EMBO reports

Bclaf1 regulates c-FLIP and protects cells from TNF induced apoptosis and tissue injury

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Dear Dr. Tang

Thank you for providing feedback on the concerns raised by the referees. Please find all referee reports copied again below my signature. Given the positive evaluation from two of the referees and taking your response to the concerns from referee 2 into account, we would like to invite you to revise your manuscript with the understanding that all referee concerns must be fully addressed and their suggestions taken on board.

Please note that all data that are relevant to the study must be included in the manuscript and that we do not permit the citation of "Data not shown". Therefore, please include the RNA-seq data and please also note that large-scale datasets need to be deposited in a relevant public database. Please see also our editorial policies regarding these points
.

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 invite you to submit your manuscript within three months of a request for revision. This would be June 23rd in your case. However, we are aware of the fact that many laboratories are not fully functional due to COVID-19 related shutdowns and we have therefore extended the revision time for all research manuscripts under our scooping protection to allow for the extra time required to address essential experimental issues. Please contact us to discuss the time needed and the revisions further.

*****
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 if the following APPLIES:

1) A data availability section is missing.
2) Your manuscript contains error bars based on n=2. Please use scatter blots showing the individual datapoints in these cases. The use of statistical tests needs to be justified.
*****

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

- 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 RNA-seq datasets of WT and Bclaf1 knockout cells.

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) We would also encourage you to include the source data for figure panels that show essential data. Numerical data should be provided as individual .xls or .csv files (including a tab describing the data). For blots or microscopy, uncropped images should be submitted (using a zip archive if multiple images need to be supplied for one panel). Additional information on source data and instruction on how to label the files are available .

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

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- the name of the statistical test used to generate error bars and 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.)
Discussion of statistical methodology can be reported in the materials and methods section, but figure legends should contain a basic description of n, P and the test applied.
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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 version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

Yours sincerely

Martina Rembold, PhD
Senior Editor
EMBO reports

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Referee #1:

The manuscript by Zhang and colleagues showed the role of the transcription factor, Bclaf1 on regulating TNF sensitivity via controlling TNF-mediated induction of the anti-apoptotic protein, cFLIP.
The manuscript is very well written, with well built experimental approach to examine the role of Bclaf1 in TNF-mediated apoptosis signalling.

The conclusions are well founded and corroborated by the experimental results and I only have a few minor comments:
1. The authors show that Bclaf1 deficiency enhances TNF-mediated apoptosis. To support the argument that the main mechanism of this is by regulating cFLIP expression, it would be good to present the effect of cFLIP knockdown on the same. Would the same level of sensitisation be achieved? Also, as the authors determined transcriptomic changes in response to Bclaf-loss, but did not include the data. Presenting the genes related to TNF signalling and top up- and downregulated genes would support the objective for the study.
2. Bcla1 is indicated as a transcriptional repressor. It would be useful to include a broader background on the known biological functions of Bclaf1 in the discussion, such as mRNA pre-processing and RNP complexes and how Bclaf1 may drive cFLIP expression (in complex with p50).
3. The manuscript has a few wording errors, which should be corrected:
Row 111: Consistently, western analysis of caspase 3 and PARP cleavage demonstrated more serious apoptosis occurred in Bclaf1 KO or knockdown cells (Figures 1A-C and Fig S1B).
Apoptosis does not have levels of seriousness. Please remove the word serious from the sentence

Row 144: Further analysis of the formation of complex IIa in HeLa and MEFs immunoprecipitation using an anti-FADD revealed that less c-FLIP was present...
Please add antibody after anti-FADD

Row 173: To examine whether Bclaf1 resides in the promoter region of c-Flip in cells, we utilized an engineered HeLa cell line, HeLa-Flag-Bclaf1, in which Flag was inserted into the Bclaf1 genome, expressing a N-terminal tagged F-Bclaf1 endogenously, and performed chromatin...
Bclaf1 probably does not always reside in the cFLIP promoter region. It would be better to use the word: can localise to the promoter....
Also, there is no Bclaf1 genome, only a Bclaf1 gene. Please correct

Row 277: TNFα engagement simultaneously activates survival and death signals. c-FLIP induced
by NF-κB is considered as the most prominent anti-apoptotic factor (Kreuz et al., 2001), and
its relative level to that of caspase 8 ultimately determines the fate of a cell.
It is a broad generalisation that the cFLIP:caspase-8 ratio is the sole determinant of cell fate. Please change the wording.

Row 287: Because p50 does not possess a transactivation domain, Bclaf1 gives its ability to activate c-Flip highly suggests...
The term: induces cFLIP expression, rather than "activate cFLIP" would be more correct.

TNFalpha is now called TNF, it would be good to replace it throughout the manuscript.

Referee #2:

Zhang et al. investigate a role for Bclaf1 in TNF-induced apoptosis using human tumor cell lines and murine cells. The authors show that TNF+CHX-induced apoptosis is enhanced in *BCLAF1* KO HeLa cells or HeLa cells treated with*BCLAF1* siRNAs. Cell death enhancement is specific to apoptosis, because TCZ stimulation (this induces necroptosis) does not enhance cell death in *BCLAF1* knockdown MEFs. These results suggest that Bclaf1-mediated cell death enhancement is relatively specific to apoptosis. Then, they find that expression of anti-apoptotic protein, $cFLIP_L$, is decreased in *BCLAF1* knockdown HeLa cells after TNF stimulation. Moreover, mRNA expression of *CFLAR* is mainly regulated by Bclaf1/p50 heterodimer using qPCR and reporter assays. Finally, the authors show that knockdown of Bclaf1 increases susceptibility to TNF-induced apoptosis of IECs of the small intestine following TNF injection in vivo. Although the study is potentially interesting, there are serious concerns about the experimental design and the authors' interpretation of the results.

Major comments:
1. The authors do not describe the detailed information of the experiments in vitro, such as concentrations of each agent and duration of incubation time.

2. Figure 1A, please indicate CHX and TNF concentrations and how long the authors treat HeLa cells with these agents. It is unclear why TNF/CHX only induces 5% of apoptosis of wild-type HeLa cells. Moreover, the authors need to show the representative FACS profile of Annexin V/PI staining in Supplementary Figures. Although the authors show that TNF/SM-164 does not increase cell death in *Bclaf1* knockdown MEFs, the authors should perform a similar experiment using HeLa cells. Moreover, the authors need to discuss why cell death enhancement by *BCLAF1* knockdown is limited to TNF/CHX stimulation. Given that treatment of cells with CHX alone induces degradation of $cFLIP_L$ and cFLIPs even in the presence of Bcfla1 (Figure S2A), it is not likely that slight change of expression level of $cFLIP_L$ may have a great impact on TNF/CHX-induced cell death.

3. Figure3A, *Bclaf1* knockdown impairs upregulation of $cFLIP_L$ and cFLIPs induced by TNF; accordingly, TNF/CHX-induced apoptosis is enhanced in *BCLAF1* knockdown cells. However, an increase in expression of $cFLIP_L$ at the protein level appears to be marginal. The reviewer wonders why such a marginal increase affects the susceptibility to TNF-induced apoptosis (lanes 1, 2, 5, and 6 from the left side).

4. Figure 3B and C, the authors show the complex II formation is enhanced in *BCLAF1* knockdown cells. Assuming that the complex II formation is induced, it is unclear why the processed form of caspase 8 is hardly detected in TNF/CHX-treated cells. The same thing holds for $cFLIP_L$; $cFLIP_L$ is recruited to the complex II and should be processed.
5. In Figure 3D and E, expression of $cFLIP_L$ completely disappears in the cells after TNF/CHX stimulation (lanes 2, 4, and 6). I have no idea why the authors claim that Bclaf1-induced anti-apoptotic function is mediated by $cFLIP_L$. The same thing holds true for Figure 5E.

6. Figure 3E, there is no cFLIPs, at least in MEFs. The reviewer has a concern about the specificity of anti-cFLIP antibody used in the present study. The authors need to show the specificity of anti-cFLIP antibody using *Cflar* siRNA. Alternatively, the authors need to verify their Western blotting with another anti-cFLIP antibody, such as Dave-2.

7. Figure 6, the authors show that Bclaf1 interacts with p50 and p65 in the nucleus following TNF stimulation. If that is the case, Bclaf1 may affect TNF-induced gene expression. However, the authors show that TNF-induced NF-κB activation is not impaired in *BCLAF1* knockdown cells. The authors need to dig into the effect of*BCLAF1* knockdown on gene expression induced by TNF, such as *IL8*.

8. Figure 7, the authors use a murine SIRS model following TNF injection. To evaluate the tissue injury, the authors need to count $CC3^+$ cells in IECs of the small intestine from control and*Bclaf1* knockdown mice and perform statistical analysis (Figure 7E). Also, injection of large amounts of TNF induce necroptosis in IECs. Thus, the authors should stain the tissue with anti-phospho-RIPK3 antibody and count numbers of necroptotic cells.

Minor comments:
1. Line 72, gene symbols of murine and human cFLIP are *Cflar* and *CFLAR*, respectively. The authors need to change *c-Flip* to *Cflar* or CFLAR throughout the manuscript.

2. Line 102, the authors need to show the data of RNA-seq analysis.

3. Line 413, 10 ng of mTNFα should be changed to be 10 μg of mTNFα.

Referee #3:

Zhang et al. investigate the role of Bclaf1 in the regulation of TNFα-induced cell death. Bclaf1 was originally identified as a Bcl2-interacting protein but seems to be expressed mainly in the nucleus of cells. Accordingly, it has been connected to the regulation of transcription and mRNA metabolism. The protein is 920 amino acids in length (calculated molecular weight is about 106 kDa) and contains several domains including a N-terminal SR domain, which is often found in proteins involved in splicing. The Tang group has published earlier that Bclaf1 is a transcriptional regulator in different biological settings (Shao et al., 2016; Qin et al., 2019; Shao et al., 2020). In the current study, they found in an RNAseq analysis of Hela cells deficient in Bclaf1 many genes related to TNFα signaling. Unfortunately, these data are not provided. Zhang et al. show that Bclaf1 knockdown cells are more sensitive to TNFα-induced, caspase-8-dependent apoptosis but not other forms of TNFα-induced cell death. They can pinpoint this effect to transcriptional upregulation of the capase-8 antagonist c-FLIP and demonstrate that this depends on the NF-κB subunit p50, which interacts with the middle domain of Bclaf1. Finally, they show a role for Bclaf1-regulated in TNFα-induced cell death and tissue damage in an in vivo mouse model using i.v. siRNA application as well as in human intestinal epithelial cells. This is a very detailed mechanistic study. There are only a few points that should be addressed before publication is warranted.

The starting point of the study is a bit unclear to me. What was the reason to perform RNAseq in Bclaf1 KO Hela cells?

Why were most of the experiments done using siRNAs when a complete knockout cell line is available? Knockdown is never complete raising the issue that remaining expression may be sufficient to mediate a certain pathway. In this line, I recommend to repeat the experiments regarding NF-κB and MAPK activation with the Hela KO cell line.

The authors use Annexin V to quantify apoptosis. Was a counterstain included (PI or 7AAD)? If so, which cells were regarded as apoptotic (AxV+ PI- or all AxV+ cells)? The authors should be aware that cells undergoing necrosis become AxV+, as well. Since the authors want to make the point that Bclaf1 regulates specifically TNFα-induced apoptosis (and not necroptosis e.g.), this is important to clarify.

On page 15 (lines 283-286) the authors state that c-FLIP upregulation by Bclaf1 depends on NF-κB, particularly on p50. However, they also show in Figure 4H that Bclaf1 is able to bind constitutively to the *CFLAR* promoter (without TNFα stimulation). Moreover, in the absence of TNFα stimulation, the expression of NF-κB in the nucleus is quite low (Figure 6A). Please discuss this point.

Figure 3F: Although re-expression of Flag-FLIP in siBclaf1 treated cells reduces caspase cleavage, the cleavage of PARP remains unaltered. Please explain.

In the discussion section (also page 15) the authors mention other transcriptional regulators of *CFLAR* such as c-Myc and Peli1. However, many more transcription factors have been described that regulate c-FLIP expression, e.g. C/EBP in B cells (Paz-Piel et al., Leukemia 2009) and NFAT (Zaichuk et al., J Exp Med 2004; Ueffing et al., Blood 2008).

The official name of the gene that encodes c-FLIP proteins is *CFLAR* in humans and *Cflar* in mice. This should be corrected throughout the manuscript.

The authors use human IECs in their final experiments. Are these primary cells? Although the source is mentioned in the materials and methods section, this point is not clear.

The sequence information for siRNA no. 2 for mouse Bclaf1 is missing in the materials and methods section.

The reference list is not formatted correctly. For instance, often the journal name is missing.

Point-by-point reply to the reviewer comments

Referee #1:

The manuscript by Zhang and colleagues showed the role of the transcription factor, Bclaf1 on regulating TNF sensitivity via controlling TNF-mediated induction of the anti-apoptotic protein, cFLIP.
The manuscript is very well written, with well built experimental approach to examine the role of Bclaf1 in TNF-mediated apoptosis signalling.

The conclusions are well founded and corroborated by the experimental results and I only have a few minor comments:

1. The authors show that Bclaf1 deficiency enhances TNF-mediated apoptosis. To support the argument that the main mechanism of this is by regulating cFLIP expression, it would be good to present the effect of cFLIP knockdown on the same. Would the same level of sensitisation be achieved? Also, as the authors determined transcriptomic changes in response to Bclaf-loss, but did not include the data. Presenting the genes related to TNF signalling and top up- and downregulated genes would support the objective for the study.

Response:

As suggested by the reviewer, we examined the effect of cFLIP knockdown on the sensitivity of cells to TNF-mediated apoptosis in comparison with that of Bclaf1 knockdown. As expected, cFLIP knockdown also enhanced apoptosis and caspase 3 activation, and the effects are stronger than that of Bclaf1 knockdown, which correlates with more profound reduction in cFLIP expression in cFLIP knockdown cells than that of Bclaf1 knockdown cells. We included the data in the revised manuscript (revised Figure 3G and H).

We have included the top 20 KEGG pathways and the heatmap of the different expression genes in TNF signaling pathway between HeLa Bclaf1-WT and KO cells in the revised Figure EV1A and B.

2. Bclaf1 is indicated as a transcriptional repressor. It would be useful to include a broader

background on the known biological functions of Bclaf1 in the discussion, such as mRNA pre-processing and RNP complexes and how Bclaf1 may drive cFLIP expression (in complex with p50).

Response:

Based on the reviewer's suggestion, we have added the following paragraph in the revised discussion.

Bclaf1 was first identified as a transcriptional repressor as it represses a reporter gene when fused to the GAL4-DNA binding domain. Subsequent studies have indicated that Bclaf1 can function either as an activator or a repressor depending on biological settings. For instance, Bclaf1 promotes transcription of p53 and inflammatory cytokines in response to DNA damage, but represses gene expression in response to RAG-mediated DNA break in early B cells. Bclaf1 has also been identified as a component of the RNA splicing complex, and promotes the stability of transcripts by regulating pre-mRNA splicing. The mechanism by which Bclaf1 enhances gene transcription is not clear. Given that the middle region of Bclaf1 is required to stimulate *CFLAR* transcription, Bclaf1 involved RNA processing activity could be excluded as it depends on its N-terminal RS rich region. However, whether Bclaf1 regulates transcription through an epigenetic mechanism, modulation of RNA polymerase activity or other mechanisms needs further investigation.

3. The manuscript has a few wording errors, which should be corrected:

Response:

We are very grateful for the reviewer for pointing out the wording errors for us. All the mistakes have been fixed as suggested by the reviewer in the revised manuscript.

Row 111: Consistently, western analysis of caspase 3 and PARP cleavage demonstrated more serious apoptosis occurred in Bclaf1 KO or knockdown cells (Figures 1A-C and Fig S1B). Apoptosis does not have levels of seriousness. Please remove the word serious from the sentence

Response:

We have removed the word "serious" from the sentence.

Row 144: Further analysis of the formation of complex IIa in HeLa and MEFs immunoprecipitation using an anti-FADD revealed that less c-FLIP was present...

Please add antibody after anti-FADD

Response:

We have fixed the mistake in the revised manuscript.

Row 173: To examine whether Bclaf1 resides in the promoter region of c-Flip in cells, we utilized an engineered HeLa cell line, HeLa-Flag-Bclaf1, in which Flag was inserted into the Bclaf1 genome, expressing a N-terminal tagged F-Bclaf1 endogenously, and performed chromatin...

Bclaf1 probably does not always reside in the cFLIP promoter region. It would be better to use the word: can localise to the promoter....

Also, there is no Bclaf1 genome, only a Bclaf1 gene. Please correct

Response:

We have corrected both inappropriate words.

Row 277: TNFα engagement simultaneously activates survival and death signals. c-FLIP induced by NF-κB is considered as the most prominent anti-apoptotic factor (Kreuz et al., 2001), and its relative level to that of caspase 8 ultimately determines the fate of a cell.

It is a broad generalisation that the cFLIP:caspase-8 ratio is the sole determinant of cell fate. Please change the wording.

Response:

We fully agree with the reviewer that the cFLIP: caspase 8 ratio is not the sole determinant in cell fate, although it may play a very important role. We have changed the wording in the revised manuscript accordingly.

Row 287: Because p50 does not possess a transactivation domain, Bclaf1 gives its ability to activate c-Flip highly suggests... The term: induces cFLIP expression, rather than "activate cFLIP" would be more correct.

Response:

We have changed the wording as suggested.

TNFalpha is now called TNF, it would be good to replace it throughout the manuscript.

Response:

We have followed the reviewer's suggestion.

Referee #2:

Zhang et al. investigate a role for Bclaf1 in TNF-induced apoptosis using human tumor cell lines and murine cells. The authors show that TNF+CHX-induced apoptosis is enhanced in BCLAF1 KO HeLa cells or HeLa cells treated with BCLAF1 siRNAs. Cell death enhancement is specific to apoptosis, because TCZ stimulation (this induces necroptosis) does not enhance cell death in BCLAF1 knockdown MEFs. These results suggest that Bclaf1-mediated cell death enhancement is relatively specific to apoptosis. Then, they find that expression of anti-apoptotic protein, $cFLIP_L$, is decreased in BCLAF1 knockdown HeLa cells after TNF stimulation. Moreover, mRNA expression of CFLAR is mainly regulated by Bclaf1/p50 heterodimer using qPCR and reporter assays. Finally, the authors show that knockdown of Bclaf1 increases susceptibility to TNF-induced apoptosis of IECs of the small intestine following TNF injection in vivo. Although the study is potentially interesting, there are serious concerns about the experimental design and the authors' interpretation of the results.

Major comments:

1. The authors do not describe the detailed information of the experiments in vitro, such as concentrations of each agent and duration of incubation time.

Response:

We thank the reviewer for pointing this out, and have now provided the detailed

information for each experiment in the revised text (page 6 and 7). We have also labeled or provided the concentrations of each agent and duration of treatment time in Figures and /or in figure legends.

2. Figure 1A, please indicate CHX and TNF concentrations and how long the authors treat HeLa cells with these agents. It is unclear why TNF/CHX only induces 5% of apoptosis of wild-type HeLa cells.

Response:

To measure apoptosis (Figure 1A), we normally treated cells with 1 μg/ml of CHX plus 10 ng/ml of TNF for 12 hours. One point we want to emphasize is that we titrated the concentrations of CHX to induce apoptosis together with TNF, and have now added the data in revised Figure EV1C. We chose 1 μg/ml of CHX because we only want to induce a mild apoptosis for Bclaf1 wild type cells. Consistent with previous reports (Micheau et al., Mol Cell Biol 2001), in our titration experiments we found the concentrations of CHX were correlated with the extent of apoptosis, and inversely correlated with the expression levels of cFLIP. Please see Figure R1 below. We apologize for not explaining this well in the manuscript.

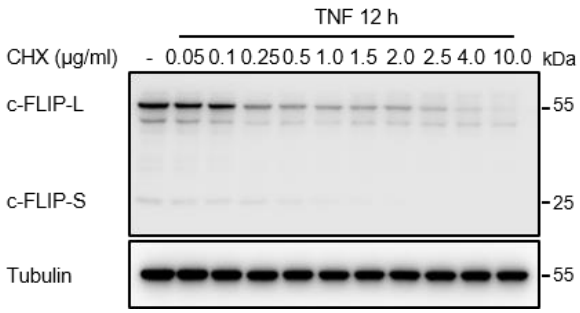

Figure R1. HeLa cells treated with increasing concentrations of CHX and 10 ng/ml of TNF for 12 hours were lysed and then analyzed by western blotting.

Moreover, the authors need to show the representative FACS profile of Annexin V/PI staining in Supplementary Figures. Although the authors show that TNF/SM-164 does not increase cell death in Bclaf1 knockdown MEFs, the authors should perform a similar experiment using HeLa cells.

Response:

The representative FACS profiles have now provided in Figures EV1D, E and J.

As suggested, we examined the effect of Bclaf1 knockdown on the apoptosis induced by TNF/SM-164 in HeLa cells, and have presented the data in the revised Figure EV1I. Consistent with data obtained in MEFs, Bclaf1 knockdown did not affect the apoptosis induced by TNF/SM-164 in HeLa cells.

Moreover, the authors need to discuss why cell death enhancement by BCLAF1 knockdown is limited to TNF/CHX stimulation.

Response:

Our data indicate that the role of Bclaf1 in regulating apoptosis depends on its involvement in transcriptional upregulation of cFLIP. It has been well established that TNF induced upregulation of cFLIP plays an important protective role against TNF induced apoptosis. For many cell types, TNF induced apoptosis can only occur when cFLIP translation is inhibited by CHX. Bclaf1 knockdown suppresses TNF induced increase in cFLIP, thus enhancing apoptosis. For FAS or TRAIL induced apoptosis, ligand engagements directly induce DISC formation and apoptosis, and we suspect that the role of Bclaf1 in these scenarios of apoptosis is minimal. However, we cannot rule out the possibility that Bclaf1 might be involved in apoptotic regulation in certain cell types where the levels of cFLIP critically determine the sensitivity of death receptor mediated apoptosis and are maintained by NF-κB signaling. The relevant discussion can be found in Page 18 of the revised manuscript.

Given that treatment of cells with CHX alone induces degradation of $cFLIP_L$ and cFLIPs even in the presence of Bcfla1 (Figure S2A), it is not likely that slight change of expression level of $cFLIP_L$ may have a great impact on TNF/CHX-induced cell death.

Response:

In the original Figure S2A (Figure EV3A in revised manuscript), to measure the half-life of cFLIP we treated cells with 50 μg/ml of CHX to completely block protein synthesis, which

is much higher than the concentration of CHX (1 μg/ml) we used to induce apoptosis. The result indicated that cFLIP is very unstable with a half-life of about 2 hours in both Bclaf1 WT and knockdown cells. In fact, cFLIP was undetectable in cells treated with 50 μg/ml of CHX/TNF, and hardly detected in cells treated with 10 μg/ml of CHX/TNF (Figure R1 above). Thus, under the condition where protein translation is mostly inhibited, the transcriptional upregulation of cFLIP by TNF fails to influence the protein level, and the cFLIP level is too low to provide any protection against apoptosis. However, on the condition that cFLIP synthesis is only partially blocked by 1 μg/ml of CHX, TNF induced upregulation of cFLIP increases the cellular level of cFLIP to a point that displays a certain level of protection against apoptosis. In this setting, Bclaf1 knockdown impairs transcriptional upregulation of cFLIP, and impacts the protein level of cFLIP, as a result, the enhanced apoptosis is observed.

3. Figure3A, Bclaf1 knockdown impairs upregulation of cFLIP$_L$ and cFLIPs induced by TNF; accordingly, TNF/CHX-induced apoptosis is enhanced in BCLAF1 knockdown cells. However, an increase in expression of cFLIP$_L$ at the protein level appears to be marginal. The reviewer wonders why such a marginal increase affects the susceptibility to TNF-induced apoptosis (lanes 1, 2, 5, and 6 from the left side).

Response:

In Figure 3A, we analyzed the dynamics of Bclaf1 knockdown induced cFLIP upregulation by treating cells with TNF for 4, 6 and 8 hours. Our data clearly showed that Bclaf1 knockdown impaired the upregulation of cFLIP at protein levels even at 4-hour time point and in a time-dependent manner. We agree with the reviewer that TNF induced increase in cFLIP at 4 hours may not play an important role in regulating apoptosis, as 4-hour treatment with TNF/CHX did not induce apoptosis. To observe visible apoptosis, at least 12 hours of treatments with TNF/CHX are required.

4. Figure 3B and C, the authors show the complex II formation is enhanced in BCLAF1 knockdown cells. Assuming that the complex II formation is induced, it is unclear why the processed form of caspase 8 is hardly detected in TNF/CHX-treated cells.

The same thing holds for cFLIP$_L$; cFLIP$_L$ is recruited to the complex II and should be processed.

Response:

In Figure 3B and C, we treated cells with TNF/CHX for 4 hours and wanted to determine if there is difference in the recruitment of cFLIP in complex II between Bclaf1 WT and knockout cells. TNF indeed induces the formation of FADD complex, which includes caspase 8, TRADD and cFLIP. However, we observe that less cFLIP was recruited to the FADD complex from Bclaf1 knockdown cells in comparison with that of control. As being pointed out by the reviewer, the process of caspase 8 is very minimal in Bclaf1 WT cells at 4-hour time point. However, we consistently observed more processed caspase 8 in Bclaf1 knock down cells in both cell lysates and FADD complexes (Figure 3C and D, Lane 4 verses lane 3).

To address the reviewer's concern, we performed two additional experiments. In the first experiment, we examined whether more processed caspase 8 can be detected in the FADD complex by extending the treatment time of TNF/CHX from 4 hours to 8 hours. Clearly, more processed caspase 8 was detected in both cell lysates and the FADD complex in Bclaf1 knockdown cells compared to that of control. However, we failed to detect cFLIP in the complex. It could be partly due to further destabilization of cFLIP at 8 hours as it has been reported that TNF stimulation activates ubiquitination pathways to degrade cFLIP, for instance Itch (Chang et al., Cell 2006). The new data have now included in the revised Figure EV2A and B. In the other experiment, in order to better observe cFLIP in the FADD complex, we treated cells with TNF for 4 hours. Without CHX treatment, cFLIP can be easily detected in the complex, and less cFLIP was present in both lysate and cFLIP complex of Bclaf1 knockdown cells. We also observed processed caspase 8 in the FADD complex with more in Bclaf1 knockdown cells than that of controls. This new data has been added in the revised Figure 3B.

5. In Figure 3D and E, expression of cFLIP$_L$ completely disappears in the cells after TNF/CHX stimulation (lanes 2, 4, and 6). I have no idea why the authors claim that Bclaf1-induced anti-apoptotic function is mediated by cFLIP$_L$. The same thing holds true

for Figure 5E.

Response:

In the original Figure 3D and E (revised Figure 3E and F), we treated cells with TNF/CHX for 12 hours and wanted to examine if there is any difference in caspase 8 activation between Bclaf1 WT and knockout or knockdown cells. We clearly see that more caspase 8 is cleaved in HeLa Bclaf1 KO (Figure 3E lane 4 and 6 verse lane 2) or Bclaf1 knock down MEFs (Figure 3F lane 4 and 6 verses lane 2). We agree with the reviewer that 12-hour treatment of cells with TNF/CHX nearly completely diminished cFLIP in Bclaf1 KO cells although some level of cFLIP still existed in Bclaf1 WT cells (Figure 3E, lane 2). We believe this is a combinational effect of long hour treatment with CHX and apoptotic process, because cFLIP is an unstable protein and apoptosis inhibits transcription. However, we think the inhibitory effect of cFLIP on apoptosis occurs much earlier than clear signs of apoptosis being presented. As stated above, in the FADD complex formed at 4 hours of TNF or TNF/CHX treatment, less cFLIP and more processed caspase 8 was present in the complex of Bclaf1 knockdown cells compared with that of controls (Figure 3B and C, lane 4 verses lane 3).

6. Figure 3E, there is no cFLIPs, at least in MEFs. The reviewer has a concern about the specificity of anti-cFLIP antibody used in the present study. The authors need to show the specificity of anti-cFLIP antibody using Cflar siRNA. Alternatively, the authors need to verify their Western blotting with another anti-cFLIP antibody, such as Dave-2.

Response:

The reviews raised a very good point. It is true that mouse does not express $cFLIP_S$ due to lack of the relevant splicing exon. However, another short isoform of cFLIP ($cFLIP_R$) exists in mouse with a similar MW as that of cFLIPs. To clarify the issue, we examined the specificity of the anti-cFLIP antibody as suggested by the reviewer. Both cFLIP-L and -S isoforms were decreased in *CFLAR* siRNA transfected HeLa indicating that the antibody can recognize both isoforms in human cells (Figure R2A below). However, as suspected by the reviewer, the indicated $cFLIP_S$ in MEFs in the original Figure 3E was indeed a non-specific band because an siRNA against *Cflar* only reduced $cFLIP_L$ but not the

smaller band (Figure R2B below). In addition, this band also cannot be detected by the anti-cFLIP antibody Dave-2 suggested by the reviewer (Figure R2C below). Thus, we repeated the experiments in the original Figure 3E and Figure 4C and probed cFLIP in MEFs with Dave-2, and replaced the data with the new data, which are presented in the new revised Figure 3F and Figure 4C.

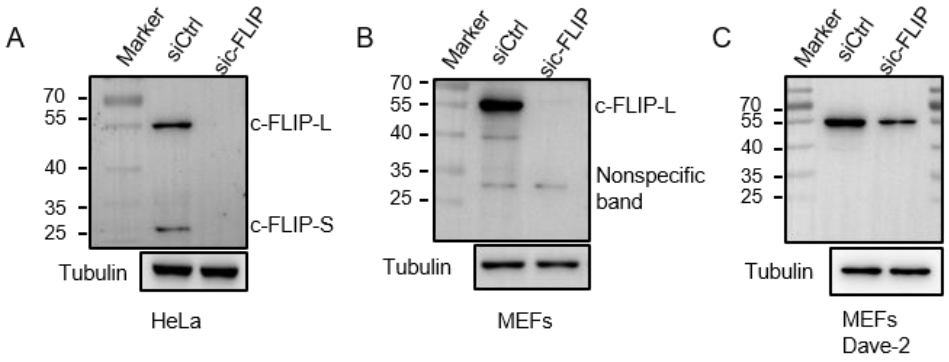

Figure R2. MEFs and HeLa cells were transfected with siCtrl or sicFLIP and then analyzed by western blotting with the indicated antibodies.

7. Figure 6, the authors show that Bclaf1 interacts with p50 and p65 in the nucleus following TNF stimulation. If that is the case, Bclaf1 may affect TNF-induced gene expression. However, the authors show that TNF-induced NF-κB activation is not impaired in BCLAF1 knockdown cells. The authors need to dig into the effect of BCLAF1 knockdown on gene expression induced by TNF, such as IL8.

Response:

We only showed and concluded that the upstreaming signaling events leading to NF-κB activation, meaning TNF induced NF-κB translocation into the nucleus, is not impaired by Bclaf1 knockdown. The data we presented mainly suggest that Bclaf1 is a novel transcriptional regulator of NF-κB in the nucleus, particularly affecting p50 arm. In addition to *CFLAR*, some of other NF-κB regulated genes induced by TNF, including *IL-8* and *CXCL10* are also transcriptionally regulated by Bclaf1 (revised Figure EV5), whereas other genes, such as *ciap*1/2 and *A20* are not (revised Figure EV3B and EV5).

8. Figure 7, the authors use a murine SIRS model following TNF injection. To evaluate the

tissue injury, the authors need to count CC3$^+$ cells in IECs of the small intestine from control and Bclaf1 knockdown mice and perform statistical analysis (Figure 7E). Also, injection of large amounts of TNF induce necroptosis in IECs. Thus, the authors should stain the tissue with anti-phospho-RIPK3 antibody and count numbers of necroptotic cells.

Response:

As suggested, we have counted CC3$^+$ cells in IECs of the small intestines from control and Bclaf1 knockdown mice and perform statistical analysis, and have included the data in the revised Figure 7E. We also performed tissue staining with an anti-phospho-RIPK3 antibody and counted necroptotic cells as suggested by the reviewer. TNF injection indeed induced necroptosis in IECs, which was not influenced by Bclaf1 knockdown (revised Figure 7F).

Minor comments:

1. Line 72, gene symbols of murine and human cFLIP are Cflar and CFLAR, respectively. The authors need to change c-Flip to Cflar or CFLAR throughout the manuscript.

Response:

We are very grateful for the reviewer's information, and have changed cFLIP gene name to *Cflar* or *CFLAR* throughout the manuscript.

2. Line 102, the authors need to show the data of RNA-seq analysis.

Response:

We have presented the top 20 KEGG pathways and the Heatmap of the different expression genes in TNF signaling pathway between HeLa Bclaf1-WT and KO cells in the revised Figure EV1A and B. The FPKM and Read Count from the RNA-seq analysis are also provided in Dataset EV1.

3. Line 413, 10 ng of mTNFα should be changed to be 10 μg of mTNFα.

Response:

We have fixed the error.

Referee #3:

Zhang et al. investigate the role of Bclaf1 in the regulation of TNFα-induced cell death. Bclaf1 was originally identified as a Bcl2-interacting protein but seems to be expressed mainly in the nucleus of cells. Accordingly, it has been connected to the regulation of transcription and mRNA metabolism. The protein is 920 amino acids in length (calculated molecular weight is about 106 kDa) and contains several domains including a N-terminal SR domain, which is often found in proteins involved in splicing. The Tang group has published earlier that Bclaf1 is a transcriptional regulator in different biological settings (Shao et al., 2016; Qin et al., 2019; Shao et al., 2020). In the current study, they found in an RNAseq analysis of Hela cells deficient in Bclaf1 many genes related to TNFα signaling. Unfortunately, these data are not provided. Zhang et al. show that Bclaf1 knockdown cells are more sensitive to TNFα-induced, caspase-8-dependent apoptosis but not other forms of TNFα-induced cell death. They can pinpoint this effect to transcriptional upregulation of the caspase-8 antagonist c-FLIP and demonstrate that this depends on the NF-κB subunit p50, which interacts with the middle domain of Bclaf1. Finally, they show a role for Bclaf1-regulated in TNFα-induced cell death and tissue damage in an in vivo mouse model using i.v. siRNA application as well as in human intestinal epithelial cells. This is a very detailed mechanistic study. There are only a few points that should be addressed before publication is warranted.

The starting point of the study is a bit unclear to me. What was the reason to perform RNAseq in Bclaf1 KO Hela cells?

Response:

In our previous study, we found Bclaf1 functions as a transcription regulator in the type I interferon signaling pathway, and can also bind to genomic DNA without any treatment (Qin et al., PloS Pathogens 2019). In an effort to examine what other pathways Bclaf1 might be involved, we performed RNAseq in Bclaf1 KO HeLa cells in comparison with that of WT cells. We have included this information in the revised manuscript page 6.

Why were most of the experiments done using siRNAs when a complete knockout cell line is available? Knockdown is never complete raising the issue that remaining expression may be sufficient to mediate a certain pathway. In this line, I recommend to repeat the experiments regarding NF-κB and MAPK activation with the Hela KO cell line.

Response:

As suggested by the reviewer, we repeated the experiments detecting NF-κB and MAPK activation in HeLa WT and Bclaf1-KO cells and got the similar results as that of knockdown cells. The new data have been included in the revised Figure 2A and C.

The authors use Annexin V to quantify apoptosis. Was a counterstain included (PI or 7AAD)? If so, which cells were regarded as apoptotic (AxV+ PI- or all AxV+ cells)? The authors should be aware that cells undergoing necrosis become AxV+, as well. Since the authors want to make the point that Bclaf1 regulates specifically TNFα-induced apoptosis (and not necroptosis e.g.), this is important to clarify.

Response:

We performed Annexin V and 7AAD co-staining for apoptosis analysis and considered total Annexin V positive cells as apoptotic cells including both Annexin V^+7AAD^- and Annexin V^+7AAD^+ cells. The reason for this is because the condition we used to induce apoptosis is rather mild, and blocking apoptosis by adding z-VAD nearly completely abolished Annexin V positive cells with the appearance of Annexin V^-7AAD^+ (Please see revised Figure EV1J). Thus, we think all Annexin V positive cells are apoptotic in our case. In addition, we also quantified Annexin V^+7AAD^- cells, and the trends were similar to that of total Annexin V^+ cells (revised Figure EV1D and E). We clarified the issue in the revised text.

On page 15 (lines 283-286) the authors state that c-FLIP upregulation by Bclaf1 depends on NF-κB, particularly on p50. However, they also show in Figure 4H that Bclaf1 is able to bind constitutively to the CFLAR promoter (without TNFα stimulation). Moreover, in the absence of TNFα stimulation, the expression of NF-κB in the nucleus is quite low (Figure

6A). Please discuss this point.

Response:

We agree with the reviewer that a portion of Bclaf1 that binds to the *CFLAR* promoter is indeed constitutive, regardless of TNF treatment or the presence of p50. Some of the binding was mediated by the basal level of p50 in the nucleus, as p50 knockdown partially reduced it (Figure 5G). However, the nature of the remaining Bclaf1 that binds the promoter is not clear, which could be mediated by other DNA binding proteins. The other possibility is that Bclaf1 may directly bind a certain DNA sequence. Our previous study has shown that Bclaf1 could bind the interferon-stimulated response element (ISRE) in an in vitro study (Qin et al., PloS Pathogens 2019). Perhaps Bclaf1 could loosely bind certain short DNA sequence in the promoter region. We have incorporated the above discussion into the revised manuscript (Page 16 and 17).

Figure 3F: Although re-expression of Flag-FLIP in siBclaf1 treated cells reduces caspase cleavage, the cleavage of PARP remains unaltered. Please explain.

Response:

The cleavage of PARP was also reduced in FIag-FLIP re-expressed cells. The original blot we used is over-exposed, and we have replaced it with a lighter exposed one to better show this result (revised Figure 3I).

In the discussion section (also page 15) the authors mention other transcriptional regulators of CFLAR such as c-Myc and Peli1. However, many more transcription factors have been described that regulate c-FLIP expression, e.g. C/EBP in B cells (Paz-Piel et al., Leukemia 2009) and NFAT (Zaichuk et al., J Exp Med 2004; Ueffing et al., Blood 2008).

Response:

We are grateful for the reviewer's information and have included these factors as transcriptional regulators of *CFLAR* in the revised manuscript (page 17).

The official name of the gene that encodes c-FLIP proteins is CFLAR in humans and Cflar in mice. This should be corrected throughout the manuscript.

Response:

We are very grateful for the reviewer's information and have corrected the gene name accordingly.

The authors use human IECs in their final experiments. Are these primary cells? Although the source is mentioned in the materials and methods section, this point is not clear.

Response:

The human IECs we used are not primary cells. We have provided the original source of this cell line in the revised materials and methods section.

The sequence information for siRNA no. 2 for mouse Bclaf1 is missing in the materials and methods section.

Response:

We thank the reviewer for pointing this out, and have added the missing sequence information in the revised materials and methods section.

The reference list is not formatted correctly. For instance, often the journal name is missing.

Response:

We have fixed the issue.

Dear Dr. Tang

Thank you for the submission of your revised manuscript to EMBO reports. I have already informed you about the referee reports that we received (copied below) to give you the chance to address the remaining concerns while I discussed the issue with the RNAseq data.

All three referees find that the study has been significantly improved during revision and recommend publication, pending that the issue with the anti-pRIPK3 staining is satisfactorily addressed.

I had noted that you have provided the Read counts and FPKM values of the RNAseq data as Dataset EV1. As you know, our editorial policies request deposition of such data in public databases. You have however informed us that the raw data is not available anymore since the company that produced these data 3 years ago has not stored it and you had not requested it back then. I have discussed the issue with our chief editor. We realize that data deposition in the absence of the raw and metadata is not possible. On the other hand, the RNAseq data do still contain valuable information and might provide guidance and clues for future experiments to the readers of your article and publication is therefore of value. We have therefore decided to move forward with the publication of your RNAseq data in the current format, i.e., in the form of the .xls file that you have provided as Dataset EV1. Please add a Data availability section at the end of Materials and Methods and add a statement along these lines: The RNAseq data obtained in this study have been provided in Dataset EV1. Raw sequences were acquired 3 years ago through a company. Since the raw and metadata have not been stored, deposition in a public database was not possible.

From the editorial side there are some further points that need to be resolved.

- Please provide the figures in higher resolution. See also https://wol-prod-cdn.literatumonline.com/pb-assets/embo-site/EMBOPress_Figure_Guidelines_061115-1561436025777.pdf

- Fig EV5 only has one panel so does not need the 'A' label.

- 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 the revised file with tracked changes with your final manuscript submission. I have also taken the liberty to make some changes to the Abstract. Could you please review it?

- 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 synopsis image that is 550x200-600 pixels large (width x height) in .png format. You can either show a model or key data in the synopsis image. 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.

Yours sincerely

Martina Rembold, PhD
Senior Editor
EMBO reports

****

Referee #1:

The authors addressed all concerns and the additional data corroborated their findings and conclusions. It is an interesting study which I recommend for publication.

Referee #2:

Although the authors have addressed almost all concerns raised by the reviewers, I still have a concern that needs to be addressed before acceptance.

Line 265-266; The authors claim that "Phospho-RIPK3 staining showed no difference in mTNF-induced necroptosis between control and Bclaf1 knockdown mice (Fig. 7). However, almost all epithelial cells are stained with anti-pRIPK3 antibody, and

numbers of pRIPK3+ cells are increased fivefold compared to CC3+ cells. Thus, I have serious concern about the specificity of anti-pRIPK3 antibody used in the study. The authors need to repeat the experiment under different experimental conditions or using different anti-pRIPK3 antibody.

Referee #3:

The authors have addressed all of my previous concerns.

Just one minor thing: since Figures 2 and 3 also contain data obtained with the CRISPR knockout cell line, "Bclaf1 knockdown" might be changed to "Bclaf1 deficiency" in the heading.

Jun Tang, Ph.D.
College of Veterinary Medicine
China Agricultural University
2 Yuanmingyuan W. Rd, Haidian District
Beijing 100193, China
Phone/Fax: 86-010-62732328
Email: jtang@cau.edu.cn

Sept 24, 2021

RE: Manuscript EMBOR-2021-52702V3,

Dear Dr. Rembold,

Thank you so much for your previous email and I am sending you a revised version of our manuscript, in which I believe we have addressed the issue with the anti-pRIPK3 staining and all the other issues you brought up. I also apologize for not being able to send our revised manuscript back sooner. One of the reasons for this is that the antibodies we purchased were out of stock in China, and it takes an unusually long time for us to finally get the antibodies.

To address the issue with the anti-pRIPK3 staining, we purchased another anti-pRIPK3 antibody, and an anti-pMLKL antibody to stain for necroptotic signals in TNF treated tissues, and observed a similar staining pattern as that shown in our original data. While waiting for the arrival of the purchased antibodies, we also performed a time course experiment using the anti-pRIPK3 antibody we used originally to stain for pRIPK3 positive cells after TNF treatment, and clearly observed a time-dependent increase in the percentage of pRIPK3 positive cells. With all these results, we are convinced that the anti-pRIPK3 antibody we used is specific. Please see point-by-point reply to the reviewer comments for details.

We hope that our revised manuscript is now acceptable for publication in *EMBO Reports*.

Best regards,

Sincerely,

Jun Tang

Response to the editor's request

I had noted that you have provided the Read counts and FPKM values of the RNAseq data as Dataset EV1. As you know, our editorial policies request deposition of such data in public databases. You have however informed us that the raw data is not available anymore since the company that produced these data 3 years ago has not stored it and you had not requested it back then. I have discussed the issue with our chief editor. We realize that data deposition in the absence of the raw and metadata is not possible. On the other hand, the RNAseq data do still contain valuable information and might provide guidance and clues for future experiments to the readers of your article and publication is therefore of value. We have therefore decided to move forward with the publication of your RNAseq data in the current format, i.e., in the form of the .xls file that you have provided as Dataset EV1. Please add a Data availability section at the end of Materials and Methods and add a statement along these lines: The RNAseq data obtained in this study have been provided in Dataset EV1. Raw sequences were acquired 3 years ago through a company. Since the raw and metadata have not been stored, deposition in a public database was not possible.

Response:

We have added the Data availability section at the end of Materials and Methods along with the statement.

From the editorial side there are some further points that need to be resolved.

- Please provide the figures in higher resolution. See also https://wol-prod-cdn.literatumonline.com/pb-assets/embo-site/EMBOPress_Figure_Guidelines_061115-1561436025777.pdf

Response:

We have fixed the issue.

- Fig EV5 only has one panel so does not need the 'A' label.

Response:

We have fixed the issue.

- 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 the revised file with tracked changes with your final manuscript submission. I have also taken the liberty to make some changes to the Abstract. Could you please review it?

Response:

Thank you very much. We have done this.

- 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 synopsis image that is 550x200-600 pixels large (width x height) in .png format. You can either show a model or key data in the synopsis image. 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.

Response:

A) This study reveals that Bclaf1 is a novel component of the anti-apoptotic program in the TNF signaling that positively regulates c-FLIP expression via p50 and protects cells from TNF induced apoptosis and tissue injury.

B)

- Bclaf1 deficiency sensitizes cells to apoptosis in response to TNF and protects intestinal epithelia of mice from the TNF-induced damage.
- Bclaf1 inhibits TNF induced apoptosis by upregulating c-FLIP.
- Bclaf1 interacts with p50 and positively regulates the transcription of *CFLAR* through p50.

C) Please see attached document.

Point-by-point reply to the reviewer comments

Referee #2:

Although the authors have addressed almost all concerns raised by the reviewers, I still have a concern that needs to be addressed before acceptance.

Line 265-266; The authors claim that "Phospho-RIPK3 staining showed no difference in mTNF-induced necroptosis between control and Bclaf1 knockdown mice (Fig. 7). However, almost all epithelial cells are stained with anti-pRIPK3 antibody, and numbers of pRIPK3+ cells are increased fivefold compared to CC3+ cells. Thus, I have serious concern about the specificity of anti-pRIPK3 antibody used in the study. The authors need to repeat the experiment under different experimental conditions or using different anti-pRIPK3 antibody.

Response:

To clarify the issue of the specificity of the anti-pRIPK3 we originally used (abcam ab222320), we performed a time course experiment by treating mice with TNF for different duration and stained the small intestine dissected at 15, 30, 60 and 120 min after TNF administration with this antibody. We clearly observed a time-dependent increase in the percentage of pRIPK3 positive cells, and by 120 min TNF treatment nearly all epithelial cells are pRIPK3 positive (please see below Figure R1A and B). Furthermore, we purchased another anti-pRIPK3 antibody (abcam ab205421, used in “The AMPK-Parkin axis negatively regulates necroptosis and tumorigenesis by inhibiting the necrosome” by Lee et al. Nature cell biology 2019), as well as an anti-pMLKL antibody (abcam ab196436, used in “ZBP1 not RIPK1 mediates tumor necroptosis in breast cancer” by Baik et al. Nature communications 2021) and stained the small intestine from control and Bclaf1 knockdown mice treated with TNF for 2 h. We obtained a similar result as that presented in Figure 7F (Figure R1C and D). pRIPK3 is just an indicator showing that the necroptotic pathway has been turned on. Whether or not these cells ultimately die from necroptosis probably depends on the intensity of the signal. Our data suggest that the necroptotic pathway in epithelial cells in vivo can be quickly activated in response to TNF, and this pathway is not affected by Bclaf1 knockdown.

Because pRIPK3 is a marker for the initiation of necroptotic pathway, not an indication of actual necroptosis, we think using “necroptotic pathway” is more appropriate than “necroptosis” in the original text “Phospho-RIPK3 staining showed no difference in mTNF induced necroptosis”. Thus, we have changed this in the text.

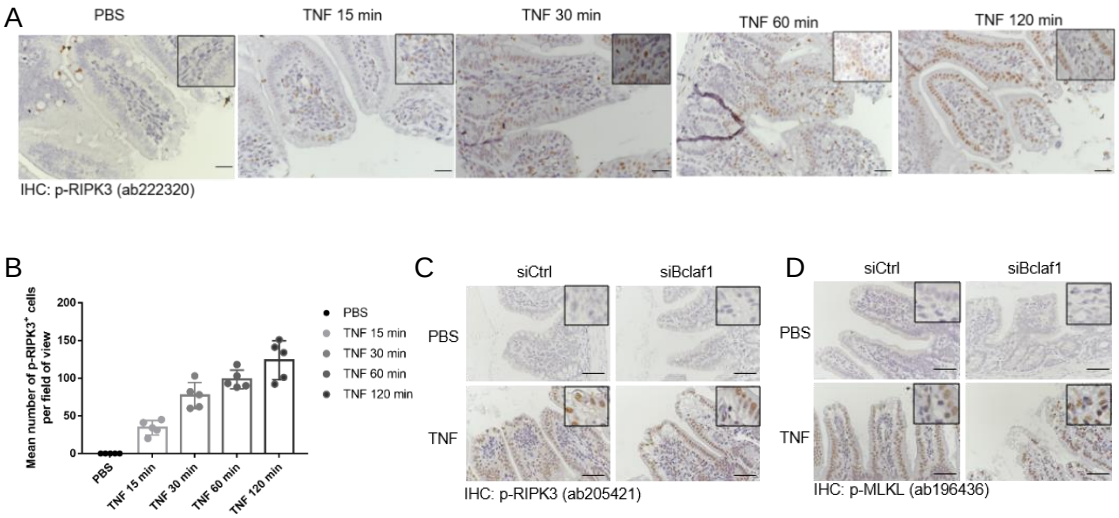

Figure R1. A, B. The mice were treated with mTNF for indicated time before sacrifice, and the small intestines were excised and processed for immunohistochemical staining with the anti-pRIPK3 antibody (ab222320) we originally used (A). pRIPK3+ positive cells in five fields per intestine were quantified (B). C, D. Mice injected with control siRNAs (siCtrl) or siRNAs against Bclaf1(siBclaf1) were treated with mTNF for two hours before sacrifice, and the small intestines were immunohistochemically stained with another anti-pRIPK3 antibody (ab205421) (C) and an anti-pMLKL (D) antibody. Scale bars: 50 μm.

Referee #3:

-

The authors have addressed all of my previous concerns.

Just one minor thing: since Figures 2 and 3 also contain data obtained with the CRISPR knockout cell line, "Bclaf1 knockdown" might be changed to "Bclaf1 deficiency" in the heading.

Response:

We have changed both wording.

2nd Revision - Editorial Decision **6th Oct 2021**

Dr. Jun Tang
China Agricultural University
State Key Laboratory of Agrobiotechnology and College of Veterinary Medicine
2 Yuanmingyuan West Rd. Haidian district,
Beijing, Beijing 100193
China

Dear Jun,

Thank you for sending the further revised files. I uploaded them to your submission and am now very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

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.

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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." Please note that the author checklist will still be published even if you opt out of the transparent process.

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.

Kind regards,

Martina

Martina Rembold, PhD
Senior Editor
EMBO reports

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Corresponding Author Name: Jun Tang
Journal Submitted to: EMBO Reports
Manuscript Number: EMBOR-2021-52702V1

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- 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.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be iustified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

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

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

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

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	At least three independent experiments were performed.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	We used 5 mice for each group.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	There were no samples excluded.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	Researchers were blinded to the type of siRNAs injected when possible.
For animal studies, include a statement about randomization even if no randomization was used.	Researchers were blinded to the type of siRNAs injected when possible.
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	Researchers were blinded to the type of siRNAs injected when possible.
4.b. For animal studies, include a statement about blinding even if no blinding was done	Researchers were blinded to the type of siRNAs injected when possible.
5. For every figure, are statistical tests justified as appropriate?	Yes,all stastitical tests are justified as appropriate.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes. Graphpad PRISM software automatically assessed the data prior to test recommendations.

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-report

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tumo

http://datadryad.org

http://figshare.com

http://www.ncbi.nlm.nih.gov/gap

http://www.ebi.ac.uk/ega

http://biomodels.net/

http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

Is there an estimate of variation within each group of data?	Yes. Graphpad PRISM software automatically assessed the data prior to test recommendations.
Is the variance similar between the groups that are being statistically compared?	Yes.

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	All the antibodies are listed in methods sections.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	HEK293T, HeLa, HepG2 cells were obtained from American Type Culture Collection (ATCC). MEFs were generated from E11.5–E13.5 embryos of mice. HIEC-6 (ATCC) were obtained from Dr. Dong Yulan from China Agricultural University. All of the cell lines were negative for mycoplasma contamination.

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

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	C57BL/6 and BALB/c mice were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. 7-week-old mice were used in our study. All animals were housed and maintained under specific pathogen-free conditions.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Animal care and protocols were approved by Animal Welfare Committee of China Agricultural University.
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	For animal studies, ARRIVE guidelines were followed.

E- Human Subjects

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

F- Data Accessibility

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

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	No.