment.
4.b. For animal studies, include a statement about blinding even if no blinding was done	N/A
5. For every figure, are statistical tests justified as appropriate?	Yes. Data of Figure 4B, D, G and Figure EV 4A, B, D are presented as the mean (± SD) of three independent experiments.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes. A two-tailed Student's t-test is used to test the difference between the samples.
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Is the variance similar between the groups that are being statistically compared?	Yes

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	All the antibody information were described in materials and methods section (Manuscript Page 14). We verified our antibodies through Research Resource Identifiers (RRIDs) (https://scicrunch.org/resources) and provided this information as Table EV2.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	All cells used in this study were purchased from ATCC and cultured in Dulbecco's modified essential medium (DMEM) containing 10% FBS and 1% penicillin-streptomycin. All cell lines were authenticated. All the cells have been tested and are free of mycoplasma contamination.

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

D- Animal Models

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

E- Human Subjects

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

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	The mass spectrometry proteomics data are deposited to PRIDE and available via ProteomeXchange with identifier PXD027017.
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	N/A
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	N/A
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	N/A

G- Dual use research of concern

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