

MAPL loss dysregulates bile and liver metabolism in mice

Vanessa Goyon, Aurele Besse-Patin, Rodolfo Zunino, Olesia Ignatenko, Mai Nguyen, Etienne Coyaude, Jonathan Lee, Bich Nguyen, Brian Raught, and Heidi McBride

DOI: N/A

Corresponding author(s): Heidi McBride (heidi.mcbride@mcgill.ca)

Review Timeline:

Transfer from Review Commons:	10th Aug 23
Editorial Decision:	6th Sep 23
Revision Received:	10th Oct 23
Accepted:	17th Oct 23

Editor: Deniz Senyilmaz Tiebe

Transaction Report: This manuscript was transferred to EMBO reports following peer review at Review Commons.

Review
COMMONS

Review #1

1. Evidence, reproducibility and clarity:

Evidence, reproducibility and clarity (Required)

In this study, the authors have investigated the impact of permanently silencing the expression of the mitochondrial anchored protein ligase (MAPL) in mice on bile acid (BA) metabolism through the alteration of ABCD3 SUMOylation. This ABC pump mediates the uptake of C27-BAs by peroxisomes and hence determines the shortening of the BA sidechain. In addition, other aspects of general metabolism have also been investigated. The study is highly relevant and contains valuable information.

****Major points****

1. Sidechain shortening is essential for the synthesis of primary C24 BAs. This study suggests that the entrance of C27 BAs in peroxisomes, which depends on ABCD3 activity, is reduced by MAPL-dependent ABCD3 SUMOylation. Thus, knocking out MAPL in mice results in enhanced BA accumulation in serum and liver, presumably by facilitated uptake of C27 by the peroxisomes and stimulation of de novo synthesis of primary BA. Indeed, a decreased C27/C24 BA ratio was found. However, the results suggest that Cyp7a1 is not the main checkpoint for the control of BA synthesis or that Fxr/Fgf15/Cyp7a1 pathway is also affected by MAPL manipulation because Cyp7a1 expression, which could be expected to be downregulated in response to enhanced BA levels, is not affected in MAPL knockout. Moreover, no change in Fgf15 was found (Suppl. Fig. 2C, 2D, 2G). The authors must discuss these surprising findings.
2. The authors discussed that the alternative acidic pathway is responsible for these changes, but Cyp27a1 was, in fact, moderately downregulated in MAPL knockout mice.
3. Serum BAs may reflect a higher BA pool. Nevertheless, this has not been assayed. Enhanced flow of C27-BA precursors into peroxisomes is consistent with increased C24-BA production and reduced intrahepatic concentration of C27-BA in MAPL knockout mice (Suppl. Table 2). However, it is not explained why C27-BA serum concentrations were increased in these animals (Suppl. Table 2 and Suppl. Fig. 2B).
4. C27-BAs have been described as more toxic species than most C24-BAs. In the liver of MAPL knockout mice, C27-BAs levels were decreased (Suppl. Table 2). Other toxic species such as DCA and CDCA were not markedly changed. Muricholic acids and ursodeoxycholic acid, which were increased, are believed to be non-toxic or even hepatoprotective. Therefore, the relationship between changes in BA

homeostasis and liver carcinogenesis should be better justified.

5. SUMOylation may affect transporters which may simulate certain cholestasis with retention in serum of BAs. Expression levels of basolateral Ntcp, Oatps, and canalicular Bsep are required to better understand BA homeostasis. Besides, biliary secretion in MAPL knockout mice would give relevant information on what is actually happening in the biliary function of these animals.

2. Significance:

Significance (Required)

The study is relevant and original.

3. How much time do you estimate the authors will need to complete the suggested revisions:

Estimated time to Complete Revisions (Required)

(Decision Recommendation)

Between 1 and 3 months

4. *Review Commons* values the work of reviewers and encourages them to get credit for their work. Select 'Yes' below to register your reviewing activity at [Web of Science Reviewer Recognition Service](#) (formerly Publons); note that the content of your review will not be visible on Web of Science.

Web of Science Reviewer Recognition

Yes

Review #2

1. Evidence, reproducibility and clarity:

Evidence, reproducibility and clarity (Required)

****Summary:****

Provide a short summary of the findings and key conclusions (including methodology and model system(s) where appropriate).

The present manuscript described novel interacting partners of a mitochondrial/peroxisomal Sumoylation ligase MAPL and describes the phenotype of a newly generated MAPL KO mouse model.

****Major comments:****

- Are the key conclusions convincing?

The authors describe in the introduction that MAPL has multiple functions, including a role in mitophagy, mitochondrial division, inflammation and cell death. New is a role in regulation of peroxisomal bile salt handling. Also the role in hepatic cell proliferation in vivo has not been demonstrated before. The individual findings are generally convincing. However, the relation between the large number of observations is not clear. The authors postulate that multiple aspects of the MAPL KO mice are related to direct effects on PMP70/ABCD3 sumoylation and/or to effects on bile salts. This connection is highly speculative and mechanistically underexplored. As MAPL function was already implicated in many processes unrelated to bile salts/ABCD3, alternative explanations are likely.

Similarly, the metabolic consequences of bile salt signalling (the authors postulate this may occur via TGR5) versus effects of the ER-stress/FGF21 pathway remain unclear.

- Should the authors qualify some of their claims as preliminary or speculative, or remove them altogether?

Yes, the title and discussion is too speculative in my opinion, in particular the claim linking ABCD3 activity to all the metabolic effects observed in the MAPL KO.

- Would additional experiments be essential to support the claims of the paper?

Request additional experiments only where necessary for the paper as it is, and do not ask authors to open new lines of experimentation.

Experimental support for an altered role of ABCD3 activity as CAUSAL for the observed phenotype is essential

- Are the suggested experiments realistic in terms of time and resources? It would help

if you could add an estimated cost and time investment for substantial experiments.

As Sumoylation sites can be predicted to some level, an MAPL-insensitive ABCD3 protein could be made and used to link effects of ABCD3 sumoylation to MAPL and consequences of MAPL deficiency. Minimally, data linking the modest effects on ABCD3 activity (for example by PMP70 knockdown in vivo) on the observed phenotype of MAPL KO is required to support the current aims.

- Are the data and the methods presented in such a way that they can be reproduced?

Yes

- Are the experiments adequately replicated and statistical analysis adequate?

Yes. However, the initial and main finding of the manuscript, the identification of ABCD3 as MAPL interacting partner is plotted somewhat vague. Seems like data is from a single experiment, while the method section suggests otherwise

****Minor comments:****

- Specific experimental issues that are easily addressable.

- Are prior studies referenced appropriately?

- Are the text and figures clear and accurate?

Abstract states: "BioID revealed the peroxisomal bile acid transporter ABCD3 as a primary MAPL interacting partner, which we show is SUMOylated in a MAPL-dependent manner." The method aspect of this sentence is too unclear as it assumes all readers know what BioID entails.

The abstract also states that increased bile salt secretion is occurring. No experimental data supporting increased hepatocellular bile salt secretion is provided, only increased serum levels, which is not the same.

How was FGF15 measured? The methods section is unclear about this, and the legends indicates this was measured by ELISA. Earlier paper suggests that FGF15 is not easily detectable and controls for the elisa should thus be included (PMID: 26039452).

Figure 1D; last lane with the duplo of the rescue with the mutant MAPL seems missing, only single value is plotted.

- Do you have suggestions that would help the authors improve the presentation of their data and conclusions?

no

2. Significance:

Significance (Required)

- Describe the nature and significance of the advance (e.g. conceptual, technical, clinical) for the field.

The individual findings/observation are very interesting. However, as the causal link and relative contribution of the multitude of processes affected in the MAPL KO remains unclear current impact is limited.

- Place the work in the context of the existing literature (provide references, where appropriate).

A highly related manuscript recently appeared: mitochondrial ubiquitin ligase MARCH5 is a dual-organelle locating protein that interacts with several peroxisomal proteins. Peroxisomal MARCH5 is required for mTOR inhibition-induced pexophagy by binding and ubiquitinating PMP70 (J Cell Biol. 2022 Jan 3; 221(1): e202103156.) This is not discussed at all. However, it supports that better scientific insight into regulation of peroxisomal processes, including the activity of ABCD3/PMP70 is very relevant to the field.

- State what audience might be interested in and influenced by the reported findings.

An audience interesting in peroxisomal function and/or bile salt signalling/toxicity

- Define your field of expertise with a few keywords to help the authors contextualize your point of view. Indicate if there are any parts of the paper that you do not have sufficient expertise to evaluate.

bile salt signalling; transport

3. How much time do you estimate the authors will need to complete the suggested revisions:

Estimated time to Complete Revisions (Required)

(Decision Recommendation)

Cannot tell / Not applicable

4. *Review Commons* values the work of reviewers and encourages them to get credit for their work. Select 'Yes' below to register your reviewing activity at [Web of Science Reviewer Recognition Service](#) (formerly Publons); note that

the content of your review will not be visible on Web of Science.

Web of Science Reviewer Recognition

Yes

Review #3

1. Evidence, reproducibility and clarity:

Evidence, reproducibility and clarity (Required)

In the manuscript „SUMOylation of ABCD3 restricts bile acid synthesis and regulates metabolic homeostasis", the authors showed that MAPL has a critical role in regulation of bile acid synthesis and loss of MAPL leads to changes in metabolism and development of liver cancer. The findings are of certain interest, However, before publication of the manuscript several short cummings have to be clarified.

****Major Comments:****

- Since levels of muricholic acid is drastically increased, the authors should investigate Cyp2c70 expression since this enzyme is responsible for muricholic acid synthesis. Is there a direct regulatory effect of MAPL on Cyp2c70?
- Does MAPL directly regulate the changes on cyp enzymes or is the regulation indirect via acting on the nuclear receptors known to regulate bile acid synthesis such as FXR, CAR, PXR. Please provide data on that.
- The authors show that Cyp4a14 is increased due to loss of MAPL. Cyp4a14 is also a downstream target of PPARα. The authors should provide data on PPARα signalling in MAPL KO mice, especially on beta oxidation. This may explain why MAPL KO mice are lean.
- The authors did not investigate bile duct proliferation and activation of cholangiocytes, features which often occur in the context of changes in bile acid homeostasis. Do MAPL KO mice show increased ductular proliferation and reactive cholangiocyte phenotype? Please provide data such as expression and staining of CK19, KI67, OPN, VCAM
- EGR1 and EGFR are key regulators in HCC and are known to be regulated by bile

acids. The authors should investigate whether these key regulators may play a role in development of HCC in MAPL KO mice.

2. Significance:

Significance (Required)

The finding that loss of MAPL is involved in regulation of bile acid synthesis is of certain interest for the field of cholestatic liver and bile duct injuries. MAPL KO mice might be an interesting model to study potential therapeutics for these diseases. Furthermore, the fact that MAPL KO mice develop spontaneous HCC is also of particular interest, since such models are quite rare.

3. How much time do you estimate the authors will need to complete the suggested revisions:

Estimated time to Complete Revisions (Required)

(Decision Recommendation)

Between 3 and 6 months

4. *Review Commons* values the work of reviewers and encourages them to get credit for their work. Select 'Yes' below to register your reviewing activity at [Web of Science Reviewer Recognition Service](#) (formerly Publons); note that the content of your review will not be visible on Web of Science.

Web of Science Reviewer Recognition

No

We would like to first thank the reviewers for their patience with us given the delays in the generation of our revised manuscript. In addition to a maternity leave taken by Dr. Goyon, we took the reviewers comments very seriously and generated significant amounts of new data to address the insightful comments and suggestions of all three reviewers. The manuscript now has 9 figures, with 7 supplemental figures and 3 tables.

Our point-by-point rebuttal is below, but the major new additions are:

- 1) Full analysis of bile acid species in the feces (to complement the liver and serum analysis provided in first submission). We also performed FDG-glucose PET analysis of the mice, which revealed significant alterations in proliferation in the gut in young MAPL KO mice. We did this in response to the concerns raised by reviewers 1 and 3 about the effects of the gut on bile acid regulation, and we discuss our findings below in response to reviewers, and within the revised manuscript.
- 2) Our initial submission reported an Illumina approach for transcriptomics in livers of male wt and KO mice. We now completed RNAseq analysis on both male and female (littermate) control and MAPL KO mice at 3 months old. This validated what we had seen in the Illumina arrays, but allowed us a deeper look into transcriptional and sex specific changes that we present in response to review and within the revised manuscript. We have also expanded our feed/fast cycle analysis of the dynamic changes in gene expression of bile acid related pathways to further document the disruption in the feedback cycles regulating bile acid synthesis in liver.
- 3) We have developed assays using primary hepatocytes from +/-MAPL mice for a better analysis of cell autonomous functions and bile acid secretion. This complements the tail-vein rescue experiments we had presented in initial submission.

Reviewer #1 (Evidence, reproducibility and clarity (Required)):

In this study, the authors have investigated the impact of permanently silencing the expression of the mitochondrial anchored protein ligase (MAPL) in mice on bile acid (BA) metabolism through the alteration of ABCD3 SUMOylation. This ABC pump mediates the uptake of C27-BAs by peroxisomes and hence determines the shortening of the BA sidechain. In addition, other aspects of general metabolism have also been investigated. The study is highly relevant and contains valuable information.

AUTHORS: We sincerely thank the reviewer for their supportive comments on the value of our work.

Major points

R1: 1) Sidechain shortening is essential for the synthesis of primary C24 BAs. This study suggests that the entrance of C27 BAs in peroxisomes, which depends on ABCD3 activity, is reduced by MAPL-dependent ABCD3 SUMOylation. Thus, knocking out MAPL in mice results in enhanced BA accumulation in serum and liver, presumably by facilitated uptake of C27 by the peroxisomes and stimulation of de novo synthesis of primary BA. Indeed, a decreased C27/C24 BA ratio was found. However, the results suggest that Cyp7a1 is not the main checkpoint for the control of BA synthesis or that Fxr/Fgf15/Cyp7a1 pathway is also affected by MAPL manipulation because Cyp7a1 expression, which could be expected to be downregulated in response to enhanced BA levels, is not affected in MAPL knockout. Moreover, no change in Fgf15 was found (Suppl. Fig. 2C, 2D, 2G). The authors must discuss these surprising findings.

AUTHORS: We entirely agree with the reviewer that the lack of feedback to downregulate bile acid synthesis through the canonical pathways was very unusual and is one of the novel aspects of our study. While the circulating bile was significantly elevated, this was not sensed by the pathways in the gut or liver to downregulate CYP7A1 through the activation of bile receptors FXR/FGF15. We have rewritten a great deal of the manuscript to be clearer about this, as we also feel the MAPL KO presents us a very unique model of bile acid dysregulation with many unexpected observations. In revision we completed an analysis of ~40 bile acid species within the feces to understand what may be happening in the gut. Interestingly, bile acid levels were decreased in feces, in contrast to the elevations seen in gut and liver (New Fig3). This prompted us to consider a block in bile acid delivery to the gut, or cholestasis. However, such a pathology is generally lethal, yet MAPL KO mice live past 2 years. Histological analysis (presented in New Fig 3) did not reveal any obstructions, necrosis or pathology in the bile canaliculi. Bomb calorimetry of the feces showed equivalent calories in KO mice (New Fig3), suggesting that digestion of food was fully intact, something that would have been altered if bile was absent. Lastly, the secondary bile acids that are generated by the microbiome were elevated in serum and liver, indicative of the successful transit of these bile species through the gut. Therefore, we conclude that the bile does reach the gut, yet appears to be significantly reabsorbed back into circulation without alerting the bile sensing pathways to secrete more FGF15. Ultimately, we have not answered the initial question, since we do not know yet how MAPL is required, directly or indirectly, for bile acid feedback loops. But we realize now that this will take significant effort to resolve mechanistically, something we will continue to work on in the next stage of our project.

R1: 2) The authors discussed that the alternative acidic pathway is responsible for these changes, but Cyp27a1 was, in fact, moderately downregulated in MAPL knockout mice.

AUTHORS: We apologize for the confusion. Yes, Cyp27A1 is moderately downregulated in MAPL KO mice, seen now within RNAseq analysis (New Fig 2C) and the western blots (Fig 3C) which we meant to say could reflect a specific feedback loop to inhibit bile acid synthesis from the acidic pathway, rather than through canonical, FXR/FGF15 mediated changes in Cyp7A1. We considered that very little is known about the regulation of the acidic pathway, and perhaps MAPL effects on bile acid metabolism may be more dominant in this loop. However, clearly it remains unresolved how the elevated bile, even in liver, goes undetected by FXR to downregulate Cyp7A1. We have tried to approach our results and discussion in a more systematic way to make these points clearer.

R1: 3) Serum BAs may reflect a higher BA pool. Nevertheless, this has not been assayed. Enhanced flow of C27-BA precursors into peroxisomes is consistent with increased C24-BA production and reduced intrahepatic concentration of C27-BA in MAPL knockout mice (Suppl. Table 2). However, it is not explained why C27-BA serum concentrations were increased in these animals (Suppl. Table 2 and Suppl. Fig. 2B).

AUTHORS: We thank the reviewer for pointing that out. We added quantification in the feces of the bile acid species (New Fig2C). Surprisingly we found decreased levels of C27 and C24 bile acids. We are speculating that some of the increased bile acid levels in the serum are due to increased synthesis/flux through the hepatocyte peroxisomes and some due to reabsorption.

R1: 4) C27-BAs have been described as more toxic species than most C24-BAs. In the liver of MAPL knockout mice, C27-BAs levels were decreased (Suppl. Table 2). Other toxic species such as DCA and CDCA were not markedly changed. Muricholic acids and ursodeoxycholic acid, which were increased, are believed to be non-toxic or even hepatoprotective. Therefore, the relationship between changes in BA homeostasis and liver carcinogenesis should be better justified.

AUTHORS: We apologize for generalizing too much in assigning elevated bile acid species as potential drivers/contributors of tumorogenesis. We have made note in the text of the reviewers points, including references to the protective nature of some bile species. We cannot yet pinpoint the precise cause of cellular transformation but have tried to balance the discussion around potential changes in 1) proliferative signaling cascades potentially linked to bile signaling, 2) ER stress, which has been linked to tumorogenesis, and 3) the protection against cell death pathways seen in MAPL KO cells.

R1: 5) SUMOylation may affect transporters which may simulate certain cholestasis with retention in serum of BAs. Expression levels of basolateral Ntcp, Oatps, and canalicular Bsep are required to better understand BA homeostasis. Besides, biliary secretion in MAPL knockout mice would give relevant information on what is actually happening in the biliary function of these animals.

AUTHORS: We thank the reviewer for an excellent point. To get a better view of the transcriptional changes of the transporters highlighted here, but also of all genes in liver, we completed RNAseq analysis. This showed no change in the mRNA levels of the transporters highlighted, so we performed qRT-PCR analysis from livers during a feed/fast experiment to determine whether the dynamic behavior of expression may be

altered upon MAPL loss (New Fig 4). Importantly, we found that the transporters expression was unchanged (except for one of the Oatp which would limit hepatocyte reabsorption). We also added bile secretion from primary hepatocytes reproducing the phenotype. This reinforces our point that MAPL loss affect primarily bile acid flux through the peroxisome and that is enough to have increased bile acid in the serum. Lastly, to test whether bile was successfully transiting into the gut we completed the bile acid analysis of feces, along with bomb calorimetry, PET analysis and histology of the gut, all of which indicate that bile flux to the gut is intact.

Reviewer #1 (Significance (Required)):

The study is relevant and original.

AUTHORS: We thank the reviewer for appreciating the strengths of our study.

Reviewer #2 (Evidence, reproducibility and clarity (Required)):

The present manuscript described novel interacting partners of a mitochondrial/peroxisomal Sumoylation ligase MAPL and describes the phenotype of a newly generated MAPL KO mouse model.

Major comments:

R2: The authors describe in the introduction that MAPL has multiple functions, including a role in mitophagy, mitochondrial division, inflammation and cell death. New is a role in regulation of peroxisomal bile salt handling. Also the role in hepatic cell proliferation in vivo has not been demonstrated before. The individual findings are generally convincing. However, the relation between the large number of observations is not clear. The authors postulate that multiple aspects of the MAPL KO mice are related to direct effects on PMP70/ABCD3 sumoylation and/or to effects on bile salts. This connection is highly speculative and mechanistically underexplored. As MAPL function was already implicated in many processes unrelated to bile salts/ABCD3, alternative explanations are likely.

AUTHORS: We appreciate these critical comments, and agree that MAPL has distinct substrates that play important roles in multiple aspects of mitochondrial (and now peroxisomal) signaling, survival and metabolism. This certainly makes it difficult to assign a given substrate to a broad set of pathologies in a KO mouse model. Our lab has been working on MAPL for over 15 years, and a major question has been the physiological function of MAPL in vivo. Our report that the primary phenotypes appear in liver, that effects global metabolism (lean phenotype, insulin sensitivity), proliferation and cancer provide the first evidence of the importance of MAPL in metabolism. Our BioID approach to identify MAPL partners led us to ABCD3, the peroxisomal bile acid transporter, in addition to the identification of established proteins of the mitochondrial and peroxisomal fission machineries. Furthermore, we provide critical new evidence that MAPLs primary role is not to regulate the degradation of its substrate partners, since they were not stabilized upon inhibition of the proteasome. We confirmed the interaction and SUMOylation of ABCD3 from liver tissue using multiple approaches (BioID, co-IP, SIM beads, glycerol gradients), therefore we do not consider the interaction between the two proteins as speculative. We agree that more will need to be done to develop structure/functional analysis of the SUMOylated bile acid transporter. Important to this study, functional data in vivo demonstrates major increases in bile acid production in liver and (new to the revision) primary hepatocytes, consistent with a role for MAPL mediated SUMOylation to gate ABCD3, the only primary bile acid transporter in peroxisomes. We have attempted to position our findings within the context of MAPL function in other pathways and broadened our discussion in terms of all mechanisms and phenotypes.

In this revised manuscript we expanded our analysis into the gut, given the important role of the gut in bile acid homeostasis. In searching for an explanation for the disruption in FXR/FGF15 responsiveness, we observed a striking proliferative phenotype in the duodenum. The limited proliferation in duodenum is consistent with previous work showing that bile acids can promote proliferation through a number of mechanisms, from the signaling of TGR5 bile receptors to YAP activation. It may also reflect cell autonomous functions of MAPL in enterocytes responsible for the suppression of proliferation, however the limitation of the proliferative phenotype to the top section of the gut does suggest a link to bile. We have included these data, along

with a full bile acid analysis of feces, in the revised manuscript given the essential role of the gut as a driver of the feedback loop for bile acid homeostasis. We hope the reviewer will now be convinced that our work places MAPL as a key metabolic regulator offering a new animal model that highlights some very unusual bile acid phenotypes, and a model of spontaneous hepatocellular carcinoma. These are unexpected phenotypes for a MAPL KO animal given all previous work into MAPL/MUL1.

R2: Similarly, the metabolic consequences of bile salt signalling (the authors postulate this may occur via TGR5) versus effects of the ER-stress/FGF21 pathway remain unclear.

AUTHORS: It is not entirely clear to us which metabolic consequences the reviewer refers to in this concern, so hopefully we will answer the question as intended. Assuming the reviewer refers to the lean, insulin sensitive phenotype of MAPL KO mice, we understand there remains some speculation in the relationship between the bile acids or FGF21 as drivers of the insulin sensitivity. The phenotype mimics FGF21 overexpression, as this hepatokine has been long linked to leanness and insulin sensitivity in mice. The curious finding was in our tail vein rescue experiments, even empty adenovirus induced ER stress, so we could not test if MAPL expression would rescue CHOP expression. Yet we had a full restoration of both FGF21 and circulating bile. This indicates that the ER stress is not the main driver of FGF21 expression (or bile secretion) in this system. Given the direct interactions we observed between MAPL and the bile acid transporter, we hypothesized that the rescue of MAPL would return the gating function of ABCD3, and perhaps that the bile acids themselves were driving FGF21 expression. This is consistent with a 2018 study demonstrating that FGF21 signaling resulted in the downregulation of bile acid synthesis (PMID: 29615519), a potential feedback loop to explain the downregulation of many bile acid enzymes seen in our RNAseq analysis. We have been more careful to state the limitations and outstanding questions in the study. It will take many additional mouse crosses, knock-in models carrying mutations in ABCD3, and many other experiments to resolve this question fully. We sincerely hope the reviewer can agree that the observations are robust and well controlled, and will open new avenues of research in the future.

R2: The title and discussion is too speculative in my opinion, in particular the claim linking ABCD3 activity to all the metabolic effects observed in the MAPL KO.

AUTHORS: We have changed the title of the manuscript to better reflect the global consequences of MAPL loss and the novelty of our findings overall.

- Would additional experiments be essential to support the claims of the paper? Request additional experiments only where necessary for the paper as it is, and do not ask authors to open new lines of experimentation.

R2: Experimental support for an altered role of ABCD3 activity as CAUSAL for the observed phenotype is essential

AUTHORS: We argue that our data has established the MAPL-dependent SUMOylation of ABCD3 in vivo using the SIM-bead pull down, our BioID identified this transporter as

the top partner of MAPL, validated with immunoprecipitation from liver in floxed and MAPLKO mice, and we observe biochemical alterations in the oligomeric state of ABCD3. Identifying the SUMO sites and generating CRISPR KI mice to confirm effects on bile acid flux would represent another year (at least) of work. We strongly believe that our study provides a number of very important new insights into bile acid metabolism with phenotypes that have not been seen before (as explained by Rev1). We understand that this will be a final documentation of the structure/function relationship between MAPL and ABCD3, but we have established this interaction and substrate/enzyme pairing between a newly identified bile acid transporter (for which very little work has been done anywhere), and an evolutionarily conserved SUMO E3 ligase.

- Are the suggested experiments realistic in terms of time and resources? It would help if you could add an estimated cost and time investment for substantial experiments.

R2: As Sumoylation sites can be predicted to some level, an MAPL-insensitive ABCD3 protein could be made and used to link effects of ABCD3 sumoylation to MAPL and consequences of MAPL deficiency. Minimally, data linking the modest effects on ABCD3 activity (for example by PMP70 knockdown in vivo) on the observed phenotype of MAPL KO is required to support the current aims.

AUTHORS: Knocking down ABCD3/PMP70 would be possible with tail vein injection. However, the loss of ABCD3 would give the opposite phenotype, where bile acid production would be lost, as already documented in Ferdinandusse et al 2015 (PMID: 25168382). ABCD3 is the only known bile acid transporter in peroxisomes so we do not agree that our phenotypes are so obviously explained by another mechanism, nor why the reviewer considers the effects on bile acid to be “modest”? We suggest, based on established paradigms for the SUMOylation of ion transporters, that the SUMOylation would gate the transporter to inhibit it. We agree this is a next step, but it is beyond the scope of this manuscript.

- Are the data and the methods presented in such a way that they can be reproduced? Yes
- Are the experiments adequately replicated and statistical analysis adequate? Yes.

R2: However, the initial and main finding of the manuscript, the identification of ABCD3 as MAPL interacting partner is plotted somewhat vague. Seems like data is from a single experiment, while the method section suggests otherwise

AUTHORS: We have repeated these experiments (BioID, co-IP, SIM bead experiments) numerous times from multiple mouse livers and cell lines. We have ensured the numbers are in the methods and legends. We would also submit that the interaction with ABCD3 is not, in fact, the main finding of the manuscript. The entire characterization of the mouse pathology, ending in the spontaneous development of hepatocellular carcinoma, will be of high interest to researchers in the fields of metabolism, liver biology and cancer, MAPL/MUL1 function, and insulin sensitivity. The BioID and interactomics was (to us) also very important for the things it did NOT find, for example, the substrates others consider to be regulated by MUL1 ubiquitination. Our experiments with MG132 clearly show that the candidate substrates identified in BioID are not targeted for degradation, including Mfn2 and others. MAPL loss did not lead to changes in mitochondrial or peroxisome mass, nor did it significantly alter the gene expression of these proteins (new RNAseq analysis). While some of these aspects

represent negative data, it is an important, in vivo demonstration that MAPL is not a key player in mitochondrial quality control. In this sense, the entire study is highly unexpected, with such clear phenotypes in global metabolism, bile acid and liver specific effects, proliferation and cancer.

Minor comments:

R2: Abstract states: "BioID revealed the peroxisomal bile acid transporter ABCD3 as a primary MAPL interacting partner, which we show is SUMOylated in a MAPL-dependent manner." The method aspect of this sentence is too unclear as it assumes all readers know what BioID entails.

AUTHORS: We apologize for the confusion and have clarified that sentence.

R2: The abstract also states that increased bile salt secretion is occurring. No experimental data supporting increased hepatocellular bile salt secretion is provided, only increased serum levels, which is not the same.

AUTHORS: We thank the reviewer for pushing us to examine bile acid secretion from primary hepatocytes isolated from MAPL KO or littermate control mice. This took us some time since the MAPL KO hepatocytes didn't seed as well as control cells, but we adapted our protocols and the cells adhered well. These experiments showed that MAPL KO hepatocytes produce 2-3X more bile acids than wild type hepatocytes over a 48 hours culture period. Therefore we have confirmed that the hepatocytes are producing more bile.

R2: How was FGF15 measured? The methods section is unclear about this, and the legends indicates this was measured by ELISA. Earlier paper suggests that FGF15 is not easily detectable and controls for the elisa should thus be included (PMID: 26039452).

AUTHORS: We quantified FGF15 by ELISA using established protocols without any difficulty, and have included all standards and controls in the excel sheets. The paper cited is from 2015, which is nearly 10 years ago so it appears that these tools have improved.

R2: Figure 1D; last lane with the duplo of the rescue with the mutant MAPL seems missing, only single value is plotted.

AUTHORS: The quantification presents n=3 biological replicates, the figure is a representative immunoblot of the 3 replicates, where only one mutant MAPL rescue is loaded. We clarified that in the legend.

Reviewer #2 (Significance (Required)):

- Describe the nature and significance of the advance (e.g. conceptual, technical, clinical) for the field.

R2: The individual findings/observation are very interesting. However, as the causal link and relative contribution of the multitude of processes affected in the MAPL KO remains unclear current impact is limited.

AUTHORS: We respectfully disagree with the reviewer that the impact is limited by the fact that we are studying an E3 ligase with multiple substrates. Clearly we understand

that MAPL has functions at mitochondria regulating DRP1 and other processes, as we have worked on MAPL for over years. The novelty and importance of this study is that it is the first full characterization of a MAPL KO mouse that presents with very unexpected phenotypes that will be used to advance the field in multiple ways. The identification of ABCD3 as a substrate represents the first in peroxisomes to be examined, and very little is known about the regulation of these transporters. Even if there are additional functions of MAPL at play in liver (which I'm sure there are), linking it to bile acid flux, is a novel finding.

- Place the work in the context of the existing literature (provide references, where appropriate).

R2: A highly related manuscript recently appeared: mitochondrial ubiquitin ligase MARCH5 is a dual-organelle locating protein that interacts with several peroxisomal proteins. Peroxisomal MARCH5 is required for mTOR inhibition-induced pexophagy by binding and ubiquitinating PMP70 (J Cell Biol. 2022 Jan 3; 221(1): e202103156.) This is not discussed at all. However, it supports that better scientific insight into regulation of peroxisomal processes, including the activity of ABCD3/PMP70 is very relevant to the field.

AUTHORS: We apologize for omitting the study of MARCH5 in our manuscript. The reviewer is correct that this highlights the unique function of MAPL in the regulation of the transporter through SUMOylation. MAPL loss does not alter the turnover or expression of ABCD3/PMP70 (or peroxisomes for that matter), which is the opposite of MARCH5.

- State what audience might be interested in and influenced by the reported findings.

An audience interesting in peroxisomal function and/or bile salt signalling/toxicity

- Define your field of expertise with a few keywords to help the authors contextualize your point of view.

Indicate if there are any parts of the paper that you do not have sufficient expertise to evaluate.

R2: bile salt signalling; transport

AUTHORS: We understand that the reviewer focused on the first figure (of 9), and perhaps did not appreciate the unique findings we have made in characterizing a very novel bile acid phenotype where the feedback loops are interrupted. The links to cancer were also not mentioned by the reviewer, something we also feel strongly about since it is consistent with the roles of MAPL in cell death pathways. The establishment of a novel animal model of HCC is of value to the community.

Reviewer #3 (Evidence, reproducibility and clarity (Required)):

R3: In the manuscript „SUMOylation of ABCD3 restricts bile acid synthesis and regulates metabolic homeostasis“, the authors showed that MAPL has a critical role in regulation of bile acid synthesis and loss of MAPL leads to changes in metabolism and development of liver cancer. The findings are of certain interest. However, before publication of the manuscript several shortcomings have to be clarified.

AUTHORS: We thank the reviewer for recognizing that the identification of MAPL as having a critical role in metabolism, bile acid signaling and cancer is of importance to the field.

Major Comments:

R3: Since levels of muricholic acid is drastically increased, the authors should investigate Cyp2c70 expression since this enzyme is responsible for muricholic acid synthesis. Is there are direct regulatory effect of MAPL on Cyp2c70?

AUTHORS: We thank the reviewer for the suggestion, we measured Cyp2c70 and found no change in MAPL KO livers, as seen in our new RNAseq analysis and qRT-PCR (New Fig4).

R3: Does MAPL directly regulate the changes on cyp enzymes or is the regulation indirect via acting on the nuclear receptors known to regulate bile acid synthesis such as FXR, CAR, PXR. Please provide data on that.

AUTHORS: We completed RNAseq analysis from livers of control and MAPLKO mice to generate a more complete picture of precise transcriptional changes in all genes. We also looked specifically at FXR, LXR, PXR and PPARa target genes using qRT-PCR approaches from livers in feed/fasting experiments to examine dynamic changes in expression. Most were unchanged and responded normally, with the exception of some PPARs target genes that were increased, supporting the metabolism necessary to handle high levels of bile acid flux.

R3: The authors show that Cyp4a14 is increased due to loss of MAPL. Cyp4a14 is also a downstream target of PPARa. The authors should provide data on PPARa signalling in MAPLKO mice, especially on beta oxidation. This may explain why MAPL KO mice are lean.

AUTHORS: See previous response

R3: The authors did not investigate bile duct proliferation and activation of cholangiocytes, features which often occur in the context of changes in bile acid homeostasis. Do MAPL. KO mice show increased ductular proliferation and reactive cholangiocyte phenotype? Please provide data such as expression and staining of CK19, Ki67, OPN, VCAM

• EGR1 and EGFR are key regulators in HCC and are known to be regulated by bile acids. The authors should investigate whether these key regulators may play a role in development of HCC in MAPL KO mice.

AUTHORS: We thank the reviewer for these suggestions. We have the Ki67 data included in Figure 8. It shows increased hepatocyte proliferation but not cholangiocytes. Moreover our histology stainings do not support any change in canicular structure. We also measured the EGF receptor activation in our model and found no change (Supplemental Figure 3). We also tried to find other indication of inflammation (as suggested), in our RNAseq dataset, we can find some known inflammatory signals like SPP1/Osteopontin, VCAM1, CCL2, CD68 being increased. However, the pathway

analyses did not reveal any increased inflammatory status, which is also supported by absence of immune cell infiltration. It is possible that some immune or inflammation remodeling is happening but not at a large scale and not following the canonical inflammatory liver diseases.

Reviewer #3 (Significance (Required)):

R3: The finding that loss of MAPL is involved in regulation of bile acid synthesis is of certain interest for the field of cholestatic liver and bile duct injuries. MAPL KO mice might be an interesting model to study potential therapeutics for these diseases. Furthermore, the fact that MAPL KO mice develop spontaneous HCC is also of particular interest, since such models are quite rare.

AUTHORS: We thank the reviewer for finding our work 'of particular interest'

Dear Heidi,

Thank you for submitting your revised manuscript to EMBO Reports, which was previously reviewed at Review Commons. It has now been seen all of the original referees.

Referees #1 and #3 find that their initial criticisms were adequately addressed. Of note, referee #3 did not provide written comments, yet he/she informed us that he/she is satisfied with the revised manuscript. However, referee #2 is still of the opinion that the causality between MAPL KO, ABCD3/PMP70 SUMOylation and bile synthesis defects remains elusive. While we appreciate the remarks of the referee and agree that establishing such causality would significantly strengthen the manuscript, we also find that the reported effects of MAPL KO on ABCD3 SUMOylation, bile metabolism and the appearance of spontaneous hepatocellular carcinoma sufficiently interesting and the advance provided sufficient for publication here.

However, I need you to address the editorial points below before I can accept the manuscript.

- We note that high throughput datasets were generated in this study (i.e., RNAseq analysis). As per our guidelines, please deposit them into public repositories and add a 'Data Availability Section' placed after the Materials & Methods section, where datasets and computer code that were generated in the reported study are listed in a structured manner. (please see <https://www.embopress.org/page/journal/14693178/authorguide#dataavailability> for further information).
- The Conflicts of Interest section needs to be renamed as "Disclosure statement and competing interests" and placed after the Acknowledgements section.
- As per our format requirements, in the reference list, citations should be listed in alphabetical order and then chronologically, with the authors' surnames and initials inverted; where there are more than 10 authors on a paper, 10 will be listed, followed by 'et al.'. Please see <https://www.embopress.org/page/journal/14693178/authorguide#referencesformat>
- In the Author Checklist, we note that the cells D43, D54 and D112 have not been filled in with a response.
- Please make the funding info a part of Acknowledgments section.
- We note that Figure 2E is currently not called out in the text.
- Suppl. Tables 1-2 should be renamed as Table EV1-EV2; their legends should be removed from the ms file and inserted in the files themselves; the callouts in the ms should be updated accordingly (<https://www.embopress.org/page/journal/14693178/authorguide#expandedview>)
- Suppl. Table 3 should be uploaded as dataset; it should also be renamed to Dataset EV1 and the callout in the ms should be uploaded accordingly. Its legend should be provided in the same Excel file in a separate sheet.
- We note that the manuscript currently has 8 Supplemental Figures. These can either be submitted as an Appendix file, which is a single pdf file with a Table of Contents. The nomenclature of the Appendix figures in the text should be Appendix Figure S1 etc. I would however encourage you to take advantage of the Expanded View figure type by converting maximum 5 of the figures into Expanded View Figures, which should be called out as Figure EV1 etc. (please see <https://www.embopress.org/page/journal/14693178/authorguide#expandedview> for further info).
- At EMBO Press, we ask the authors to provide the source data that were used to generate the main figures. We note that currently no source data have been submitted. Please refer to the email from our Source Data Coordinator Dr. Hannah Sonntag dated 23.08.2023 for details.
- During our routine figure checks we noted the potential re-use of western blot Figure 3G MAPL-Flag & Figure 6J Ad - MAPL-Flag, which is only allowed if the figures are derived from the same experiment, in which case it should be clearly stated in the figure legends.
- During our routine checks we noted that statistical analysis was applied in Fig S7C, where n=2, which is not optimal. Therefore, I recommend removing the statistics from that panel (please see <https://www.embopress.org/page/journal/14693178/authorguide#statisticalanalysis>)
- Papers published in EMBO Reports include a 'synopsis' and 'bullet points' to further enhance discoverability. Both are displayed on the html version of the paper and are freely accessible to all readers. The synopsis includes a short standfirst summarizing the study in 1 or 2 sentences (max 35 words) that summarize the paper and are provided by the authors and streamlined by the handling editor. I would therefore ask you to include your synopsis blurb and 3-5 bullet points listing the key experimental findings.
- In addition, please provide an image for the synopsis. This image should provide a rapid overview of the question addressed in the study but still needs to be kept fairly modest since the image size cannot exceed 550 (width) x 300-600 (height) pixels.
- Our production/data editors have asked you to clarify several points in the figure legends (see attached document). Please incorporate these changes in the attached word document and return it with track changes activated.

Thank you again for giving us to consider your manuscript for EMBO Reports, I look forward to your minor revision.

Kind regards,

Deniz

--

Deniz Senyilmaz Tiebe, PhD
Editor
EMBO Reports

Referee #1:

The authors have appropriately addressed all my comments, questions, and criticisms.

Referee #2:

I provided a lengthy review several months ago. The main criticism has not been addressed and still interferes with publication or subsequent impact on the field.

The main comments is that the MAPL KO has an interesting phenotype, but the causal link to PMP70 and even to impaired bile salt synthesis or signaling in general remains very weak and unclear. Without such insight, the connection between the large set of data is absent making it a difficult story, largely descriptive.

The authors have addressed all minor editorial requests.

Dear Heidi,

Thank you for submitting your revised manuscript. I have now looked at everything and all looks fine. Therefore, I am very pleased to accept your manuscript for publication in EMBO Reports.

Congratulations on a nice study!

Kind regards,

Deniz

--

Deniz Senyilmaz Tiebe, PhD

Scientific Editor

EMBO Reports

--

At the end of this email I include important information about how to proceed. Please ensure that you take the time to read the information and complete and return the necessary forms to allow us to publish your manuscript as quickly as possible.

As part of the EMBO publication's Transparent Editorial Process, EMBO reports publishes online a Review Process File to accompany accepted manuscripts. As you are aware, this File will be published in conjunction with your paper and will include the referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript.

If you do NOT want this File to be published, please inform the editorial office within 2 days, if you have not done so already, otherwise the File will be published by default [contact: emboreports@embo.org]. If you do opt out, the Review Process File link will point to the following statement: "No Review Process File is available with this article, as the authors have chosen not to make the review process public in this case."

Should you be planning a Press Release on your article, please get in contact with emboreports@wiley.com as early as possible, in order to coordinate publication and release dates.

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

THINGS TO DO NOW:

You will receive proofs by e-mail approximately 2-3 weeks after all relevant files have been sent to our Production Office; you should return your corrections within 2 days of receiving the proofs.

EMBO Press participates in many Publish and Read agreements that allow authors to publish Open Access with reduced/no publication charges. Check your eligibility: <https://authorservices.wiley.com/author-resources/Journal-Authors/open-access/affiliation-policies-payments/index.html>

Please inform us if there is likely to be any difficulty in reaching you at the above address at that time. Failure to meet our deadlines may result in a delay of publication, or publication without your corrections.

All further communications concerning your paper should quote reference number EMBOR-2023-57972V2 and be addressed to emboreports@wiley.com.

Should you be planning a Press Release on your article, please get in contact with emboreports@wiley.com as early as possible, in order to coordinate publication and release dates.

Rev_Com_number: RC-2022-01335

New_manu_number: EMBOR-2023-57972V2

Corr_author: McBride

Title: MAPL loss dysregulates bile and liver metabolism in mice

EMBO Press Author Checklist

Corresponding Author Name: Heidi McBride
Journal Submitted to: Embo Reports
Manuscript Number: EMBOR-2023-57972V1

USEFUL LINKS FOR COMPLETING THIS FORM

[The EMBO Journal - Author Guidelines](#)
[EMBO Reports - Author Guidelines](#)
[Molecular Systems Biology - Author Guidelines](#)
[EMBO Molecular Medicine - Author Guidelines](#)

Reporting Checklist for Life Science Articles (updated January

This checklist is adapted from Materials Design Analysis Reporting (MDAR) Checklist for Authors. MDAR establishes a minimum set of requirements in transparent reporting in the life sciences (see Statement of Task: [10.31222/osf.io/9sm4x](#)). Please follow the journal's guidelines in preparing your

Please note that a copy of this checklist will be published alongside your article.

Abridged guidelines for figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- ideally, figure panels should include only measurements that are directly comparable to each other and obtained with the same assay.
- plots include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical
- if n<5, the individual data points from each experiment should be plotted. Any statistical test employed should be justified.
- Source Data should be included to report the data underlying figures according to the guidelines set out in the authorship guidelines on Data

2. Captions

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

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements.
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.
- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
 - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ^2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
 - are tests one-sided or two-sided?
 - are there adjustments for multiple comparisons?
 - exact statistical test results, e.g., P values = x but not P values < x;
 - definition of 'center values' as median or average;
 - definition of error bars as s.d. or s.e.m.

Please complete ALL of the questions below.
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
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	Materials and methods
DNA and RNA sequences	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Short novel DNA or RNA including primers, probes: provide the sequences.	Yes	Supplementary Table
Cell materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Cell lines: Provide species information, strain. Provide accession number in repository OR supplier name, catalog number, clone number, and/ OR RRID.	Yes	Material and methods
Primary cultures: Provide species, strain, sex of origin, genetic modification status.	Yes	Material and methods
Report if the cell lines were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Yes	Material and methods
Experimental animals	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Laboratory animals or Model organisms: Provide species, strain, sex, age, genetic modification status. Provide accession number in repository OR supplier name, catalog number, clone number, OR RRID.	Yes	Material and methods
Animal observed in or captured from the field: Provide species, sex, and age where possible.	Not Applicable	
Please detail housing and husbandry conditions.	Yes	Material and methods, results
Plants and microbes	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Plants: provide species and strain, ecotype and cultivar where relevant, unique accession number if available, and source (including location for collected wild specimens).	Not Applicable	
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)
If your work benefited from core facilities, was their service mentioned in the acknowledgments section?	Yes	Acknowledgments
Design	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Study protocol		

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

Sample definition and in-laboratory replication	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
In the figure legends: state number of times the experiment was replicated in laboratory.	Yes	figure legends
In the figure legends: define whether data describe technical or biological replicates .	Yes	figure legends

Ethics

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 approval (IRB or equivalent committee(s), provide reference number for approval. Include a statement of compliance with ethical regulations.	Yes	Material and methods
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	Data availability
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?	Not Applicable	
If publicly available data were reused, provide the respective data citations in the reference list .	Yes	references