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The IκB kinase complex is a regulator of mRNA stability

Nadine Mikuda, Marina Kolesnichenko, Patrick Beaudette, Oliver Popp, Bora Uyar, Wei Sun, Ahmet Bugra Tufan, Björn Perder, Altuna Akalin, Wei Chen, Philipp Mertins, Gunnar Dittmar, Michael Hinz and Claus Scheidereit.

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 24th January 2018

Thank you for submitting your manuscript for consideration by the EMBO Journal and my apologies for the extended duration of the review period. Your study was sent to three referees and their comments are shown below. As you will see from the reports, our referees all express interest in the findings reported in your manuscript but they also raise a number of concerns - both conceptual and technical - that you will have to address before they can support publication in The EMBO Journal.

Should you be able to address these criticisms in full, and provided that the original conclusion still holds true, we would be happy to consider a revised manuscript. I should remind you that it is EMBO Journal policy to allow a single round of revision only and that, therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses in this revised version. I do realize that addressing all the referees' criticisms will require a lot of additional time and effort and be technically challenging. I would therefore also understand if you wish to publish the manuscript rapidly and without any significant changes elsewhere, in which case please let us know so we can withdraw it from our system.

For the revised manuscript, I would particularly ask you to focus on the following points:

-> Please elaborate on the generality of IKK as a regulator of mRNA stability e.g. downstream of TNF stimulation as suggested by ref #1

-> Provide evidence that IKK-dependent phosphorylation of the EDC4 phospho sites identified occurs in cells (refs #1 and #2)

-> Improve the overall data presentation and include information on statistics and number of replica experiments performed (ref #3)

-> Provide additional data to test the directness of the interactions as well as the epistasis between

IKK and EDC4 as outlined by ref #2

REFEREE REPORTS

Referee #1:

This is an interesting manuscript that identifies a new and previously uncharacterized function for IKKbeta as an NF-kB independent regulator of mRNA stability. Given that so many studies using, for example, IKK knockout mice or IKK inhibitors assume that the effects seen result from NF-kB regulation, the data presented here is of great significance to those with an interest in this area. Moreover, the manuscript more widely addresses issues of importance to our understanding of the inflammatory and DNA damage responses.

The data presented is generally robust and well controlled. However there are some areas where either some additional confirmation of experimental data is required or where by extending the analysis the data shown will be better placed in the context of the field.

Major comments

(1) The authors show that even in unstimulated cells, IKK has an important role destabilizing transcripts. Given IKK kinase activity will be low under such conditions, they speculate that this effect might result from the physical interact between IKK and P body components. Can they confirm whether this is the case by performing an experiment such as that in Fig S5h and determining whether inclusion of an IKKbeta inhibitor has the same effect in unstimulated cells as shRNA depletion of IKKbeta?

(2) The authors should confirm that IKK dependent phosphorylation of the EDC4 phospho sites identified occurs in cells. This could be through phospho-proteomic analysis of endogenous or exogenously expressed EDC4 (+/- IKK inhibitor) or the use of phospho specific antibodies.

(3) The focus of the manuscript is on the effect of ionising radiation (IR) on IKK regulation of transcripts. However, similar stabilization of transcripts appears to occur after TNF stimulation. Given the important role that IKK plays after inflammatory cytokine stimulation, can the authors determine the effect of inhibiting IKK activity on representative NF-kB independent transcripts following TNF stimulation?

(4) The authors note that many of the IKK regulated transcripts contain AU rich elements in their 3' UTRs. However, this is not investigated. Can the authors confirm using an exogenously expressed reporter that it is the AU rich elements that confer IKK dependence on mRNA stability?

Other comments

(5) Was the quantification of experiments determining the levels of P-bodies in cells performed using blinded samples? This should be clarified.

(6) Figure 4A is hard to understand and poorly described in the legend. e.g. what is the y-axis of the two graphs. What are the blobs in the mRNA diagram and why are they significant? Also the pale pink lines on the right hand graph do not show up well.

(7) The orange circles in Figs 4b-e are not described in the legend.

(8) Some of the Supplementary data should be included in with the main figures of the paper since it is interesting, very relevant and not really supplementary. In particular some of the data looking at stability looking at IL-8 (Fig. S5) and other targets (Fig. S6). Furthermore, the inclusion of at least one table in the main manuscript listing the transcripts (or a number of representative examples) where IKK regulates mRNA stability in a more user friendly form that the supplementary excel

spread sheets would be of great help to the reader.

(9) Some of the Fig S5 panels are wrongly cited in the text (see top of page 7). Furthermore the manuscript sometimes references supplementary tables that do not seem to exist (e.g. Table S15 page 8), while Table S7 refers to Table S11. There may be other examples I have missed

Referee #2:

The manuscript by Mikuda et al. reports on a role for the IkB kinase (IKK) in mRNA stability through the phosphorylation of the P-body component EDC4. The paper is potentially interesting. However, before publication, several points need to be addressed.

1. None of the imunoprecipitations was performed in the presence of RNAse A, therefore the interactions described in this manuscript can be all indirect and mediated by RNA. Given that the interaction with EDC4 seems to be very weak, the IPs need to be repeated in the presence of RNase A. given that the authors can express the proteins in bacteria, the interactions could be validated using recombinant protein fragments.
2. What is the relevance of S583 and S855, are they phosphorylated in vivo? These serines were not mutated individually.
3. SG formation does not follow or is linked to P-body formation.
4. I do not see why the authors conclude that mRNAs show a epistatic regulation by IKK and EDC4. Some mRNAs are commonly regulated by IKK and EDC4 but the effects can be independent of each other. Are the effects additive? This could be tested for some of the common targets by double knockdown.
5. The authors need to analyze the stability of additional common targets using complementation assays in EDC4 depleted cells and complement with EDC4 wt or mutant.

Additional comments.

1. The authors have changed the names of the proteins. For example DCP1 and DCP2: should be mRNA decapping protein 1 and 2. EDC4: is enhancer of decapping 4. But not Processing (P)-body scaffold. This should be corrected.
2. XRN1 is an exonuclease. Therefore it is incorrect to write that it performs nucleolytic cleavage. It degrades RNA by exonucleolytic digestion/degradation.
3. The authors are not very well informed of the literature regarding P-bodies. It has been shown that P-body integrity is not required for mRNA degradation. It is therefore incorrect to state that P-bodies contain mRNAs destined for degradation. Degradation occurs even in the absence of detectable P-bodies. Furthermore, what is the evidence that EDC4 provides a scaffold for RBPs?
4. P-body expression should be replaced by either P-body assembly or formation.
5. The quality of western blots and IPs needs to be improved. The authors show cropped panels and it is not clear whether the inputs and IPs were analyzed in the same gel and whether the signals can be compared. Also what fraction of the input and pellet has been analyzed? Fig. 1D,G panels are of different size and panel 1D (input) is of low technical quality. Fig. 1G, transfections should be adjusted so that the protein fragments are expressed at similar levels and can be compared. Also panels are cut too close to the bands.Finally what are ther additional bands recognized by the antibodies?
6. All fluorescent images should be quantified as is done in Figure 3A,B.

Referee #3:

Proteins interacting with IKK in response to irradiation were identified, and the P-body scaffold

protein was selected for further analysis. The authors map several phosphorylation sites in EDC4 and establish that IKK can phosphorylate these sites in vitro. mRNAs destabilized and stabilized by IR in an IKK-dependent manner were identified using RNA seq, and mRNAs whose stability was altered by KD of IKK or EDC4 were identified and classified according to GO terms. Much of the data are solid and informative, and carefully presented. While a positive correlation between EDC4 phosphorylation and P-body formation is apparent, the assertion that IKK-mediated phosphorylation of EDC4 causes P-body formation is not adequately supported by the data. In addition, a number of other flaws distracted from the overall message.

Major concerns:
Their data support their conclusion that IR/TNFa stimulate P-body formation in an IKK/EDC4-dependent manner. Interactions between IKK and EDC4 the molecular level are supported by structure-function in vitro kinase data, and point mutations eliminating phosphorylation sites in EDC4 prevent TNFa-induced interactions between EDC4 and IKKgamma. The schematics in Figs 1 and 2 are clear and informative. Induction of P-bodies by IR and its dependence on IKKbeta (Fig 3a and b) are convincing. The authors show that EDC4 KD impairs PB formation (Sup 2p) (although the western blot in Sup 2q showing the KD efficiency is very poor), as has been reported by others. However, the proposed causal link between EDC4 phosphorylation and P-body formation is not convincingly established (Fig 3c). The authors generate cell lines in which endogenous EDC4 is knocked down and replaced with EDC4-WT or an SA mutant, then treat these cell lines to induce P-bodies. They state that "introduction of the EDC4 SA mutant led to a low basal level of P-body formation and could not restore their amplification (Fig. 3c).". However, Fig 3c data is not quantified and is not convincing. From the raw images, it appears that the SA-EDC4 cells display more P-bodies in the non-irradiated cells than in the irradiated ones, which is the opposite of what the authors state in the text. This experiment needs to be repeated 3 times and data quantified as in 3a and 3b. It is also very important to show that the levels of EDC4 (WT and SA) in these clonal cell lines are similar, and expressed at close to normal endogenous levels. Other data obtained using these cells is supposed to be shown in Fig S5d; however, it seems to be in Fig S5h and I instead. While there are clear effects on the stability of IL-8 mRNA, what is not clear is whether these cells are really expressing comparable levels of EDC4 wt/SA, and whether endogenous EDC4 is reduced in both. Using CAS9 to delete endogenous EDC4 might be required, given that the antibodies used to blot EDC4 seem poor (S2-q).
The authors also show that "a fraction" (a very minor fraction) of IKK localizes to P-bodies (sup 2c, d), but how do they know that the antibodies are really monospecific for IKK? Does the P-body signal diminish in the IKK KD cells? Alternatively, can a tagged form of IKK be shown to localize to P-bodies?

Specific points:

Fig 2b (raw MS data) is not explained, labeled, or legible. What do the colors indicate? Axis, units are unclear and font is so small as to be illegible. Was this analysis repeated more than once? This panel would be more appropriate in the supplemental data, while the data in Sup 1a would be more appropriate in Fig 2.

Fig 3c data is not quantified and is not convincing. This needs to be repeated 3 times and data quantified as in 3a and 3b. From the raw images, it appears that the SA-EDC4 cells display more P-bodies in the non-irradiated cells than in the irradiated ones, which is the opposite of what the authors state in the text. It is also very important to show that the levels of EDC4 (WT and SA) in these clonal cell lines are similar, and expressed at close to normal endogenous levels.

Fig. 4f, g. Axes are labeled "semantic space." This is confusing.

Fig 5. It would be helpful to explain what the various symbols in this model are supposed to represent. The model appears to show mRNAs that are capped and have polyA tails in P-bodies. However, others have shown that PABP and polyA (detected by FISH) are usually absent from P-bodies. Do the authors have any evidence that polyA is present in these P-bodies? Can they show that mRNAs stabilized (or destabilized) by IKK are recruited to P-bodies during IR?

Sup 1b. X-axis is labeled "-log p-value"; this is not clear. Is it negative, or is the dash not a negative sign? It would be informative if

Sup 1c: Only a single timepoint at 40' post-IR shows an increased interaction between DDX6 and IKK. Is this reproducible? It would be more convincing if a time course showing at least 2 elevated points were shown.

Sup 2a,b- Legend indicates that DDX6-staining is red, but what is shown in the green channel? Similarly, what is shown in the red channel in Sup 2h, i, and j? Blot in 2q is not convincing.

FIG S5d. The Result section refers to "S5d middle and lower panels," which are not present in Fig S5d. This probably refers to S5h and j, but this sort of mistake is not reassuring.

" To determine whether mRNA stability of IL-8 depends on the phosphorylation state of EDC4, EDC4-depleted cells were reconstituted with the wt EDC4 or the phospho-deficient EDC4 SA mutant. Reconstitution with the wt EDC4 but not EDC4 SA rescued stabilisation of IL-8 mRNA in irradiated cells (Fig. S5d, middle panel). Expression and stability of IL-8 mRNA in cells expressing EDC4 SA resembled that of EDC4 and IKKβ knockdown cells (Fig. S5d, lower panel)."

Point-by-point response to the referees' comments

The IκB kinase complex is a regulator of mRNA stability

Mikuda, N.*, Kolesnichenko, M.*, Beaudette, P., Popp, O., Uyar, B., Sun, W., Tufan, A.B., Perder, B., Akalin, A., Chen, W., Mertins, P., Dittmar, G., Hinz, M., and Scheidereit, C.**

We would like to thank the three expert referees for their insightful and constructive comments and suggestions. As you find outlined below, we were able to address all the referees' comments. With a set of new experiments we could fully support our previous conclusions.
We provide several new figure panels and supplementary figures, an additional supplementary table, as well as figures for reviewer inspection only (Fig. 1R-4R). Due to the additional experiments, we now include four new authors. All changes introduced into the manuscript (and supplementary material) are marked by font color blue.
The four major points to be focused on, according to the advice of the editors, are answered below, followed by our point-by-point response to the individual referees. The comments of the referees are in italics.
We hope that the referees are satisfied with our comprehensive revision, which further improved the manuscript, and that a positive decision can now be reached.

General points summarized by editors

1. Please elaborate on the generality of IKK as a regulator of mRNA stability e.g. downstream of TNF stimulation as suggested by ref #1

Referee 1 stated that "Given the important role that IKK plays after inflammatory cytokine stimulation, can the authors determine the effect of inhibiting IKK activity on representative NF-κB independent transcripts following TNF stimulation". In order to determine the generality of IKK as a regulator of mRNA stability, including downstream of TNF stimulation, we repeated Actinomycin D chase experiments with additional NF-κB-independent and -dependent targets, as recommended by the referee (new Fig.S7d). We analyzed IL-8, BAMBI, NFKBIA (as a control), JunB and DUSP1 in U2-OS cells bearing dox-inducible IKKβ shRNA constructs. We show that like IR, TNFα treatment leads to stabilization of the abovementioned transcripts (new Fig.S7d-e). Knockdown of IKKβ leads to increased stability of transcripts already in untreated cells and therefore does not provide further stabilization following TNFα treatment. All these findings therefore confirm that IKK is a general regulator of RNA stability.

With our other data we also demonstrated regulation by TNFα; including TNFα-induced IKKβ-EDC4 interaction (Fig. 1c; Fig. S1c), TNFα-induced EDC4 phosphorylation (Fig. 2 and new Fig. S2), TNFα-increased P-body numbers (Fig. S3h) and TNFα-stabilized IL-8 mRNA (Fig. S5f).

2. Provide evidence that IKK-dependent phosphorylation of the EDC4 phospho sites identified occurs in cells (refs #1 and #2)

We have previously identified phosphorylation sites of EDC4 through *in vitro* kinase assays with endogenous IKK and the purified recombinant sub-regions of EDC4 followed by mass spectrometry analysis. This led to the identification of serines 583 and 855 as IKK substrates. To increase coverage we also used the dbPTM database to predict further potential IKK phosphosites and showed experimentally that serines 107 and 405 were phosphorylated by IKK in *in vitro* kinase assays.

We thank the referees for the suggestion to confirm that IKK serves as an EDC4 kinase also in cells. We now generated CRISPR knockout cell lines of IKK beta and of EDC4 and following immunoprecipitation with anti-EDC4 antibody, subjected the cell lysates from TNF treated cells and controls to mass spectrometry analysis. We have identified the previously detected site (serine 583) (new Fig. S2a, and data not shown). Not all previously identified sites were however detected. This could be due to limited peptide coverage (no peptides covering the Ser 107 and Ser 405 were detected in the previous MS analysis of recombinant EDC4 domains nor in the MS analysis of endogenous full length EDC4; it also appears that these regions are only poorly (Ser 107) or not at all (Ser 405) covered in other phosphoproteomic studies referenced in the PhosphoSitePlus.org database, suggesting poor suitability with MS analysis of these protein regions). However, re-identification of phospho-serine 583, together with our previous kinase assay data, clearly demonstrates that EDC4 is an IKK substrate in intact cells. Importantly, we also observed that Ser 583 phosphorylation was enhanced by TNFα stimulation (new Fig. S2b). No enhancement was seen in IKKβ knockout cells, while EDC4 knockout cells did not produce detectable signals, as expected, confirming the specificity of the measurements (new Fig. S2b). Three of the four sites presented in the manuscript (Ser 107, Ser 583 and Ser 855, Fig. 2 and new Fig. S2) were also reported as EDC4 phosphosites in the PhosphoSitePlus.org database (new Fig. S2c).

Our new results confirm our initial conclusion that IKK phosphorylates EDC4 in response to TNFα treatment.

3. Improve the overall data presentation and include information on statistics and number of replica experiments performed (ref #3)

We have redone several experiments and improved data presentation. We have shown that knockdown or chemical inhibition of IKK leads to loss of induced P-body assembly (*i.e*. increase in numbers following stimulation) (Fig. 3B and Fig.

S3l-o). Knockdown of EDC4 via shRNA or siRNA led to a loss of P-body assembly (Figure 3c and Figure S3p-r). We now repeated these experiments additionally using CRISPR EDC4 knockout cells and quantitated foci using Image J software (blind count as requested) (Fig. R1). Our new data support our initial observation that EDC4 is required for P-body formation and that IKK is necessary for P-body induction.

Referee 3 pointed out that data presentation in Figure 3C, depicting overexpression of SA mutant in the EDC4 knockdown, is not convincing and needs to be quantified. The referee may agree that the increase in foci upon IR is clearly higher in WT-EDC4 compared to EDC4-SA reconstituted cells. However, to alleviate the referees' concern, we performed additional experiments in CRISPR EDC4 knockout cell lines, where we also overexpressed the SA EDC4 construct. We furthermore used different tags on the plasmid to ascertain that neither the plasmid nor the Tag prevents foci formation in the SA mutant. Each experiment was performed with at least three replicates (Fig. R1 and 2). Ectopic overexpression also led to larger protein clusters, observed in images as points, as opposed to much smaller foci (see Fig. R2). Image J software is not technically able to differentiate between points and foci (see Fig. R1). We therefore used manual blind count of foci but not aggregates, using at least 200 cells per condition with three biological replicates. We observed no significant difference between untreated wt EDC4 knockout or knockdown cell lines and untreated and treated EDC4 knockdown or knockout cell lines overexpressing EDC4 SA mutant (Figure 3C and Fig. R1). These data support our initial observation that intact phospho sites on EDC4 are required for P-body assembly in response to stimulus.

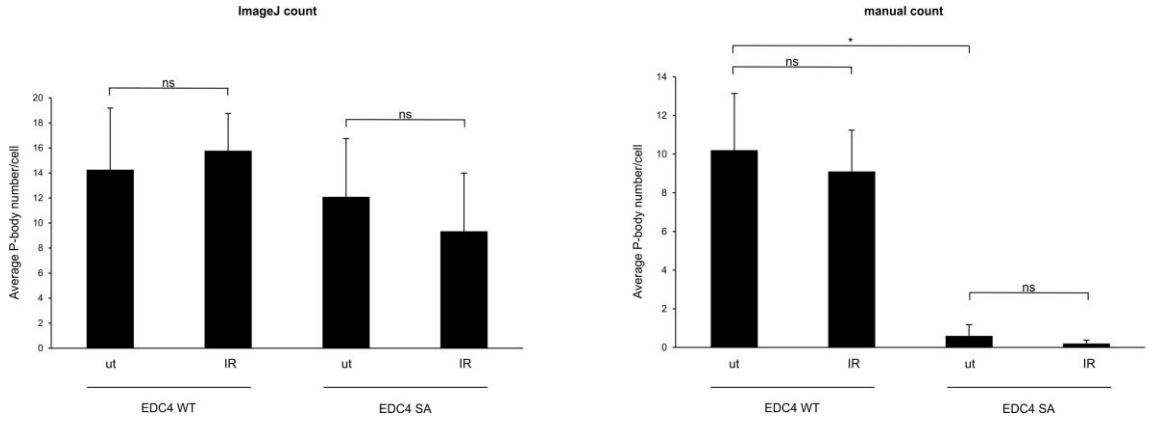

Fig. R1 No significant change observed in the number of foci between EDC4 wt and EDC4 SA cells. U2-OS CRISPR EDC4 KO cells were transiently transfected with plasmids encoding phosphosite-mutated EDC4 (SA) or WT EDC4 (EDC4 WT). Foci were visualized by immunofluorescence. Left panel: ImageJ quantification of foci. Right panel: blind manual count of foci. ns showing no significant difference between conditions.

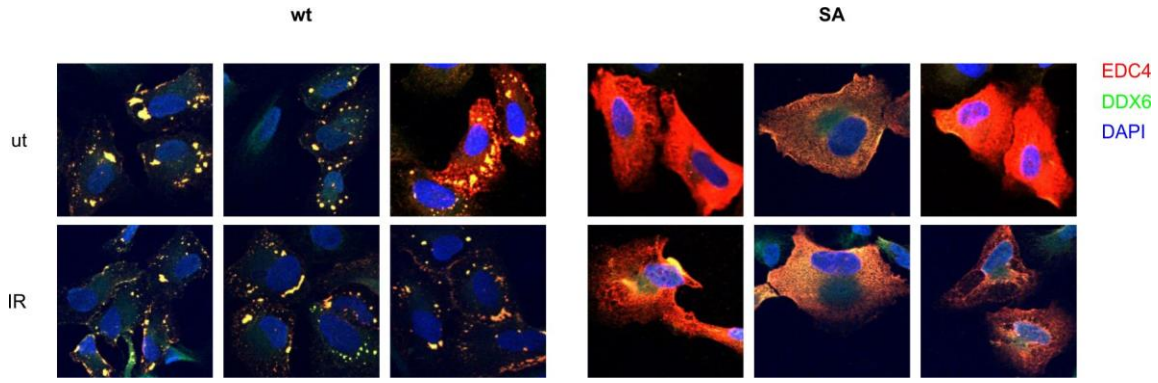

Fig. R2 Representative immunofluorescence images of U2-OS cells transfected as in R1. EDC4 in red. DDX6 in green and DAPI in blue. EDC4 and DDX6 display formation of foci shown as yellow separate points of foci in the EDC4 WT expressing cells, while displaying more diffuse expression in the EDC4 SA cells.

4. Provide additional data to test the directness of the interactions as well as the epistasis between IKK and EDC4 as outlined by ref #2

Referee 2 pointed out that protein-protein interaction between IKK and EDC4 could be mediated via RNA. In order to rule out this possibility we repeated all IPs in the presence of RNAse A and RNAse T1, using either irradiation (IR) or TNFα as stimuli (new Fig.S1C). Induced interaction observed between IKK and EDC4 was observed following IR or TNFα treatment and this interaction was not altered in the presence of RNase, confirming our initial observation that IKK and EDC4 interact directly.
We performed Duolink Proximity Ligation Assay to determine and quantify protein interaction between EDC4 and IKKβ under endogenous conditions. We could indeed demonstrate a significant increase in interaction between these proteins in the cytoplasm, following either irradiation (IR) or TNFα stimulation (new Fig. 1D and 1E).

Referee 2 asked whether some targets are regulated by EDC4 and by IKKβ independently, or whether the effects of the proteins are additive. We therefore performed single or combined siRNA knockdowns of EDC4 and IKKβ (new Fig. 7e). RNA stability assays were performed using IR treatment as stimulus and Actinomycin D was used to inhibit transcription, as described previously in the manuscript. Representative targets (IL-8, BAMBI, NFKBIA, JunB and DUSP1) were analyzed by qRT-PCR. We demonstrated that combined knockdown of IKK and EDC4 compared to one or both of the single knockdowns did not further affect RNA stability of the transcripts analyzed, therefore suggesting that IKK and EDC4 regulate stability along the same axis, without additive effect on stability.

Specific Points by Referees:

Referee #1:

This is an interesting manuscript that identifies a new and previously uncharacterized function for IKKbeta as an NF-kB independent regulator of mRNA stability. Given that so many studies using, for example, IKK knockout mice or IKK inhibitors assume that the effects seen result from NF-kB regulation, the data presented here is of great significance to those with an interest in this area. Moreover, the manuscript more widely addresses issues of importance to our understanding of the inflammatory and DNA damage responses.

The data presented is generally robust and well controlled. However there are some areas where either some additional confirmation of experimental data is required or where by extending the analysis the data shown will be better placed in the context of the field.

We thank the referee for her/his positive evaluation of our work. We have now performed additional experiments to support our findings and extended the analysis of the data as recommended.

Major comments

(1) The authors show that even in unstimulated cells, IKK has an important role destabilizing transcripts. Given IKK kinase activity will be low under such conditions, they speculate that this effect might result from the physical interact between IKK and P body components. Can they confirm whether this is the case by performing an experiment such as that in Fig S5h and determining whether inclusion of an IKKbeta inhibitor has the same effect in unstimulated cells as shRNA depletion of IKKbeta?

We thank the referee for this suggestion. As the referee mentioned, IKK could regulate RNA stability in unstimulated cells either through physical interaction (which could be independent of its activity as a kinase) or due to low residual kinase activity. Indeed we did detect low basal phosphorylation of IKK (data not shown) and low basal kinase activity also unstimulated cells (Figure S2c).
As suggested, we analyzed the effect of inhibition of IKK kinase activity using an IKK inhibitor (BMS345541) on stability of the IL-8 transcript. Inhibition of IKK kinase activity resulted in lower basal expression of the IL-8 transcript, however it also precluded its degradation, following IR (Fig. R3). These results suggest that at least for the IL-8 transcript, IKK kinase activity is required for destabilization under basal conditions.

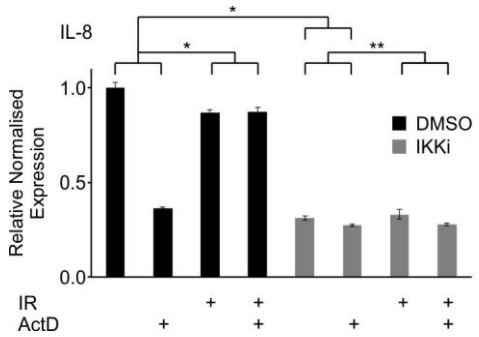

Fig. R3 inhibition of the IKK kinase activity leads to loss of stabilization of IL-8 mRNA in response to IR. U2-OS cells were treated with the IKK inhibitor BMS345541 and RNA stability was analyzed by Actinomycin D chase experiment, as described previously. DMSO treated cells (control) shown in black and IKKi treated cells in grey.

(2) The authors should confirm that IKK dependent phosphorylation of the EDC4 phospho sites identified occurs in cells. This could be through phospho-proteomic analysis of endogenous or exogenously expressed EDC4 (+/- IKK inhibitor) or the use of phospho specific antibodies.

We are thankful for the useful suggestion. Since no adequate phospho-specific antibodies exist, we performed mass spectrometry analysis on immunoprecipitated endogenous EDC4 from cellular lysates from CRISPR knockout cell lines of IKKβ and of EDC4 plus controls. To determine which sites are phosphorylated, cells were treated with TNF to identify sites that are phosphorylated in an IKK-dependent manner. We have previously detected site Ser 583 on EDC4 as a substrate of IKK through *in vitro* kinase assay, followed by mass spectrometry analysis. Our new analysis of phosphorylation sites in cells, validated this site as an IKK substrate (new Fig. S2a) and we show that the phosphorylation at this site is stimulated by TNFα only in cells expressing IKKβ. As control, the signal was not detected in cells lacking EDC4 (new Fig. S2b). These sites were likewise predicted by the PhosphoSitePlus.org (Figure S2c). Therefore IKK serves as an EDC4 kinase in cells and phosphorylates EDC4 on the abovementioned site.
We refer the referee also to our response to the editors' main point 2 (above) regarding this query.

(3) The focus of the manuscript is on the effect of ionising radiation (IR) on IKK regulation of transcripts. However, similar stabilization of transcripts appears to occur after TNF stimulation. Given the important role that IKK plays after inflammatory cytokine stimulation, can the authors determine the effect of inhibiting IKK activity on representative NF-kB independent transcripts following TNF stimulation?

We agree with the referee that it is important to determine whether TNFα would similarly lead to stabilization/destabilization of transcripts in an IKK-dependent manner. In order to determine the generality of IKK as a regulator of mRNA stability, including downstream of TNFα stimulation, we repeated Actinomycin D time course experiments with additional NF-κB-independent and -dependent targets, as recommended by the referee (new Fig.S7d). We analyzed DUSP1, IL-8, BAMBI and JunB in the Scr siRNA treated versus the IKKβ siRNA treated cells. Cells were treated with TNFα and mRNA stability was analyzed. We show that like IR, TNFα treatment leads to stabilization of the abovementioned transcripts. Knockdown of IKKβ leads to increased stability of transcripts already in untreated cells and therefore does not provide further stabilization following TNFα treatment. These findings therefore confirm that IKK is a general regulator of RNA stability. We refer to our response to the editor's main point 1 (above).

(4) The authors note that many of the IKK regulated transcripts contain AU rich elements in their 3' UTRs. However, this is not investigated. Can the authors confirm using an exogenously expressed reporter that it is the AU rich elements that confer IKK dependence on mRNA stability?

This is a very interesting point and we plan to address this more in depth in our future studies. As suggested, to determine whether mRNAs containing AU rich elements in the 3' UTR would be destabilized by IKK or by EDC4, we used a pEZX-MT01 dual renilla luciferase reporter plasmid, containing either no AU rich elements (control), or 5 or 7 repeats of the motif (Figure S8A). As expected, AU rich elements led to reduced expression of the luciferase in the unstimulated cells. Irradiation, IKK or EDC4 knockdown however, led to an increase in luciferase expression (new Fig. S8A). These data indicate that IKK and EDC4, at least in the context of this system, destabilize AU rich containing transcripts.

It could therefore be proposed that IKK phosphorylation of EDC4 by IKK may lead to conformational change of the protein and alter its affinity for additional RNA binding partners that differentially regulate stability of AU rich containing transcripts.

<u>Other comments</u>

(5) Was the quantification of experiments determining the levels of P-bodies in cells performed using blinded samples? This should be clarified.

Yes, student assistants who counted the number of P-body foci (either manually or with Image J software) were given sample numbers only but not sample names.

(6) Figure 4A is hard to understand and poorly described in the legend. e.g. what is the y-axis of the two graphs. What are the blobs in the mRNA diagram and

why are they significant? Also the pale pink lines on the right hand graph do not show up well.

We thank the referee for pointing this out and we improved the legend and changed the color in the graph.

(7) The orange circles in Figs 4b-e are not described in the legend.

We respectfully point the referees to the legend 4f. We have now underlined each category to make it more prominent.

(8) Some of the Supplementary data should be included in with the main figures of the paper since it is interesting, very relevant and not really supplementary. In particular some of the data looking at stability looking at IL-8 (Fig. S5) and other targets (Fig. S6). Furthermore, the inclusion of at least one table in the main manuscript listing the transcripts (or a number of representative examples) where IKK regulates mRNA stability in a more user friendly form that the supplementary excel spread sheets would be of great help to the reader.

We thank the referee for this very kind appraisal of the data. Due to space constraints, qRT-PCR analysis of individual targets should stay in supplements.

(9) Some of the Fig S5 panels are wrongly cited in the text (see top of page 7). Furthermore the manuscript sometimes references supplementary tables that do not seem to exist (e.g. Table S15 page 8), while Table S7 refers to Table S11. There may be other examples I have missed

We are sorry to have overlooked this and are grateful to the referee for pointing this out. We have now changed the labels.

We appreciate the referee's insightful suggestions and hope that our amended manuscript addressed the issues raised.

Referee #2:

The manuscript by Mikuda et al. reports on a role for the IkB kinase (IKK) in mRNA stability through the phosphorylation of the P-body component EDC4. The paper is potentially interesting. However, before publication, several points need to be addressed.

We thank the referee for the overall positive evaluation of the manuscript. We have addressed the points raised below.

1. None of the immunoprecipitations was performed in the presence of RNAse A, therefore the interactions described in this manuscript can be all indirect and

mediated by RNA. Given that the interaction with EDC4 seems to be very weak, the IPs need to be repeated in the presence of RNase A. given that the authors can express the proteins in bacteria, the interactions could be validated using recombinant protein fragments.

We agree with the referee and took into account this possibility that RNA could be mediating interaction between the two proteins. Therefore, to determine whether EDC4 and IKK interact through RNA, we treated cell lysates with an RNase cocktail (Rnase A and T1), confirmed degradation of RNA by running the samples on an agarose gel, and subsequently performing immunoprecipitation, as before (new Fig. S1c). No difference in interaction was observed in the absence of RNA. To further determine the nature of interaction between EDC4 and IKK, we performed Duolink ligation assay which allowed us to quantitate the interaction between the proteins, visualize their cellular localization and determine the directness of interaction. We saw a significant increase in interaction between these proteins the cytoplasm, following either irradiation (IR) or TNFα stimuli (new Fig.1d). These data therefore confirm that IKK and EDC4 interact directly. Please also see our comments to the editor's general point 4 (above).

2. What is the relevance of S583 and S855, are they phosphorylated in vivo? These serines were not mutated individually.

We thank the referee for this valid point. Because no phospho-specific antibody is available, we performed mass spectrometry analysis on endogenous, immunoprecipiated EDC4 from wild type cells and IKKβ knockout cells. We were able to show that Ser 583 is phosphorylated in cells. Possibly, due to incomplete coverage of peptides, we did not detect Ser 855. Nevertheless we identified several other sites (Ser 6, Ser 486, Thr 693, Thr 821) in cells that are also phosphorylated in a TNFα- and IKKβ-dependent manner (not shown). The roles of individual phosphorylation sites on EDC4 regulation of mRNA stability and interaction with other proteins, will be pursued as part of a future project.
We also refer to our response to general point 2 of the editor.

3. SG formation does not follow or is linked to P-body formation.

We agree with the referee that SG formation does not follow or is linked to P-body formation and therefore we removed this sentence in the text.

4. I do not see why the authors conclude that mRNAs show a epistatic regulation by IKK and EDC4. Some mRNAs are commonly regulated by IKK and EDC4 but the effects can be independent of each other. Are the effects additive? This could be tested for some of the common targets by double knockdown.

We are thankful to the referee for pointing this out and we now rephrased the text. To determine whether some targets show regulation by IKKβ and EDC4

with or without additive effect of the other protein, we performed siRNA knockdown of EDC4, or IKKβ or of both IKK and EDC4. We then performed Actinomycin D chase and analyzed representative targets by qRT-PCR. We demonstrated that knockdown of IKK and EDC4 together did not further affect RNA stability of the transcripts analyzed, therefore suggesting that IKK and EDC4 regulate stability along the same axis, without additive effect on stability (Fig. S7e).

5. The authors need to analyze the stability of additional common targets using complementation assays in EDC4 depleted cells and complement with EDC4 wt or mutant.

We thank the referee for this recommendation and now show more common targets of IKK and EDC4. In addition to the experiments we performed with the shRNA knockdown we now used CRISPR knockout EDC4 cells and reconstituted them with EDC4 WT or SA mutant. We now analyzed common targets of IKK/EDC4 (IL-8, BAMBI, DUSP1, JunB) and also included an IKK/EDC4 independent (concerning stability), but NF-κB-dependent target (NFKBIA) as a control (Fig. S7c). Our new data show that rescue of EDC4 expression through complementation with the WT EDC4, largely restores the responsiveness of the RNA stabilization to stimulus. However, complementation with the SA EDC4 mutant does not have this effect. In summary, using additional targets we demonstrated that phosphorylation of EDC4 is necessary for control of RNA stability of the abovementioned transcripts.

Additional comments:

1. The authors have changed the names of the proteins. For example DCP1 and DCP2: should be mRNA decapping protein 1 and 2. EDC4: is enhancer of decapping 4. But not Processing (P)-body scaffold. This should be corrected.

We appreciate this correction and changed the text accordingly.

2. XRN1 is an exonuclease. Therefore it is incorrect to write that it performs nucleolytic cleavage. It degrades RNA by exonucleolytic digestion/degradation.

We thank the referee for his/her help and we made appropriate changes in the text.

3. The authors are not very well informed of the literature regarding P-bodies. It has been shown that P-body integrity is not required for mRNA degradation. It is therefore incorrect to state that P-bodies contain mRNAs destined for degradation. Degradation occurs even in the absence of detectable P-bodies. Furthermore, what is the evidence that EDC4 provides a scaffold for RBPs?

We have now changed the text and included appropriate citation (Chung-Te

Chung et al. NAR 2014), showing that DCP1 and DCP2 assemble on the EDC4 scaffold. Also reviewed in (Jonas S. and Izaurralde E. Genes&Dev 2013).

4. P-body expression should be replaced by either P-body assembly or formation.

We are thankful for this suggestion and have changed the wording to P-body assembly.

5. The quality of western blots and IPs needs to be improved. The authors show cropped panels and it is not clear whether the inputs and IPs were analyzed in the same gel and whether the signals can be compared. Also what fraction of the input and pellet has been analyzed? Fig. 1D,G panels are of different size and panel 1D (input) is of low technical quality. Fig. 1G, transfections should be adjusted so that the protein fragments are expressed at similar levels and can be compared. Also panels are cut too close to the bands. Finally what are ther additional bands recognized by the antibodies?

Signals in inputs and co-immunoprecipitationss need not to be compared directly crosswise, but relative to each other in input or IP samples, respectively. We make no points regarding quantitated differences but show convincing interaction between recombinant EDC4 and IKKγ fragments, which are schematically summarized in the new Fig. S1g and h (previously Fig.1e and h).

We have now improved presentation of some images (Fig. 4a, Fig. S1 and Fig. S3s). For immunoprecipitation, 1/100 of the total loaded protein was used for Input, corresponding to 20 micrograms.
We replaced some of the panels and included several images for referee consideration only (Figs. 1-4R).
The additional bands detected represent different isoforms of EDC4, because knockout of the protein eliminates those bands. The IKKγ(NEMO) antibody similarly detects several isoforms of IKKγ.

6. All fluorescent images should be quantified as is done in Figure 3A,B.

We agree with this comment and have now repeated the experiments and quantified relevant images, using blind count and as described in materials and methods.

Referee 2 raised valid points and provided useful suggestions and we appreciate his/her constrictive criticism of our work.

Referee #3:

Proteins interacting with IKK in response to irradiation were identified, and the P-body scaffold protein was selected for further analysis. The authors map several

phosphorylation sites in EDC4 and establish that IKK can phosphorylate these sites in vitro. mRNAs destabilized and stabilized by IR in an IKK-dependent manner were identified using RNA seq, and mRNAs whose stability was altered by KD of IKK or EDC4 were identified and classified according to GO terms. Much of the data are solid and informative, and carefully presented. While a positive correlation between EDC4 phosphorylation and P-body formation is apparent, the assertion that IKK-mediated phosphorylation of EDC4 causes P-body formation is not adequately supported by the data. In addition, a number of other flaws distracted from the overall message.

We thank the referee for the overall positive appraisal of our work. In order to further improve it, we addressed the suggestions and provided additional evidence to support our findings.

Major concerns:

Their data support their conclusion that IR/TNFa stimulate P-body formation in an IKK/EDC4-dependent manner. Interactions between IKK and EDC4 the molecular level are supported by structure-function in vitro kinase data, and point mutations eliminating phosphorylation sites in EDC4 prevent TNFa-induced interactions between EDC4 and IKKgamma. The schematics in Figs 1 and 2 are clear and informative. Induction of P-bodies by IR and its dependence on IKKbeta (Fig 3a and b) are convincing. The authors show that EDC4 KD impairs PB formation (Sup 2p) (although the western blot in Sup 2q showing the KD efficiency is very poor), as has been reported by others. However, the proposed causal link between EDC4 phosphorylation and P-body formation is not convincingly established (Fig 3c).

We thank the referee for the positive evaluation. We have now included additional images and quantitated P-body numbers in cells overexpressing EDC4 WT or the SA mutant both manually (blind count) and with ImageJ (Please see images R1-2).

The authors generate cell lines in which endogenous EDC4 is knocked down and replaced with EDC4-WT or an SA mutant, then treat these cell lines to induce P-bodies. They state that "introduction of the EDC4 SA mutant led to a low basal level of P-body formation and could not restore their amplification (Fig. 3c).". However, Fig 3c data is not quantified and is not convincing. From the raw images, it appears that the SA-EDC4 cells display more P-bodies in the non-irradiated cells than in the irradiated ones, which is the opposite of what the authors state in the text. This experiment needs to be repeated 3 times and data quantified as in 3a and 3b. It is also very important to show that the levels of EDC4 (WT and SA) in these clonal cell lines are similar, and expressed at close to normal endogenous levels.

We agree with the referees' suggestion that data shown could be improved by

quantification. We therefore performed blind count on experiments performed in triplicates. We repeated the experiment in CRISPR EDC4 knockout cell lines, where we also overexpressed the SA EDC4 construct. We furthermore used different tags on the plasmid to ascertain that neither the plasmid nor the Tag prevents foci formation in the SA mutant. As expected, ectopic overexpression led to protein aggregation, observed in images as points, as opposed to much smaller foci (see Fig. R2, above). Image J software is not technically able to differentiate between points and foci (see Fig. R1). We therefore used manual blind count of foci but not aggregates, using at least 200 cells per condition with three biological replicates. We observed no significant difference between unstimulated and irradiated EDC4 knockout cell lines reconstituted with the WT EDC4. But we confirmed a clear difference in the number of P-body foci in cells reconstituted with wildtype EDC4 versus the SA mutant (Figure 3C and Fig. R1). These data support our initial observation that intact phospho sites on EDC4 are required for P-body amplification in response to stimulus.

Other data obtained using these cells is supposed to be shown in Fig S5d; however, it seems to be in Fig S5h and I instead.

We thank the referee for noticing this error and we have corrected it in the manuscript.

While there are clear effects on the stability of IL-8 mRNA, what is not clear is whether these cells are really expressing comparable levels of EDC4 wt/SA, and whether endogenous EDC4 is reduced in both. Using CAS9 to delete endogenous EDC4 might be required, given that the antibodies used to blot EDC4 seem poor (S2-q).

This is a very good point and we have made EDC4 CRISPR knockout cells and overexpressed wt and mutant EDC4 in these cells (see new Fig. S7c). A Western blot showing comparable levels of EDC4 wt and SA is now included for inspection by the referee (Figure R4).

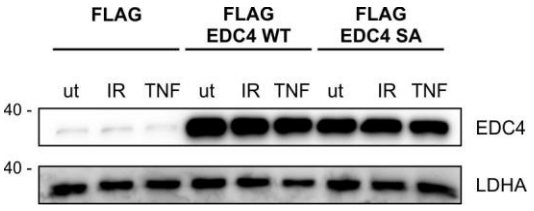

Fig. R4 Expression of FLAG-EDC4 and FLAG-EDC4 SA in U2-OS cells versus endogenous EDC4. SDS-PAGE western blot analysis of the expression of EDC4 constructs or empty vector under different stimulation conditions compared to expression of endogenous EDC4. LDHA, loading control.

The authors also show that "a fraction" (a very minor fraction) of IKK localizes to P-bodies (sup 2c, d), but how do they know that the antibodies are really monospecific for IKK? Does the P-body signal diminish in the IKK KD cells? Alternatively, can a tagged form of IKK be shown to localize to P-bodies?

It is true that antibodies are not always monospecific. We have performed analysis additionally with other antibodies and showed localization of IKKγ and IKKβ to P-bodies (Fig. S3c and d).

Specific points:

Fig 2b (raw MS data) is not explained, labeled, or legible. What do the colors indicate? Axis, units are unclear and font is so small as to be illegible. Was this analysis repeated more than once? This panel would be more appropriate in the supplemental data, while the data in Sup 1a would be more appropriate in Fig 2.

We improved the legend description. This PDF file can be enlarged on the screen with a high resolution. We had now to additionally incorporate MS analysis of endogenous phosphorylated EDC4 into the supplementary figure section, which confirmed the *in vitro* MS data and out of space consideration would prefer to keep the current panel distribution between main and supplementary figures.

Fig 3c data is not quantified and is not convincing. This needs to be repeated 3 times and data quantified as in 3a and 3b. From the raw images, it appears that the SA-EDC4 cells display more P-bodies in the non-irradiated cells than in the irradiated ones, which is the opposite of what the authors state in the text. It is also very important to show that the levels of EDC4 (WT and SA) in these clonal cell lines are similar, and expressed at close to normal endogenous levels.

Please see our response to this point in this reviewer's comments above.

Fig. 4f, g. Axes are labeled "semantic space." This is confusing.

The x and y axis are so called semantic coordinates given by the REVIGO program and designated as such in the data presentations in publications.

Fig 5. It would be helpful to explain what the various symbols in this model are supposed to represent. The model appears to show mRNAs that are capped and have polyA tails in P-bodies. However, others have shown that PABP and polyA (detected by FISH) are usually absent from P-bodies. Do the authors have any evidence that polyA is present in these P-bodies? Can they show that mRNAs stabilized (or destabilized) by IKK are recruited to P-bodies during IR?

This is a good point and we will consider it in future studies. Here we did not analyze polyA presence in P-bodies. Figure 5 is a simplified schematic. As

outlined above, we do not want to claim that the role of IKK in mRNA stability control is necessarily linked to P-body formation.

*Sup 1b. X-axis is labeled "-log p-value"; this is not clear. Is it negative, or is the dash not a negative sign? It would be informative i*f

This is the standard presentation of GO term analysis.

Sup 1c: Only a single timepoint at 40' post-IR shows an increased interaction between DDX6 and IKK. Is this reproducible? It would be more convincing if a time course showing at least 2 elevated points were shown.

We are not claiming any detailed insight into DDX6-IKKγ interaction. This data is used to confirm the interactions of other P-body components with IKKγ.

Sup 2a,b- Legend indicates that DDX6-staining is red, but what is shown in the green channel? Similarly, what is shown in the red channel in Sup 2h, i, and j?

The stain is EDC4. We thank the referee for noting this and we completed the legend correspondingly.

Blot in 2q is not convincing.

We acknowledge the referees' comment and we believe that the blot clearly shows strong reduction of EDC4 expression upon knockdown and actin as a loading control.

FIG S5d. The Result section refers to "S5d middle and lower panels," which are not present in Fig S5d. This probably refers to S5h and j, but this sort of mistake is not reassuring.

" To determine whether mRNA stability of IL-8 depends on the phosphorylation state of EDC4, EDC4-depleted cells were reconstituted with the wt EDC4 or the phospho-deficient EDC4 SA mutant. Reconstitution with the wt EDC4 but not EDC4 SA rescued stabilisation of IL-8 mRNA in irradiated cells (Fig. S5d, middle panel). Expression and stability of IL-8 mRNA in cells expressing EDC4 SA resembled that of EDC4 and IKKβ knockdown cells (Fig. S5d, lower panel)."

We apologize for this mistake and corrected this point in the revised version.

We hope that we have sufficiently answered the referee's questions and thank the referee again for the constructive suggestions, which helped to further improve the manuscript.

2nd Editorial Decision 17th July 2018

Thank you for submitting a revised version of your manuscript. It has now been seen by two of the original referees whose comments are shown below.

As you will see they both find that all criticisms have been sufficiently addressed and recommend the manuscript for publication. However, before we can officially accept the manuscript there are a few editorial issues concerning text and figures that I need you to address in a final revision.

REFEREE REPORTS

Referee #1:

This revised version of the manuscript addresses the issues I raised in my first review. I have no additional concerns

Referee #3:

The authors have addressed the points raised to my satisfaction.

2nd Revision - authors' response 1st October 2018

Thank you very much for the decision letter. We have now completed all open points that you finally raised (paraphrased below) with our response in bold.

Accepted 8th October 2018

Thank you for submitting a revised version of your manuscript to The EMBO Journal, I am pleased to inform you that your study has now been officially accepted for publication here. However, before we can go on to transfer your manuscript files to our production team I have one remaining formatting issue that I need you to address.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Scheidereit C.
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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 justified
- 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.

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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?	Samples sizes were empirically determined in respect of previous experiments. And at least two independent experiments were conducted containing technical triplicates per experiment.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	Sample size determined based on variation from previous experiments
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	NA
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.	samples were collected at the same time. Blinded counting was performed.
For animal studies, include a statement about randomization even if no randomization was used.	NA
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.	Blinding of the investigator and all marker association analysed using immunofluorescence data was quantified by an automated ImageJ software.
4.b. For animal studies, include a statement about blinding even if no blinding was done	NA
5. For every figure, are statistical tests justified as appropriate?	yes
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	NA
Is there an estimate of variation within each group of data?	yes
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).	Supplier information/catalog number/clone number are provided in Materials and Methods for the antibodies used.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Cells were obtained from ATCC and checked for mycoplasma.

* 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.	NA
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	NA
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.	NA

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
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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	Data availability statement can be found in materials and methods.
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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