EMBO reports

CMTM6 suppresses cell-surface expression of death receptor FAS in mice but not in humans

Peter Draber, Tereza Semberova, Michaela Pribikova, Veronika Cimermanova, Tijana Trivic, Rafik Haderbache, Darina Paprckova, Luca Christen, Helena Kissiova, and Ondrej Stepanek

Corresponding author(s): Peter Draber (peter.draber@unil.ch)

Review Timeline:

Submission Date:	10th May 25
Editorial Decision:	1st Jul 25
Revision Received:	9th Nov 25
Editorial Decision:	10th Dec 25
Revision Received:	18th Dec 25
Accepted:	13th Jan 26

Editor: Martina Rembold

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.)

Dear Dr. Draber

Thank you for the submission of your research manuscript to our journal. We have now received the full set of referee reports that is copied below.

As you will see, the referees acknowledge that the findings are interesting and that the conclusions are overall supported by the data presented but they also raise a number of partially overlapping concerns and have suggestions how to further strengthen the data that need to be addressed. The concern from referee #2 regarding absence of in vivo relevance ("A key limitation is that the in vivo impact of the Cmtm6-Fas interaction is inferred...") should be addressed in the discussion, but I think that the suggestion to analyse T cell activation and survival using the available KO mice (point 2) would significantly strengthen your study and provide physiological context.

Please let me know in case you disagree, and we can discuss the exact revision requirements further, also in a video chat, if you like.

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

We realize that it is difficult to revise to a specific deadline. In the interest of protecting the conceptual advance provided by the work, we recommend a revision within 3 months (October 1st). Please discuss the revision progress ahead of this time with the editor if you require more time to complete the revisions.

I am also happy to discuss the revision further via e-mail or a video call, if you wish.

You can either publish the study as a short report or as a full article. For short reports, the revised manuscript should not exceed 27,000 characters (including spaces but excluding materials & methods and references) and 5 main plus 5 expanded view figures. The results and discussion sections must further be combined, which will help to shorten the manuscript text by eliminating some redundancy that is inevitable when discussing the same experiments twice. For a normal article there are no length limitations, but it should have more than 5 main figures and the results and discussion sections must be separate. In both cases, the entire materials and methods must be included in the main manuscript file.

==========
IMPORTANT NOTE:

We perform an initial quality control of all revised manuscripts before re-review. Your manuscript will FAIL this control and the handling will be delayed IN CASE the following APPLIES:

1) A data availability section providing access to data deposited in public databases is missing. If you have not deposited any data, please add a sentence to the data availability section that explains that.

2) Your manuscript contains statistics and error bars based on n=2. Please use scatter blots in these cases. No statistics should be calculated if n=2.

When submitting your revised manuscript, please carefully review the instructions that follow below. Failure to include requested items will delay the evaluation of your revision.

==========

When submitting your revised manuscript, we will 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).
Please download our Figure Preparation Guidelines (figure preparation pdf) from our Author Guidelines pages https://www.embopress.org/page/journal/14693178/authorguide for more info on how to prepare your figures.

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/14693178/authorguide>). Please insert information in the checklist that is also reflected in the manuscript. The completed author checklist will also be part of the RPF.

5) Please note that all corresponding authors are required to supply an ORCID ID for their name upon submission of a revised manuscript (<https://orcid.org/>). Please find instructions on how to link your ORCID ID to your account in our manuscript tracking system in our Author guidelines (<https://www.embopress.org/page/journal/14693178/authorguide#authorshipguidelines>)

6) 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. See detailed instructions regarding expanded view here: <https://www.embopress.org/page/journal/14693178/authorguide#expandedview>

- 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.

7) Before submitting your revision, primary datasets (and computer code, where appropriate) produced in this study need to be deposited in an appropriate public database (see < https://www.embopress.org/page/journal/14693178/authorguide#dataavailability>).

Specifically, we would kindly ask you to provide public access to the mass spectrometry datasets.

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

The accession numbers and database should be listed in a formal "Data Availability " section (placed after Materials & Method) that follows the model below (see also < https://www.embopress.org/page/journal/14693178/authorguide#dataavailability>). Please note that the Data Availability Section is restricted to new primary data that are part of this study.

Data availability

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

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

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

8) At EMBO Press we ask authors to provide source data for the main manuscript figures. You will receive a separate email with instructions for providing source data with your revised manuscript, including how to upload and organize the files.

Additional information on source data and instruction on how to label the files are available <https://www.embopress.org/page/journal/14693178/authorguide#sourcedata>

9) The journal requires a statement specifying whether or not authors have competing interests (defined as all potential or actual interests that could be perceived to influence the presentation or interpretation of an article). In case of competing interests, this must be specified in your disclosure statement. Further information: https://www.embopress.org/competing-interests

10) Figure legends and data quantification:
The following points must be specified in each figure legend:

- the name of the statistical test used to generate error bars and P values,
- the EXACT p-values,

- the number (n) of independent experiments (please specify technical or biological replicates) underlying each data point,
- the nature of the bars and error bars (s.d., s.e.m.)

- If the data are obtained from n {less than or equal to} 5, show the individual data points in addition to the SD or SEM.
- If the data are obtained from n {less than or equal to} 2, use scatter blots showing the individual data points.

Discussion of statistical methodology can be reported in the materials and methods section, but figure legends should contain a basic description of n, P and the test applied.

See also the guidelines for figure legend preparation:
https://www.embopress.org/page/journal/14693178/authorguide#figureformat

- Please also include scale bars in all microscopy images and define their size in the legend, not the image.

11) 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 <https://www.embopress.org/page/journal/14693178/authorguide#referencesformat>.

12) All Materials and Methods need to be described in the main text using our 'Structured Methods' format. According to this format, the Methods section includes a Reagents and Tools Table (listing key reagents, experimental models, software and relevant equipment and including their sources and relevant identifiers) followed by a Methods and Protocols section describing the methods, ideally using a step-by-step protocol format. The aim is to facilitate adoption of the methodologies across labs. Please download and fill our Reagents and Tools Table template (.docx), which you can find in our author guidelines:
https://www.embopress.org/page/journal/14693178/authorguide#structuredmethods.
When submitting your revised manuscript, please do not include the Reagents and Tools Table in the Methods section of the manuscript but upload it as a separate file choosing the file type "Reagent Table".
An example of a Method paper with Structured Methods can be found here:
https://www.embopress.org/doi/10.15252/msb.20178071.

13) As part of the EMBO publication's Transparent Editorial Process, EMBO Reports publishes online a Review Process File to accompany accepted manuscripts. 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.

You are able to opt out of this by letting the editorial office know (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."

We would also welcome the submission of cover suggestions, or motifs to be used by our Graphics Illustrator in designing a cover.

I look forward to seeing a revised form of your manuscript when it is ready and please let me know if you have questions or comments regarding the revision.

Yours sincerely,

Martina Rembold, PhD
Senior Editor
EMBO reports

==========

Referee #1:

CMTM6 and CMTM4, members of a four transmembrane domain containing protein family, are known to regulate the plasma membrane expression of several proteins, including PD-L1, IL17 receptor C, and EGFR. In this manuscript, the authors report

the cell death receptor FAS as a novel interactor of CMTM6 in mouse cells, but interestingly, this interaction is lost in human cells. This finding reveals species-specific features of CMTM6 that has implications on preclinical models evaluating the therapeutic potential of CMTM6. This study is well executed at the biochemical level, but functional aspects can be enhanced.
Major:
1. One major finding of the current study is that CMTM6 downregulates FAS surface expression, rendering tumor cells more resistant to FASL-induced apoptosis. This finding contrasts the reported roles of CMTM6 on stabilizing PD-L1 expression, through promoting its recycling to the plasma membrane (PMID: 28813417). Can the authors please explore the molecular mechanisms how CMTM6 regulates FAS expression/localization? For example, imaging the subcellular localization of CMTM6 and/or FAS, together with the markers for endosomes and lysosomes would provide valuable insight, as done previously for PD-L1 (PMID: 28813417).
2. This study heavily relies on co-IP experiments, with little functional data, even in the in vitro setting. Specifically, there is a lack of functional data in human cells. Can the authors please examine FASL-induced cell death differ in Hela cells with/without CMTM6 expression?
3. The manuscript would benefit from analyzing the evolutionary divergence of the FAS:CMTM6 interaction. A FAS sequence alignment across species could reveal when this interaction emerged. Given that CD58 is absent in mice, did FAS evolution track with CD58 appearance?
4. Figure 2E,F, I see actin signals in the IP samples? Does it mean that FAS binds to actin? If so how does this alter the interpretation of the data?
Minor:
1. Fig. 2A: What about other CMTM family proteins? Was CMTM6 the only member that was enriched by FAS?
2. Figure S4D presents important data that CMTM6 does not affect FAS expression in human cells. This data deserves to be shown in a main figure.

Referee #2:

In this manuscript, Semberova et al. uncover an interesting species-specific difference in the interaction between mouse Cmtm6 and the death receptor Fas. Using quantitative proteomics and a series of well-controlled validation experiments, the authors demonstrate that mouse Cmtm6 physically associates with Fas and suppresses its surface expression in stromal cells. Consistently, in Cmtm6 knockout mice, Fas levels are markedly elevated at the cell surface across the major T cell subsets. Functionally, the increased surface expression of Fas in Cmtm6-deficient mouse MEFs leads to enhanced sensitivity to FasL-mediated apoptosis.
By contrast, the study finds that human FAS neither interacts with CMTM6 nor regulates its expression. Through elegant domain-swapping and mutagenesis experiments, the authors identify 3 amino acid differences in the extracellular/transmembrane junction that explain the loss of such interaction in human cells. This mechanistic insight is a notable strength of the study.
Overall, the findings are novel and significant for the immunology communities. CMTM6 is well known for its role in stabilizing immune checkpoint proteins PD-L1 and CD58. However, the net immunological consequences of CMTM6 loss vary depending on the model system, in part due to species-specific differences, such as the absence of a CD58 homolog in mice. The discovery that Cmtm6 regulates Fas in mice but not in humans provides an important mechanistic insight for these divergent outcomes. It also underscores the need for caution when interpreting data from murine models, especially syngeneic tumor models, in translational contexts.
A key limitation is that the in vivo impact of the Cmtm6-Fas interaction is inferred rather than directly demonstrated. The authors rely primarily on cell-based assays and previously published results to provide functional context.
Overall, this manuscript is well-written, and the experimental work is carefully executed, with high-quality data presented throughout. The study provides compelling new insight into CMTM6 biology and offers a valuable caution against assuming cross-species equivalence in immune regulation. I believe that, with some minor revisions, the manuscript will be further strengthened.

Specific Comments
1. One might have expected Cmtm6 to promote Fas surface expression as it does for PD-L1 and CD58, but here it does the opposite, which makes the finding intriguing. The authors propose a plausible explanation that Cmtm6 may promote internalization of Fas, and its absence allows Fas to accumulate at the cell surface. The paper would be further strengthened by providing experimental support for this hypothesis. While not strictly required, the authors could, for example, assess colocalization of Fas and Cmtm6 in endosomal/lysosomal compartments, or directly measure Fas internalization rates in wild-type vs Cmtm6-deficient cells.
2. Surface Fas levels are elevated across all major T cell subsets in Cmtm6 knockout mice. This is an important finding, as it may help reconcile discrepancies in the reported functional outcomes of Cmtm6 deficiency across different models. Given that the necessary resources (e.g., Cmtm6 knockout mice) are available, it would be highly informative to investigate whether the increased Fas expression impacts T cell activation, survival, or function. For example, elevated Fas levels could render T cells more susceptible to activation-induced cell death, potentially impairing their functionality. Such functional assessments would provide valuable insight into the consequences of Cmtm6 loss in the immune compartment in mouse models.

CMTM6 suppresses cell-surface expression of death receptor FAS in mice but not in humans

Semberova et al.

Responses to Reviewers

Referee #1:

CMTM6 and CMTM4, members of a four transmembrane domain containing protein family, are known to regulate the plasma membrane expression of several proteins, including PD-L1, IL17 receptor C, and EGFR. In this manuscript, the authors report the cell death receptor FAS as a novel interactor of CMTM6 in mouse cells, but interestingly, this interaction is lost in human cells. This finding reveals species-specific features of CMTM6 that has implications on preclinical models evaluating the therapeutic potential of CMTM6. This study is well executed at the biochemical level, but functional aspects can be enhanced.

We thank the Reviewer for assessing our work positively and for providing valuable suggestions on how to improve the manuscript further.

Major:

1. One major finding of the current study is that CMTM6 downregulates FAS surface expression, rendering tumor cells more resistant to FASL-induced apoptosis. This finding contrasts the reported roles of CMTM6 on stabilizing PD-L1 expression, through promoting its recycling to the plasma membrane (PMID: 28813417). Can the authors please explore the molecular mechanisms how CMTM6 regulates FAS expression/localization? For example, imaging the subcellular localization of CMTM6 and/or FAS, together with the markers for endosomes and lysosomes would provide valuable insight, as done previously for PD-L1 (PMID: 28813417).

The question about how CMTM6 suppresses FAS membrane expression was also raised by Reviewer #2 in question 1. Based on the suggestions from both Reviewers, we performed two types of experiments to address this issue.

First, we analyzed whether CMTM6 promotes FAS internalization. Cells were labeled on ice with anti-FAS antibody and subsequently incubated at 37°C for several hours. The remaining surface FAS was subsequently detected using a fluorescently labeled secondary antibody. *Cmtm6*$^{-/-}$ MEFs had a substantially decreased rate of FAS internalization compared to WT cells (newly added Fig. 5G). We confirmed these data by showing that *Cmtm6*$^{-/-}$ MEFs reconstituted with CMTM6 had enhanced FAS

internalization compared to cells transduced with EV (newly added Fig. EV5B). These data showed that CMTM6 promotes FAS internalization, which is in accord with increased surface FAS expression in CMTM6-deficient cells compared to control cells.

Subsequently, we performed an analysis of the subcellular localization of FAS and CMTM6. We expressed CMTM6-mCherry and FAS-EGFP in *Cmtm6*$^{-/-}$ cells and showed the colocalization of both proteins in recycling endosomes, detected by staining with transferrin receptor (newly added Fig. 5H). Altogether, these data demonstrated that CMTM6 enhances FAS internalization and therefore suppresses its surface expression.

2. This study heavily relies on co-IP experiments, with little functional data, even in the in vitro setting. Specifically, there is a lack of functional data in human cells. Can the authors please examine FASL-induced cell death differ in Hela cells with/without CMTM6 expression?

Human FAS does not interact with CMTM6 (Fig. 6A-C), and its surface expression is unchanged in HeLa WT versus HeLa *CMTM6*^{KO} (Fig. 7A). In accord, HeLa WT and *CMTM6*^{KO} cells do not differ in their sensitivity towards FASL-induced cell death (newly added Fig. EV5F). These data further strengthen our conclusion that CMTM6 suppresses FAS expression and FASL-induced cell death in mouse cells, but not in human cells.

3. The manuscript would benefit from analyzing the evolutionary divergence of the FAS:CMTM6 interaction. A FAS sequence alignment across species could reveal when this interaction emerged. Given that CD58 is absent in mice, did FAS evolution track with CD58 appearance?

To establish when the interaction between FAS and CMTM6 evolved, we first needed to find a reliable predictor of this interaction.

In our mapping of the interaction between mouse FAS and CMTM6, we showed the critical role of arginine localized just above the transmembrane domain of mouse FAS. Mutation of this residue to asparagine completely abolished the interaction of mouse FAS with CMTM6 (Fig. 6H). Interestingly, the comparison of the transmembrane and juxtamembrane regions of mouse FAS with mouse PD-L1, human PD-L1, or human CD58 proteins showed that there is very little overlap in amino acid sequence between these strong CMTM6 interactors. There was, however, one notable exception: very strong conservation of arginine above the plasma membrane (newly added Fig. 7B). To verify that this residue is universally required for CMTM6 interaction, we performed a detailed mutagenesis screen of amino acids at the boundary of the extracellular and transmembrane domains of PD-L1. This experiment showed that mutation R238A nearly abolished the interaction with CMTM6 (newly added Fig. 7C). Therefore, the emergence of arginine positioned above the plasma membrane seems as a critical requirement for CMTM6 interaction.

The analysis of the FAS transmembrane domain from various mammalian species showed that arginine above the transmembrane domain is very rare, as it is lacking even in rats. Interestingly, this

residue is not present in the Asian lineage mouse *M. caroli*, but appears in the Palearctic mouse group, which includes *M. spretus*, *M. musculus*, and *M. spicilegus* (newly added Fig 7D). These mouse species diverged approximately 3-6 million years ago, indicating that the evolution of FAS and CMTM6 interaction is a relatively recent evolutionary event.

4. Figure 2E and F, I see actin signals in the IP samples? Does it mean that FAS binds to actin? If so how does this alter the interpretation of the data?

We apologize for the confusing actin staining in immunoprecipitation (IP) samples. We employed actin staining as a standard loading control for lysates in our immunoblotting experiments. However, actin is notoriously sticky and binds directly to immunoprecipitation beads. This is well documented in the 'Crapome database', where it was shown using mass spectrometry that actin is a widespread contamination in immunoprecipitation experiments (Mellacheruvu et al., 2014, Nat Methods, PMID: 23921808).

In accord, we detected actin in immunoprecipitation samples when we used a control antibody, which was of the same intensity as when we used the anti-FAS antibody. This shows that detection of actin in IP samples is due to background staining, not due to specific binding to FAS. In contrast, CMTM6 was strongly isolated in samples subjected to immunoprecipitation with the anti-FAS antibody, while there was no signal using the control antibody.

We removed actin staining from panels 2E and 2F, since these experiments contain only one lysate sample separated into two IP samples using control and FAS antibody. The actin staining, therefore, did not provide any additional information.

Minor:

1. Fig. 2A: What about other CMTM family proteins? Was CMTM6 the only member that was enriched by FAS?

We observed that mouse FAS is strongly associated with CMTM6 and very weakly with CMTM4 (Fig. 1B-C). Mass spectrometry analysis of exogenously expressed mouse FAS-SF showed that CMTM6 is its strongest interactor in nonstimulated cells. We detected only a very weak signal from CMTM4, while no other CMTM family member was associated with mouse FAS-SF (Table EV2). Importantly, we could not detect an association of endogenous mouse FAS with CMTM4 in various cell lines and MEFs.

To further show that endogenous mouse FAS binds strongly to CMTM6, but not CMTM4, we isolated bone marrow-derived macrophages from WT or *Cmtm6*$^{-/-}$ mice. We confirmed the interaction between mouse FAS and CMTM6 in WT cells (newly added Fig. 2H), and we showed that deficiency of CMTM6 promotes FAS membrane expression also in these primary cells (newly added Fig. EV5A). However, we did not detect interaction between FAS and CMTM4 in WT or *Cmtm6*$^{-/-}$ BMDMs (newly

added Fig. 2H). Combined, these data show that in the endogenous system, FAS binds strongly to CMTM6, but not other members of the CMTM family.

2. Figure S4D presents important data that CMTM6 does not affect FAS expression in human cells. This data deserves to be shown in a main figure.

We moved this panel to the main figure (Fig. 7A).

Referee #2:

In this manuscript, Semberova et al. uncover an interesting species-specific difference in the interaction between mouse Cmtm6 and the death receptor Fas. Using quantitative proteomics and a series of well-controlled validation experiments, the authors demonstrate that mouse Cmtm6 physically associates with Fas and suppresses its surface expression in stromal cells. Consistently, in Cmtm6 knockout mice, Fas levels are markedly elevated at the cell surface across the major T cell subsets. Functionally, the increased surface expression of Fas in Cmtm6-deficient mouse MEFs leads to enhanced sensitivity to FasL-mediated apoptosis.

By contrast, the study finds that human FAS neither interacts with CMTM6 nor regulates its expression. Through elegant domain-swapping and mutagenesis experiments, the authors identify 3 amino acid differences in the extracellular/transmembrane junction that explain the loss of such interaction in human cells. This mechanistic insight is a notable strength of the study.

Overall, the findings are novel and significant for the immunology communities. CMTM6 is well known for its role in stabilizing immune checkpoint proteins PD-L1 and CD58. However, the net immunological consequences of CMTM6 loss vary depending on the model system, in part due to species-specific differences, such as the absence of a CD58 homolog in mice. The discovery that Cmtm6 regulates Fas in mice but not in humans provides an important mechanistic insight for these divergent outcomes. It also underscores the need for caution when interpreting data from murine models, especially syngeneic tumor models, in translational contexts.

A key limitation is that the in vivo impact of the Cmtm6-Fas interaction is inferred rather than directly demonstrated. The authors rely primarily on cell-based assays and previously published results to provide functional context.

Overall, this manuscript is well-written, and the experimental work is carefully executed, with high-quality data presented throughout. The study provides compelling new insight into CMTM6 biology and offers a valuable caution against assuming cross-species equivalence in immune regulation. I believe that, with some minor revisions, the manuscript will be further strengthened.

We thank the Reviewer for their positive evaluation of our data and for the suggestions on how to improve the manuscript further.

Specific Comments

1. One might have expected Cmtm6 to promote Fas surface expression as it does for PD-L1 and CD58, but here it does the opposite, which makes the finding intriguing. The authors propose a plausible explanation that Cmtm6 may promote internalization of Fas, and its absence allows Fas to accumulate at the cell surface. The paper would be further strengthened by providing experimental support for this hypothesis. While not strictly required, the authors could, for example, assess colocalization of Fas and Cmtm6 in endosomal/lysosomal compartments, or directly measure Fas internalization rates in wild-type vs Cmtm6-deficient cells.

The question about how CMTM6 suppresses FAS membrane expression was also raised by Reviewer #1 in question 1. Based on the suggestions from both Reviewers, we performed two types of experiments to address this issue.

First, we analyzed whether CMTM6 promotes FAS internalization. Cells were labeled on ice with anti-FAS antibody and subsequently incubated at 37°C for several hours. The remaining surface FAS was subsequently detected using a fluorescently labeled secondary antibody. *Cmtm6*$^{-/-}$ MEFs had a substantially decreased rate of FAS internalization compared to WT cells (newly added Fig. 5G). We confirmed these data by showing that *Cmtm6*$^{-/-}$ MEFs reconstituted with CMTM6 had enhanced FAS internalization compared to cells transduced with EV (newly added Fig. EV5B). These data showed that CMTM6 promotes FAS internalization, which is in accord with increased surface FAS expression in CMTM6-deficient cells compared to control cells.

Subsequently, we performed an analysis of the subcellular localization of FAS and CMTM6. We expressed CMTM6-mCherry and FAS-EGFP in *Cmtm6*$^{-/-}$ cells and showed the colocalization of both proteins in recycling endosomes, detected by staining with transferrin receptor (newly added Fig. 5H). Altogether, these data demonstrated that CMTM6 enhances FAS internalization and therefore suppresses its surface expression.

2. Surface Fas levels are elevated across all major T cell subsets in Cmtm6 knockout mice. This is an important finding, as it may help reconcile discrepancies in the reported functional outcomes of Cmtm6 deficiency across different models. Given that the necessary resources (e.g., Cmtm6 knockout mice) are available, it would be highly informative to investigate whether the increased Fas expression impacts T cell activation, survival, or function. For example, elevated Fas levels could render T cells more susceptible to activation-induced cell death, potentially impairing their functionality. Such functional assessments would provide valuable insight into the consequences of Cmtm6 loss in the immune compartment in mouse models.

To address whether CMTM6 ablation impacts the T cell activation in vivo, we infected WT and *Cmtm6*$^{-/-}$ mice intravenously with *Listeria monocytogenes* expressing ovalbumin (OVA) antigen (newly

added Fig. 4A). After 10 days, we isolated the spleens and analyzed T cells via flow cytometry (Fig. 4B and gating strategy in Fig. EV4A). We noted a markedly decreased proportion of effector $CD4^+$ T cells (Fig. 4C), while the emergence of effector and OVA-specific $CD8^+$ T cells was not significantly impacted (Fig. 4D). In contrast to $CD8^+$ cells, $CD4^+$ effectors are highly sensitive to FAS-mediated activation-induced cell death (AICD). Therefore, the decreased proportion of effector $CD4^+$ T cells upon infection in *Cmtm6*$^{-/-}$ mice compared to WT littermates is in accord with enhanced FAS expression and presumably increased AICD of these cells.

Importantly, we noticed that, similarly to naïve cells, effector T cells isolated from *Cmtm6*$^{-/-}$ mice had substantially increased expression of surface FAS compared to WT mice (Fig. 4E-I). In contrast, the surface levels of FASL were not different between WT and *Cmtm6*$^{-/-}$ mice (EV4B-F). These data further demonstrate that CMTM6 is a potent negative regulator of FAS expression in murine T cells.

Dear Dr. Draber

Thank you for the submission of your revised manuscript to EMBO reports. We have now received the full set of referee reports that is copied below.

As you will see, both referees are very positive about the study and recommend publication.

From the editorial side, there are a few things that we need before we can proceed with the official acceptance of your study:

- Please provide up to 5 keywords on the title page.

- Please rename to Conflict of Interest section to Disclosure and Competing Interests Statement.

- Regarding the Author Contributions, we now use CRediT to specify the contributions of each author in the journal submission system. Therefore, please remove the Author Contributions from the manuscript file and make sure that the author contributions in our online manuscript tracking system are correct and up-to-date. The information you specified in the system will be automatically retrieved and typeset into the article. You can enter additional information in the free text box provided, if you wish. See also our guide to authors https://www.embopress.org/page/journal/14693178/authorguide#authorshipguidelines.

- Figure S3 (line 809) is a wrong callout and needs correction.

- Tables EV1 - EV3 are complex datasets and should therefore be updated to Dataset EV1-Dataset EV3 in all places: source file names, legends, titles in the system, callouts in the manuscript.

- Materials and Methods should be renamed to Methods

- Please provide the specific URLs for datasets (ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifiers PXD070246, PXD070298, and PXD070359) in the data availability statement.

- Please address the following points in the figure legends:
a) Please provide the information related to n in the legends of figures 1B.
b) Please define the error bars in the legends of figures 1B; 2A; 5A-G; 7A; EV-5A,B,F.
c) Please define the asterisk in the legend of figure 6A,B.

- Animal models: please provide the reference number of the approval of mouse work by the Czech Academy of Sciences.

- Finally, EMBO Reports papers are accompanied online by
A) a short (1-2 sentences) summary of the findings and their significance,
B) 2-3 bullet points highlighting key results and
C) a schematic summary figure that provides a sketch of the major findings (not a data image).
Please provide the summary figure as a separate file in PNG or JPG format at a size of 550x300-600 pixels (width x height). Please note that the size is rather small and that text needs to be readable at the final size. Please send us this information along with the revised manuscript.

We look forward to seeing a final version of your manuscript as soon as possible.

With kind regards,

Martina Rembold, PhD
Senior Editor
EMBO reports

==========

Referee #1:

The authors have addressed our concerns fully

Referee #2:

My comments have been satisfactorily addressed.

All minor editorial requests have been addressed by the authors.

Dr. Peter Draber
University of Lausanne
Department of Immunobiology
Ch. des Boveresses 155
Epalinges, Switzerland 1066
Switzerland

Dear Dr. Draber,

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

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.

You may qualify for financial assistance for your publication charges - either via a Springer Nature fully open access agreement or an EMBO initiative. Check your eligibility: https://link.springer.com/journal/44319/how-to-publish-with-us

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

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

Yours sincerely,

Martina Rembold, PhD
Senior Editor
EMBO reports

>>> Please note that it is EMBO Reports policy for the transcript of the editorial process (containing referee reports and your response letter) to be published as an online supplement to each paper. If you do NOT want this, you will need to inform the Editorial Office via email immediately. More information is available here: https://link.springer.com/partners/embo-press/editorial-policies#Peer%20review

EMBO Press Author Checklist

Corresponding Author Name: Peter Draber	
Journal Submitted to: EMBO Reports	
Manuscript Number: EMBOR-2025-61917V1	

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	In the Materials and Methods section and in the Reagents and Tools table.

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	In the Materials and Methods section and in the Reagents and Tools table.

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	In the Materials and Methods section, in the Reagents and Tools table, and in the Source data.

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	The cell lines were provided by our colleagues as specified.
Primary cultures: Provide species, strain, sex of origin, genetic modification status.	Yes	The information about animal strains from which MEFs and BMDMs originated is included.
Report if the cell lines were recently **authenticated** (e.g., by STR profiling) and tested for mycoplasma contamination.	Yes	We used common cell lines, and we did not perform further authentication. The cells were regularly checked for mycoplasma contamination.

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

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	In the Acknowledgement section.

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	In the 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?	Not Applicable	
Include a statement about **blinding** even if no blinding was done.	Not Applicable	
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	The statistical tests employed are specified in the Figure legends. The tests used for normality assessment are specified in the Materials and Methods section.

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	In the Figure legends.
In the figure legends: define whether data describe **technical or biological replicates**.	Yes	In the 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	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	In the Materials and Methods section.
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**.	Not Applicable	