EMBO Molecular Medicine

ERK5 suppression overcomes FAK inhibitor resistance in mutant KRAS-driven non-small cell lung cancer

Georgia Konstantinidou, Chiara Pozzato, Gonçalo Outeiro-Pinho, Mirco Galiè, and Giorgio Ramadori

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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.)

6th Nov 2023

Decision on your manuscript EMM-2023-18934

Dear Prof. Konstantinidou,

Thank you for the submission of your research manuscript to our editorial offices. I have now had the opportunity to read it and to discuss it with the other members of our editorial team. I am afraid we all agree that the manuscript is not well suited for publication in EMBO Molecular Medicine and have therefore decided not to proceed with its handling and peer review.

The study identifies ERK5 as a mediator of FAK resistance in mutant KRAS-driven non-small cell lung cancer. We do recognize potential interest the findings, however, translational implications of your work are not further developed, limiting the overall clinical insights that are key for publication in EMBO Molecular Medicine. In particular, ERK5 inhibition as a potential co-targeting strategy to counteract FAK inhibitor resistance in NSCLC requires further investigation in an appropriate in vivo model. Therefore, I am afraid that we cannot offer further consideration to your article.

That being said, I discussed your work with editors at our sister journal Life Science Alliance (www.life-science-alliance.org). Life Science Alliance is an open access journal launched in partnership between EMBO, Rockefeller, and Cold Spring Harbor Laboratory Presses, and it publishes work that is of high value to the respective communities across all areas in the life sciences. I am glad to say that Life Science Alliance is interested in considering your work for publication and would like to send it out for formal peer-review.

I am very sorry to disappoint you on this occasion and I hope you will view the encouragement of a transfer favorably. If this is the case, please use the link below to transfer the manuscript directly; no re-formatting is required.

Yours sincerely,

Zeljko Durdevic

Zeljko Durdevic
Editor
EMBO Molecular Medicine

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Authors' Correspondence **6th Nov 2023**

Dear Dr Durdevic,
Thank you for evaluating our manuscript. We are currently performing the co-targeting of ERK5 and FAK in FAK-resistant lung tumors in our lung adenocarcinoma mouse model. Would that be sufficient to accept a resubmission of our manuscript and have it send out for review?
Thanks a lot in advance,
Best regards,
Georgia

Editor's Correspondence **6th Nov 2023**

Dear Georgia,

Thank you for the inquiry. We would welcome resubmission of the manuscript with the co-targeting of ERK5 and FAK in FAK-resistant lung tumors in a lung adenocarcinoma mouse model. Of course, if the results support potential clinical translation.

Best wishes,
Zeljko

19th Mar 2024

Dear Prof. Konstantinidou,

Thank you for the submission of your manuscript to EMBO Molecular Medicine, and please accept my apologies for the delay in getting back to you, which is due to the fact that one referee needed more time to complete his/her review. We have now received feedback from the three reviewers who agreed to evaluate your manuscript.

As you will see from the reports pasted below, while the referee #2 supports publication of the manuscript, referees #1 and #3 recognize potential interest and clinical relevance of the study but also raise important concerns that should be addressed in a mjor revision of the current manuscipt. Addition of experiments using a human LUAD xenograft model as suggested by the referee #3 is encouraged, however and as mentioned by the referee (point 5) this can also be discussed as a limitation of the study. If you would like to discuss further the points raised by the referees, I am available to do so via email or video. Let me know if you are interested in this option.

Further consideration of a revision that addresses reviewers' concerns in full will entail a second round of review. EMBO Molecular Medicine encourages a single round of revision only and therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript. For this reason, and to save you from any frustrations in the end, I would strongly advise against returning an incomplete revision.

We would welcome the submission of a revised version within three to six months for further consideration. Please let us know if you require longer to complete the revision.

I look forward to receiving your revised manuscript.

Yours sincerely,

Zeljko Durdevic

Zeljko Durdevic
Editor
EMBO Molecular Medicine

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When submitting your revised manuscript, please carefully review the instructions that follow below. We perform an initial quality control of all revised manuscripts before re-review; failure to include requested items will delay the evaluation of your revision.

We require:

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). For guidance, download the 'Figure Guide PDF': (https://www.embopress.org/page/journal/17574684/authorguide#figureformat).

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.

4) A complete author checklist, which you can download from our author guidelines (https://www.embopress.org/page/journal/17574684/authorguide#submissionofrevisions). Please insert information in the checklist that is also reflected in the manuscript. The completed author checklist will also be part of the RPF.

5) Please note that all corresponding authors are required to supply an ORCID ID for their name upon submission of a revised manuscript.

6) It is mandatory to include a 'Data Availability' section after the Materials and Methods. Before submitting your revision, primary datasets produced in this study need to be deposited in an appropriate public database, and the accession numbers and database listed under 'Data Availability'. Please remember to provide a reviewer password if the datasets are not yet public (see https://www.embopress.org/page/journal/17574684/authorguide#dataavailability).

In case you have no data that requires deposition in a public database, please state so in this section. Note that the Data Availability Section is restricted to new primary data that are part of this study.

7) For data quantification: please specify the name of the statistical test used to generate error bars and P values, the number (n) of independent experiments (specify technical or biological replicates) underlying each data point and the test used to calculate p-values in each figure legend. The figure legends should contain a basic description of n, P and the test applied. Graphs must include a description of the bars and the error bars (s.d., s.e.m.). See also 'Figure Legend' guidelines: https://www.embopress.org/page/journal/17574684/authorguide#figureformat

8) At EMBO Press we ask authors to provide source data for the main manuscript figures. Our source data coordinator will contact you to discuss which figure panels we would need source data for and will also provide you with helpful tips on how to upload and organize the files.

9) 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 .

10) We replaced Supplementary Information with Expanded View (EV) Figures and Tables that are collapsible/expandable online. A maximum of 5 EV Figures can be typeset. EV Figures should be cited as 'Figure EV1, Figure EV2" etc... in the text and their respective legends should be included in the main text after the legends of regular figures.

- For the figures that you do NOT wish to display as Expanded View figures, they should be bundled together with their legends in a single PDF file called *Appendix*, which should start with a short Table of Content. Appendix figures should be referred to in the main text as: "Appendix Figure S1, Appendix Figure S2" etc.

- Additional Tables/Datasets should be labeled and referred to as Table EV1, Dataset EV1, etc. Legends have to be provided in a separate tab in case of .xls files. Alternatively, the legend can be supplied as a separate text file (README) and zipped together with the Table/Dataset file.

See detailed instructions here:

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11) The paper explained: EMBO Molecular Medicine articles are accompanied by a summary of the articles to emphasize the major findings in the paper and their medical implications for the non-specialist reader. Please provide a draft summary of your article highlighting

- the medical issue you are addressing,

- the results obtained and

- their clinical impact.

This may be edited to ensure that readers understand the significance and context of the research. Please refer to any of our published articles for an example.

12) For more information: There is space at the end of each article to list relevant web links for further consultation by our readers. Could you identify some relevant ones and provide such information as well? Some examples are patient associations, relevant databases, OMIM/proteins/genes links, author's websites, etc...

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14) A Conflict of Interest statement should be provided in the main text.

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Please also suggest a striking image or visual abstract to illustrate your article as a PNG file 550 px wide x 300-800 px high.

EMBO Molecular Medicine has a "scooping protection" policy, whereby similar findings that are published by others during review or revision are not a criterion for rejection. Should you decide to submit a revised version, I do ask that you get in touch after three months if you have not completed it, to update us on the status.

***** Reviewer's comments *****

Referee #1 (Comments on Novelty/Model System for Author):

Results are novel, and experiments performed with high technical quality and adequate model systems. Results could have clinical impact to treat patients with lung cancer.

Referee #1 (Remarks for Author):

In this manuscript, Pozzato and colleagues investigate possible mechanisms that lead to FAK inhibitor resistance in KRAS mutant LUAD. In particular, the authors identify ERK5 as mediator of FAK inhibitor resistance providing a possible new therapeutic strategy for patients with LUAD. The research is original, of potential therapeutic value and the experiments are adequately performed. The results are convincing and nicely add to our comprehension of important and potentially targetable mechanisms in mutant Kras tumors. I have a few specific comments:

1. Figure 1: there is no validation of p53 knockdown, which should be assessed by WB. Because p53 expression is generally low in cells, the efficiency of knockdown should be tested in p53-stimulating conditions. Also, reviewer lacks information about the strategy used to knock down p53. If this was through shRNA, how many different shRNAs were used?

2. In a previous study by the authors (Konstantinidou, 2013), Y397 was found as an important phosphorylated residue for FAK function in Kras mutant lung tumor cells. How would a FAK Y397F mutant compare to the other mutations studied here, in terms of tumor cell proliferation / colony formation as shown in Fig. 1G-J?

3. The effect of blocking together ERK5 and CDK5 pharmacologically on tumor development agrees with the importance of the identified residues, S732 and S910 on FAK. Thus, what would be the result for the expression of combined mutations in these residues (S732A,S910A) for HBEC-FAK KO cell proliferation and colony formation, as reported in Fig. 1G-J?

4. To establish the connection between ERK5, CDK5 and FAK more firmly, a FAK KO (using CRISPR/Cas9 like the one used in HBEC cells for Fig. 1) should be done in the used tumor cells, A549, A427 and H460. In these FAK KO conditions, what would be the effect of combined XMD8-92 and seliciclib compared to what was obtained in FAK expressing cells (Fig. 2A)? In addition, or an alternative would be to generate stable cells expressing either FAK S732A,S910A or FAK S732E,S910E to mimic non-phosphorylatable or, respectively, constitutively phosphorylated FAK, and to test the action of the drugs in this context.

Minor comments:

- Specify the time point used in Figure S1A for relative cell number count. Was it calculated at day 2 or 4? To be in line with the other figures of the paper, it would be better to provide proliferation curves with both times, or at least at 4 days.

- Did the mice show any side effects due to the treatments?

- Reviewer feels that treating KP mice at 12 weeks post-tumor initiation is not treating late-stage tumors, thus this should be removed from the text (p. 11). Unless staging is actually performed.

- In the Discussion, the authors should add information about the therapeutic window such combination treatments could have or not in the clinics. Are toxicities expected?

- Because kinase inhibitors are never specific, is there information about the selectivity of these three compounds (ERKi, FAKi, CDK5i) and potential off-targets known from the literature? This should be discussed.

Referee #2 (Comments on Novelty/Model System for Author):

Overall, this manuscript identifies two new Focal Adhesion Kinase (FAK) interactors, namely ERK5 and CDK5, as relevant players in FAK-dependent mainteinance of KRAS mutant NSCLC. The biochemical data and the in vitro and in vivo experiments are well conducted.The results are reliable and innovative.

Referee #2 (Remarks for Author):

In this manuscript the authors identify two new Focal Adhesion Kinase (FAK) interactors, namely ERK5 and CDK5, as relevant players in FAK-dependent mainteinance of KRAS mutant NSCLC.The results are well decsribed, starting from the identification of FAK serine phosphorylation 732 and 910 as critical for the proliferation of mutant KRAS-transformed lung cells.These two sites can be phosphorylated by CDK5 and ERK5, respectively. They show that inhibition of ERK5 and CDK5 synergistically suppress FAK function, proliferation and survival of NSCLC cells. Moreover they also demonstrate that the pharmacological inhibition of ERK5 and CDK5 inhibits tumor progression in a mouse model of KrasG12D-driven lung adenocarcinoma. Finally, pharmacological inhibition of ERK5 combined to FAK inhibition has the potential to overcome resistance to FAK inhibitors, enhancing the antitumor response.

Overall, the data presented are solid, based on biochemical and cellular assays, going from the in vitro to the in vivo conditions.The data are highly significative.

Referee #3 (Remarks for Author):

EMM-2023-18934-V2-Q
Pozzato et al. "ERK5 suppression overcomes FAK inhibitor resistance in mutant KRAS driven non-small cell lung cancer "

The authors study the role of development of resistance to FAK inhibition in KRAS mutant preclinical models of human lung cancer using genetically manipulated immortalized normal human lung epithelial cells (HBECs), patient derived tumor cell lines to identify two "interactors" ERK5 and CDK5 as potential therapeutic targets. Using the HBEC model system they show that in the presence of oncogenic KRAS ERK5 and CDK5 are required to provide resistance to FAK genetic depletion or pharmacologic inhibition. Importantly inhibition of ERK5 and CDK5 suppress FAK function leading to programmed cell death resulting from "ROS-induced DNA damage." In a genetically engineered mouse model (GEMM) of KRAS lung adenocarcinoma (LUAD) drug targeting of ERK5 and CDK5 inhibited tumor formation in vivo. ERK5 pharmacologic inhibition prevented development of FAK inhibitor resistance with increased anti-tumor responses in preclinical studies. They conclude: " Therefore, we propose ERK5 inhibition as a potential co-targeting strategy to counteract FAK inhibitor resistance in NSCLC. "

Comments to the authors:

The manuscript is reviewed in the context that FAK inhibition has shown dramatic results in KRAS driven preclinical models of LUAD, and while it has been tested in the clinic with some indication of effect, resistance has universally developed. If ways, especially pharmacologic approaches, to enhance FAK inhibitory treatment could be developed this would generate immediate clinical trials given the limitations of even our best oncogenic KRAS targeting drugs. It is also reviewed in the context of the knowledge of FAK inhibition in LUAD leading to alterations in DNA repair (e.g. after radiation, see authors Tang et al. 2016 citation), the massive efforts going into develop CDK5 inhibitors for other diseases (such as neurologic function), and what is known about the specificity of ERK5 and CDK5 available inhibitors.

1. The work is all technically well done and clearly presented. The interactions of ERK5 and CDK5 with FAK and the potential for combined targeting of ERK5/CDK5 (alone or together) to overcome FAK inhibitor resistance in KRAS mutant LUAD is clear.
2. The first major issue is that there is no human tumor xenograft data presented. While the GEMM in vivo data are interesting an important, clearly the approach did not appear to be curative (remove all tumors). However, equally important, for planning for clinical translation, investigators will want to see anti-tumor activity in human KRAS mutant LUAD xenografts with FAK inhibition

combined with targeting ERK5, CDK5 alone and together. As part of this, given the role of LKB1/STK11 mutations co-occurring with KRAS mutations and the potential for targeting FAK in preclinical models (see JCI Insight 2017 Gilbert-Ross et al. PMC5333956) it would be important to include at least one KRAS mutant LKB1 mutant xenograft in these tests.
3. Similarly, given current clinical translation of targeted therapy in patients for KRASG12C and KRASG12D mutant LUAD, it would be important to know the results comparing targeting oncogenic KRAS, FAK, and ERK5 or CDK5 in one of the human tumor xenograft models.
4. A major issue is the specificity of the two drugs used in these studies - XMD8-92 and inhibitor of ERK5 which also targets BRD4, and seliciclib (roscovitine) which also inhibits CDK1, CDK2, CDK7 in addition to CDK5. The issue here is whether the anti-tumor effects seen with pharmacologic inhibition are do to ERK5 and CDK5 targeting or to one of the other targets these drugs hit. There could be several ways to address this key issue - including showing that BRD4 knockdown does not give the same results as XMD8-92, failure of exogenous BRD4 to "rescue" the XMD8092 effects, the use of some more specific CDK5 inhibitors (e.g. GFB-128-11, or CDK5 inhibitor small peptide). Alternatively, inducible knockdown of ERK5, CDK5 could be generated to be used in vivo in a xenograft that was then treated with the FAK inhibitor to show the effect occurred with functional genomic ablation even if we do not yet have very specific ERK5 or CDK5 inhibitors.
5. All of these issues can also be mentioned in the Discussion section as "limitations of the current study"
6. Given the importance for their studies of identifying additional ERK5 inhibitors with specificity the authors may want to cite some of the current literature (e.g. Modulation of ERK5 Activity as a Therapeutic Anti-Cancer Strategy. Duncan C. Miller, et al., J. Med. Chem. 2023, 66, 4491−4502) including the problem of paradoxical activation of ERK5 by ERK5 inhibitors (e.g. Lochhead et al. Nature Comm 2020.), and the use of ERK5 inhibition to sensitize tumors to TRAIL targeted therapy (Espinosa-Gil et al. CDD, 2023).

Referee #1 (Comments on Novelty/Model System for Author):

Results are novel, and experiments performed with high technical quality and adequate model systems. Results could have clinical impact to treat patients with lung cancer.

Thank you for recognizing the novelty of the findings and technical quality of our experiments.

Referee #1 (Remarks for Author):

In this manuscript, Pozzato and colleagues investigate possible mechanisms that lead to FAK inhibitor resistance in KRAS mutant LUAD. In particular, the authors identify ERK5 as mediator of FAK inhibitor resistance providing a possible new therapeutic strategy for patients with LUAD. The research is original, of potential therapeutic value and the experiments are adequately performed. The results are convincing and nicely add to our comprehension of important and potentially targetable mechanisms in mutant Kras tumors. I have a few specific comments:

1. Figure 1: there is no validation of p53 knockdown, which should be assessed by WB. Because p53 expression is generally low in cells, the efficiency of knockdown should be tested in p53-stimulating conditions. Also, reviewer lacks information about the strategy used to knock down p53. If this was through shRNA, how many different shRNAs were used?

We thank the reviewer for the comments. We used shRNA strategy to knockdown p53. The p53 steady-state level in HBEC cells is enough to be detected by wb without stimulation. We used one shRNA that has been previously validated and published by the lab of Didier Trono (PMID: 16432520). We have added this information in the materials and methods as well as reagents excel file. We have added the wb with p53 knockdown in Supplementary Fig. 1A and below (Fig. 1A) for your perusal.

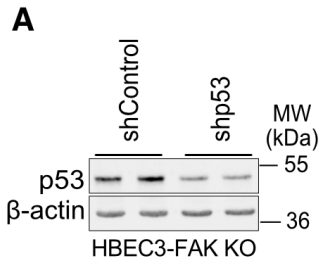

Figure 1. knockdown of p53 in HBEC3-FAK KO cells. (A) Immunoblot for the indicated targets of HBEC3-FAK KO cells previously transduced with a lentivirus expressing shControl or a shRNA against human p53.

2. In a previous study by the authors (Konstantinidou, 2013), Y397 was found as an important phosphorylated residue for FAK function in Kras mutant lung tumor cells. How would a FAK Y397F mutant compare to the other mutations studied here, in terms of tumor cell proliferation / colony formation as shown in Fig. 1G-J?

In fact, the reviewer is right and the mutant FAK^{Y397F} was our "positive control" for the mutations experiments. The effect of FAK^{Y397F} mutant closely recapitulates the effect of the empty vector (FAK KO) in the presence of mutKRAS as they both reduced cell proliferation and colony forming capacity in the presence of mutKRAS, please see Fig. 2A-2C below (manuscript Fig. S1C and S1E).

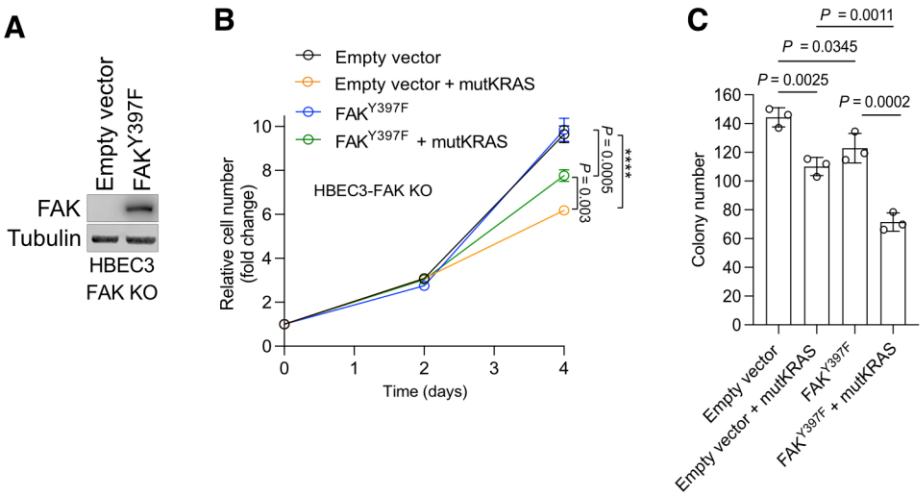

Figure 2. Expression of a loss-of-function point mutant of FAK at position 397 (FAK^{Y397F}), reduces cell proliferation and colony forming capacity in presence of mutKRAS. (A) Immunoblot for the indicated targets of HBEC FAK KO cells transduced with a retrovirus expressing empty vector (pWZL hygro) or a vector carrying FAK^{Y397F} followed by transduction with a plasmid expressing mutKRAS. **(B)** Relative cell number of HBEC FAK KO cells transduced as described in (A). **(C)** Colony formation of HBEC FAK KO cells transduced as described in (A).

3. The effect of blocking together ERK5 and CDK5 pharmacologically on tumor development agrees with the importance of the identified residues, S732 and S910 on FAK. Thus, what would be the result for the expression of combined mutations in these residues (S732A,S910A) for HBEC-FAK KO cell proliferation and colony formation, as reported in Fig. 1G-J?

We thank the reviewer for suggesting to perform this experiment. We mutagenized the human WT FAK to generate the S732A,S910A double mutation. However, we noticed by immunoblot that the protein levels of FAK upon ectopic expression of S732A/S910A double mutant plasmid was lower compared to the WT FAK plasmid expression and other FAK single point mutants FAK (Y397F) (Fig. 3A and 3B below), which was not the case for other mutants as shown in the manuscript Fig. S1F of the manuscript. We think that this double mutation may somehow affect the overall stability of FAK, which needs careful further investigation and goes beyond the scope of this manuscript. Of note, this was not the case for other FAK point mutations or drug treatments as the total FAK protein levels was unchanged (Figs. S1F and main fig. 2F). Accordingly, ectopic expression of mutKRAS in the S732A/S910A double mutant FAK expressing cells led to reduced proliferation (Fig. 3C below). However, the reduced proliferation might be due to the low total FAK levels and not due to the lower pTyr397-FAK protein levels. We report these results below for the reviewer`s perusal only.

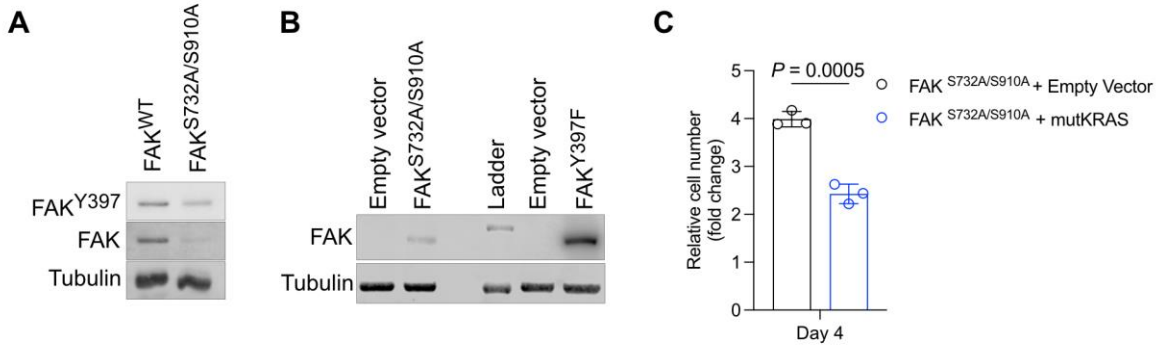

Figure 3. Expression of a double loss-of-function point mutant of FAK at positions 732 and 910 ($FAK^{S732A/S910A}$) reduces the stability of FAK. (A) Immunoblot for the indicated targets of HBEC FAK KO cells transduced with a retrovirus expressing wild type (WT) FAK or a vector carrying $FAK^{S732A/S910A}$. **(B)** Immunoblot for the indicated targets of HBEC FAK KO cells transduced with a retrovirus expressing an empty vector (pWZL hygro) or a vector carrying $FAK^{S732A/S910A}$ or a vector carrying a loss-of-function point mutant of FAK, FAK^{Y397F} **(C)** Relative cell number of HBEC FAK KO cells transduced with $FAK^{S732A/S910A}$ and with a plasmid expressing mutKRAS.

4. To establish the connection between ERK5, CDK5 and FAK more firmly, a FAK KO (using CRISPR/Cas9 like the one used in HBEC cells for Fig. 1) should be done in the used tumor cells, A549, A427 and H460. In these FAK KO conditions, what would be the effect of combined XMD8-92 and seliciclib compared to what was obtained in FAK expressing cells (Fig. 2A)? In addition, or an alternative would be to generate stable cells expressing either FAK S732A,S910A or FAK S732E,S910E to mimic non-phosphorylatable or, respectively, constitutively phosphorylated FAK, and to test the action of the drugs in this context.

We thank the reviewer for the comments. As FAK is a synthetic vulnerability of KRAS mutant cancer cells, we are unable to knockout FAK via CRISPR/Cas9 in NSCLC cells already harbouring KRAS mutations (A549, A427 and H460) to generate stable cell lines as this procedure requires time and they don`t survive enough for follow-up studies. This is why we engineered the HBEC cell line and built up step-by-step the genetic alterations to study the dependency of these cells on FAK (in these experiments we first knocked out FAK and then we expressed mutKRAS). We attempted to use the cell line that we generated as part of the comment/answer #3 above (HBEC-FAK KO cells harbouring S732A/S910A), but they are also not healthy after mutKRAS expression which includes 3 weeks of selection, precluding additional cytotoxic experiments with drugs on top of it.

Minor comments:

- Specify the time point used in Figure S1A for relative cell number count. Was it calculated at day 2 or 4? To be in line with the other figures of the paper, it would be better to provide proliferation curves with both times, or at least at 4 days.

Thank you for the comment. The proliferation on the panel in S1A (now appears as S1B) was calculated at day 4 as the other figures. We have added this information in the figure legend.

- Did the mice show any side effects due to the treatments?

The mice in the XMD8-92/ Seliciclib cohort all lost some weight at the beginning (up to almost 8%) until day 4 after starting the treatment, including the vehicle control suggesting a vehicle effect rather than toxicity due to the drugs. See panel A in Fig. below and manuscript Fig. S3A.

The XMD8-92/VS4718 cohort did not show any reduction in body weight when given alone or any other parameters based on our score sheets. However, during the combination of XMD8-92 + VS4718 treatment 3 mice showed a moderate reduction in body weight (up to almost 8%,) at day 6-9. After that, some regained weight or remained stable until the end of the treatment. See panel B below and Fig. S6A of the new manuscript.

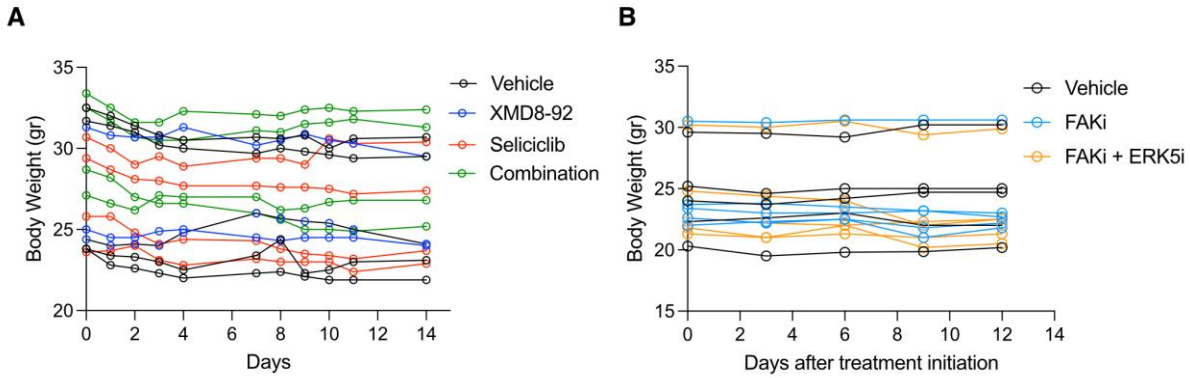

Figure 4. Body weights of mice during treatments with the indicated pharmacological inhibitors. (A) Treatment with XMD8-92, seliciclib or combination of both. **(B)** Treatment with VS4718, XMD8-92 or combination of both drugs.

- Reviewer feels that treating KP mice at 12 weeks post-tumor initiation is not treating late-stage tumors, thus this should be removed from the text (p. 11). Unless staging is actually performed.

We agree and have eliminated this sentence from the manuscript.

- In the Discussion, the authors should add information about the therapeutic window such combination treatments could have or not in the clinics. Are toxicities expected?

Based on our preclinical trial data, the combination was well tolerated despite the i.p. injections and vehicle that seemed to moderately affect the XMD8-92/Seliciclib cohorts (refer to the above point of the body weights). As Seliciclib is orally available we expect that they are even better tolerated in patients. Indeed, Seliciclib is very well tolerated as single drug. Moreover, it has also been tested in a Phase I trial in combination with Gemcitabine and Cisplatin in NSCLC patients. The maximum tolerated dose was 800 mg in combination with 1000 mg/m2 Gemcitabine and 75 mg/m2 Cisplatin. Interestingly, for this drug combination the level of haematological toxicity observed was low (PMID: 20822897). Unfortunately, there are no MEK5/ERK5 inhibitors in clinical testing at the moment. Therefore, it is difficult to predict the therapeutic window that these combination therapies may have in the clinic. We have discussed these issues in the discussion section.

- Because kinase inhibitors are never specific, is there information about the selectivity of these three compounds (ERKi, FAKi, CDK5i) and potential off-targets known from the literature? This should be discussed.

We agree with the statement that kinase inhibitors are never specific and this is also the case with the inhibitors used in this study namely, XMD8-92 (inhibits also BRD4 and LRRK2), Seliciclib (inhibits also other CDKs) and VS4718 (PYK2). It is however, important to note that XMD8-92 and Seliciclib based on our results in lung cancer don`t impact cell proliferation or cell death when used alone. This is also confirmed with the use of shRNAs (manuscript Fig. 2G-2I). This suggests that any single drug off-target effect is not functionally important in this context. We only observed anti-proliferative and pro-apoptotic effects when we combined these drugs and shRNAs together. Moreover, from our experiments in Fig. 4, we could pinpoint that the functional consequences of the combination of XMD8-92 and Seliciclib, which is identical with that of the FAK inhibitor, are restricted to the induction of ROS and DNA damage, as the cell proliferation defect was totally rescued by the antioxidant compound SOD2. Altogether, these results suggest that the known off-target effects of these compounds may not be relevant in the context of lung cancer. We have discussed these issues in the discussion section as a limitation of our studies.

Referee #2 (Comments on Novelty/Model System for Author):

Overall, this manuscript identifies two new Focal Adhesion Kinase (FAK) interactors, namely ERK5 and CDK5, as relevant players in FAK-dependent mainteinance of KRAS mutant NSCLC. The biochemical data and the in vitro and in vivo experiments are well conducted.The results are reliable and innovative.

Referee #2 (Remarks for Author):

In this manuscript the authors identify two new Focal Adhesion Kinase (FAK) interactors, namely ERK5 and CDK5, as relevant players in FAK-dependent mainteinance of KRAS mutant NSCLC.The results are well decsribed, starting from the identification of FAK serine phosphorylation 732 and 910 as critical for the proliferation of mutant KRAS-transformed lung cells.These two sites can be phosphorylated by CDK5 and ERK5, respectively. They show that inhibition of ERK5 and CDK5 synergistically suppress FAK function, proliferation and survival of NSCLC cells. Moreover they also demonstrate that the pharmacological inhibition of ERK5 and CDK5 inhibits tumor progression in a mouse model of KrasG12D-driven lung adenocarcinoma. Finally, pharmacological inhibition of ERK5 combined to FAK inhibition has the potential to overcome resistance to FAK inhibitors, enhancing the antitumor response.
Overall, the data presented are solid, based on biochemical and cellular assays, going from the in vitro to the in vivo conditions.The data are highly significative.

Thank you for recognizing the significance of our findings and technical quality of our experiments.

Referee #3 (Remarks for Author):

EMM-2023-18934-V2-Q
Pozzato et al. "ERK5 suppression overcomes FAK inhibitor resistance in mutant KRAS driven non-small cell lung cancer "

The authors study the role of development of resistance to FAK inhibition in KRAS mutant preclinical models of human lung cancer using genetically manipulated immortalized normal human lung epithelial cells (HBECs), patient-derived tumor cell lines to identify two "interactors" ERK5 and CDK5 as potential therapeutic targets. Using the HBEC model system they show that in the presence of oncogenic KRAS ERK5 and CDK5 are required to provide resistance to FAK genetic depletion or pharmacologic inhibition. Importantly inhibition of ERK5 and CDK5 suppress FAK function leading to programmed cell death resulting from "ROS-induced DNA damage." In a genetically engineered mouse model (GEMM) of KRAS lung adenocarcinoma (LUAD) drug targeting of ERK5 and CDK5 inhibited tumor formation in vivo. ERK5 pharmacologic inhibition prevented development of FAK inhibitor resistance with increased anti-tumor responses in preclinical studies. They conclude: " Therefore, we propose ERK5 inhibition as a potential co-targeting strategy to counteract FAK inhibitor resistance in NSCLC. "

Comments to the authors:

The manuscript is reviewed in the context that FAK inhibition has shown dramatic results in KRAS driven preclinical models of LUAD, and while it has been tested in the clinic with some indication of effect, resistance has universally developed. If ways, especially pharmacologic approaches, to enhance FAK inhibitory treatment could be developed this would generate immediate clinical trials given the limitations of even our best oncogenic KRAS targeting drugs. It is also reviewed in the context of the knowledge of FAK inhibition in LUAD leading to alterations in DNA repair (e.g. after radiation, see authors Tang et al. 2016 citation), the massive efforts going into develop CDK5 inhibitors for other diseases (such as neurologic function), and what is known about the specificity of ERK5 and CDK5 available inhibitors.

1. The work is all technically well done and clearly presented. The interactions of ERK5 and CDK5 with FAK and the potential for combined targeting of ERK5/CDK5 (alone or together) to overcome FAK inhibitor resistance in KRAS mutant LUAD is clear.

We thank the reviewer for recognizing the quality of our work.

2. The first major issue is that there is no human tumor xenograft data presented. While the GEMM in vivo data are interesting an important, clearly the approach did not appear to be curative (remove all tumors). However, equally important, for planning for clinical translation, investigators will want to see anti-tumor activity in human KRAS mutant LUAD xenografts with FAK inhibition combined with targeting ERK5, CDK5 alone and together.

We thank the reviewer for the suggestions. As the reviewer stated in point #5 we agree that these above points could be added as study limitations due to the use of an already high

number of animals used for our study. Given the general relevance of the KRAS-driven GEMM model in recapitulating the tumor microenvironment of lung cancer and predict response to therapy we are confident that the treatment combination will have an impact in the clinic.

The reviewer stated that “our approach did not appear to be curative”. I would like to add here that we evaluated the impact of this therapy at the end of the 2 weeks treatment. Even with this treatment scheme we observed a high percentage of tumor cell apoptosis induction in all mice treated with FAKi + ERK5i out of which one mouse had no detectable tumors (Fig. 6D and 6F). Therefore, the results show a trend towards tumor regression.

As part of this, given the role of LKB1/STK11 mutations co-occurring with KRAS mutations and the potential for targeting FAK in preclinical models (see JCI Insight 2017 Gilbert-Ross et al. PMC5333956) it would be important to include at least one KRAS mutant LKB1 mutant xenograft in these tests.

The human cell lines A549, A427 and H460 that we used in vitro harbour a deletion in *STK11*. Therefore, our findings (development of resistance upon FAKi and response to ERK5i and CDK5i), at least based on our in vitro observations, are expected to also apply for the *STK11* deleted tumors that co-occur with KRAS mutations.

3. Similarly, given current clinical translation of targeted therapy in patients for KRASG12C and KRASG12D mutant LUAD, it would be important to know the results comparing targeting oncogenic KRAS, FAK, and ERK5 or CDK5 in one of the human tumor xenograft models.

We thank the reviewer for this suggestion. We agree that comparing the efficacy of these 3 treatments is crucial. Despite the FDA approval of highly specific inhibitors targeting $KRAS^{G12C}$ (sotorasib and adagrasib) in the clinic, clinical trial data evidence that not all patients show partial response, owing to the development of drug resistance (Awad, Liu et al. 2021). Notably, recent findings indicated that non-genetic acquired resistance to sotorasib involves alterations in focal adhesions (Mohanty, Nam et al. 2023). In fact, we plan to perform these experiments, including to assess whether in $KRAS^{G12C}$ inhibitor-resistant cells and tumors in mice, FAK and ERK5 targeting may be also effective therapeutic strategy both to potentiate the efficacy of KRAS inhibitors and to prevent the development of non-genetic acquired drug resistance. However, the generation of necessary tools and the correct execution of these experiments requires time that goes beyond the scope of the current manuscript. As suggested, we have discussed this in the discussion section.

4. A major issue is the specificity of the two drugs used in these studies - XMD8-92 and inhibitor of ERK5 which also targets BRD4, and seliciclib (roscovitine) which also inhibits CDK1, CDK2, CDK7 in addition to CDK5. The issue here is whether the anti-tumor effects seen with pharmacologic inhibition are due to ERK5 and CDK5 targeting or to one of the other targets these drugs hit. There could be several ways to address this key issue - including showing that BRD4 knockdown does not give the same results as XMD8-92, failure of exogenous BRD4 to "rescue" the XMD8092 effects, the use of some more specific CDK5 inhibitors (e.g. GFB-128-11, or CDK5 inhibitor small peptide). Alternatively, inducible knockdown of ERK5, CDK5 could be generated to be used in vivo in a xenograft that was then treated with the FAK inhibitor to show the

effect occurred with functional genomic ablation even if we do not yet have very specific ERK5 or CDK5 inhibitors.

In our studies in vitro, when we used XMD8-92 or Seliciclib alone, we did not observe a biologically significant antitumoral effect (Fig. 2A-2D). Contrary, we observed a potent anti-proliferative and pro-apoptotic effect only when we combined these 2 inhibitors. This suggests that direct inhibition of the off-targets (BRD4 or CDK1/2/7) does not have any impact on the proliferation or survival of lung cancer cells. Moreover, in Fig. 2G-2H we showed that shRNAs against ERK5 and CDK5 can recapitulate the anti-proliferative and pro-apoptotic effects of XMD8-92 and seliciclib in vitro. We agree that the limitation of our study is the lack of xenografts with inducible knockdown of ERK5, CDK5. We have discussed this in the text of Fig. 2 and in the discussion section.

5. All of these issues can also be mentioned in the Discussion section as "limitations of the current study".

As mentioned above, we have discussed these issues in the discussion session.

6. Given the importance for their studies of identifying additional ERK5 inhibitors with specificity the authors may want to cite some of the current literature (e.g. Modulation of ERK5 Activity as a Therapeutic Anti-Cancer Strategy. Duncan C. Miller, et al., J. Med. Chem. 2023, 66, 4491−4502) including the problem of paradoxical activation of ERK5 by ERK5 inhibitors (e.g. Lochhead et al. Nature Comm 2020.), and the use of ERK5 inhibition to sensitize tumors to TRAIL targeted therapy (Espinosa-Gil et al. CDD, 2023).

As suggested, we have included these references in the results and discussion sections.

20th Aug 2024

Dear Prof. Konstantinidou,

Thank you for the submission of your revised manuscript to EMBO Molecular Medicine. I am pleased to inform you that we will be able to accept your manuscript pending the following final amendments:

1) Figures:
- Please reduce number of EV figures to max. 5, e.g by fusing 2 or more EV figures with one panel. Also, in figures with one panel labeling "A" should be omitted. Please update the callouts in the text accordingly.
- Please correct EV figure nomenclature to "Figure EV1" etc. in the legends.
2) In the main manuscript file, please do the following:
- Please address all comments suggested by our data editors listed below:
o Data availability statement:
1. Please note that the specific URLs for GSE255628 and GSE255643 datasets are not provided in the data availability statement.
o Figure legends:
1. Please note that the exact p values are not provided in the legends of figures 1e-i; 2a-d, h; 4d-e, g; 5h; 6c, g; EV 1c, g-j; EV 2a.
2. Please indicate the statistical test used for data analysis in the legend of figure EV 5a.
3. Please note that in figure EV 4a; there is a mismatch between the annotated p values in the figure legend and the annotated p values in the figure file that should be corrected.
4. Please note that information related to n is missing in the legends of figures 2b-d; EV 1b.
5. Please note that for heatmap present in figure 5e a numbered scale bar is not provided. This needs to be rectified.
- Correct order of manuscript sections to: Abstract, The Paper Explained, Introduction, Results, Discussion, Methods, Acknowledgements, Disclosure and competing interests statement, References, Figure legends, Tables and their legends, Expanded View Figure legends.
- Add up to 5 keywords.
- Correct callout Supplementary reagents Table 1 to Reagents Table.
- Rename "Competing interests" to "Disclosure and competing interests statement". We updated our journal's competing interests policy in January 2022 and request authors to consider both actual and perceived competing interests. Please review the policy https://www.embopress.org/competing-interests and update your competing interests if necessary.
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- Remove "For more information".
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6) As part of the EMBO Publications transparent editorial process initiative (see our Editorial at http://embomolmed.embopress.org/content/2/9/329), EMBO Molecular Medicine will publish online a Review Process File (RPF) to accompany accepted manuscripts. This file will be published in conjunction with your paper and will include the anonymous referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript. Let us know whether you agree with the publication of the RPF and as here, if you want to remove or not any figures from it prior to publication. Please note that the Authors checklist will be published at the end of the RPF.
7) Please provide a point-by-point letter INCLUDING my comments as well as the reviewer's reports and your detailed responses (as Word file).

I look forward to reading a new revised version of your manuscript as soon as possible.

Yours sincerely,

Zeljko Durdevic

Zeljko Durdevic
Editor
EMBO Molecular Medicine

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4) a letter INCLUDING the reviewer's reports and your detailed responses to their comments (as Word file).

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You are also welcome to suggest a striking image or visual abstract to illustrate your article. If you do please provide a jpeg file 550 px-wide x 300-600px high.

10) A Conflict of Interest statement should be provided in the main text

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12) Include a Reagents and Tools Table as part of the Methods section, which can be downloaded from our author guidelines (https://www.embopress.org/page/journal/17574684/authorguide#structuredmethods)

*Additional important information regarding Figures

Each figure should be given in a separate file and should have the following resolution:
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Figures are not edited by the production team. All lettering should be the same size and style; figure panels should be indicated by capital letters (A, B, C etc). Gridlines are not allowed except for log plots. Figures should be numbered in the order of their appearance in the text with Arabic numerals. Each Figure must have a separate legend and a caption is needed for each panel.

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***** Reviewer's comments *****

Referee #1 (Remarks for Author):

The authors have responded satisfactorily to my comments.

Referee #3 (Comments on Novelty/Model System for Author):

While more xenograft tests of patient derived models would have added to the value of the manuscript, the data and models as presented are adequate.

Referee #3 (Remarks for Author):

The authors have responded appropriately to all of the reviewers' comments including providing addition experimental data and extensive editing, including discussion of "limitations of the current study" in the Discussion section.

The authors addressed the minor editorial issues.

manuscript. Please be aware that in the proof stage minor corrections only are allowed (e.g., typos).

We have provided the image as 550 px wide x 400px high. The synopsis text has been checked.

6) As part of the EMBO Publications transparent editorial process initiative (see our Editorial at http://embomolmed.embopress.org/content/2/9/329), EMBO Molecular Medicine will publish online a Review Process File (RPF) to accompany accepted manuscripts. This file will be published in conjunction with your paper and will include the anonymous referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript. Let us know whether you agree with the publication of the RPF and as here, if you want to remove or not any figures from it prior to publication. Please note that the Authors checklist will be published at the end of the RPF.

We agree with the publication of the RPF and will not remove any figures from it prior publication.

7) Please provide a point-by-point letter INCLUDING my comments as well as the reviewer's reports and your detailed responses (as Word file).

We provided a point-by-point letter to all the reviewers and editor comments.

29th Aug 2024

Dear Prof. Konstantinidou,

We are pleased to inform you that your manuscript is accepted for publication and is now being sent to our publisher to be included in the next available issue of EMBO Molecular Medicine.

Your manuscript will be processed for publication by EMBO Press. It will be copy edited and you will receive page proofs prior to publication. Please note that you will be contacted by Springer Nature Author Services to complete licensing and payment information.

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If you have any questions, please do not hesitate to contact the Editorial Office. Thank you for your contribution to EMBO Molecular Medicine.

Yours sincerely,
Zeljko Durdevic

Zeljko Durdevic
Editor
EMBO Molecular Medicine

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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.
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- plots include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical
- if n<5, the individual data points from each experiment should be plotted. Any statistical test employed should be justified.
- Source Data should be included to report the data underlying figures according to the guidelines set out in the authorship guidelines on Data

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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.
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- 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:
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  - 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.

Materials

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

Antibodies	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
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	This information is reported in the Reagents Table

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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.	Yes	This information is reported in the Reagents Table
Primary cultures: Provide species, strain, sex of origin, genetic modification status.	Not Applicable	
Report if the cell lines were recently **authenticated** (e.g., by STR profiling) and tested for mycoplasma contamination.	Yes	This information is in the Materials and Methods section

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Animal observed in or captured from the field: Provide species, sex, and age where possible**.**	Not Applicable	
Please detail **housing and husbandry conditions**.	Yes	This information is in the Materials and Methods section

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Microbes: provide species and strain, unique accession number if available, and source.	Not Applicable	

Human research participants	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If collected and within the bounds of privacy constraints report on age, sex and gender or ethnicity for all study participants.	Not Applicable	

Core facilities	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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Design

Study protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If study protocol has been **pre-registered, provide DOI in the manuscript**. For clinical trials, provide the trial registration number **OR** cite DOI.	Not Applicable	
Report the **clinical trial registration number** (at ClinicalTrials.gov or equivalent), where applicable.	Not Applicable	

Laboratory protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Provide DOI OR other citation details if **external detailed step-by-step protocols** are available.	Not Applicable	

Experimental study design and statistics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Include a statement about **sample size** estimate even if no statistical methods were used.	Yes	This information is in the figure legends section
Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. **randomization procedure**)? If yes, have they been described?	Yes	This information is in the Materials and Methods section
Include a statement about **blinding** even if no blinding was done.	Yes	This information is in the Materials and Methods section
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	This information is in the Materials and Methods section

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In the figure legends: state number of times the experiment was **replicated** in laboratory.	Yes	This has been already stated in the Statistics and Reproducibility (Materials and Methods) section
In the figure legends: define whether data describe **technical or biological replicates**.	Yes	In the Figure legends

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Ethics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Studies involving **human participants**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval.	Not Applicable	
Studies involving **human participants**: 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.	Not Applicable	
Studies involving **human participants:** For publication of **patient photos**, include a statement confirming that consent to publish was obtained.	Not Applicable	
Studies involving experimental **animals**: State details of **authority granting ethics approva**l (IRB or equivalent committee(s), provide reference number for approval. Include a statement of compliance with ethical regulations.	Yes	Stated in the Materials and Methods section
Studies involving **specimen and field samples:** State if relevant **permits** obtained, provide details of authority approving study; if none were required, explain why.	Not Applicable	

Dual Use Research of Concern (DURC)	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Could your study fall under dual use research restrictions? Please check biosecurity documents and list of **select agents and toxins** (CDC): https://www.selectagents.gov/sat/list.htm	Not Applicable	
If you used a select agent, is the security level of the lab appropriate and reported in the manuscript?	Not Applicable	
If a study is subject to dual use research of concern regulations, is the name of the **authority granting approval and reference number** for the regulatory approval provided in the manuscript?	Not Applicable	

Reporting

The MDAR framework recommends adoption of discipline-specific guidelines, established and endorsed through community initiatives. Journals have their own policy about requiring specific guidelines and recommendations to complement MDAR.

Adherence to community standards	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
State if relevant guidelines or checklists (e.g., **ICMJE, MIBBI, ARRIVE, PRISMA**) have been followed or provided.	Not Applicable	
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.	Not Applicable	
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.	Not Applicable	

Data Availability

Data availability	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Have **primary datasets** been deposited according to the journal's guidelines (see 'Data Deposition' section) and the respective accession numbers provided in the Data Availability Section?	Yes	RNA seq has been deposited in GEO and plasmids in Addgene. See Materials and Methods section and Reagents Table.
Were **human clinical and genomic datasets** deposited in a public access-controlled repository in accordance to ethical obligations to the patients and to the applicable consent agreement?	Not Applicable	
Are **computational models** that are central and integral to a study available without restrictions in a machine-readable form? Were the relevant accession numbers or links provided?	Yes	In Materials and Methods section and Reagents Table.
If publicly available data were reused, provide the respective **data citations in the reference list**.	Not Applicable	