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The transcription factor FBI-1 inhibits SAM68-mediated BCL-X alternative splicing and apoptosis

Pamela Bielli, Roberta Busa, Savino M. Di Stasi, Manuel J. Munoz, Flavia Botti, Alberto R. Kornblihtt and Claudio Sette

Corresponding author: Claudio Sette, University of Rome Tor Vergata

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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.)

Transfer Note:

Please note that this manuscript was originally submitted to the EMBO Journal where it was peer-reviewed and revised. It was then transferred to EMBO reports with the original referees' comments and the authors' replies attached. (Please see below)

Correspondence – decision letter and referee comments, revised manuscript EMBO Journal 24 September 2013

Thank you for submitting your manuscript for consideration by The EMBO Journal and my apologies for the unusually long review period in this case.

Your revised study has now been seen by the original three referees whose comments are included below. As you will see, while they all praise the novelty of your findings and acknowledge the efforts you have made to address the original concerns raised, they still find that the presented data

do sufficiently support the proposed model and that especially the second half of the manuscript remains inconclusive. Consequently, they do not offer strong support for publication in The EMBO Journal at the current stage.

I will not repeat all their individual criticisms here, but it becomes clear that the referees are not convinced that the proposed bridging role for HDACs suffices to explain the mechanism underlying the FBI-1/Sam68 axis in alternative splicing. Given these serious concerns raised by all three referees as well as the extent and unpredictable outcome of the additional experiments required to fully delineate the role for transcription and HDAC activity, I am afraid we cannot offer to publish the manuscript in The EMBO Journal.

However, since the referees do highlight the novelty and impact of the initial finding, I have taken the liberty to discuss your manuscript and the referee reports with an editor from our sister journal EMBO Reports, which as you may know specializes in publishing short reports of high general interest but without requirements for extensive mechanistic insight. The responsible editor there, Esther Schnapp, would be very interested in publishing your manuscript based on the current referee reports following satisfactory minor revisions as outlined below:

- You need to remove the model in figure 8 and reduce data presentation in figures 6 and 7 to simply show that HDAC inhibition affects the ability of FBI-1 to affect splicing. As requested by ref #2 this would let you tone down the conclusion about the underlying mechanism.

- In additional communication with our editorial office, ref#3 directly suggested removal of the HDAC and transcription data. Since data addressing transcriptional elongation rate as a mediator of the FBI-1 effects on alternative splicing was already postponed to a subsequent study, we would recommend that you only mention a role for HDACs here and include a more detailed analysis in a future study.

- As requested by ref #1 (remark #1) you should demonstrate that alternative splicing of Bcl-X is the driver for Sam68-induced apoptosis in FBI-deficient prostate cancer cells.

- Given the altered format requirements at EMBO Reports, you will also have to shorten the manuscript text prior to resubmission. The revised manuscript may not exceed 30,000 characters (including spaces and references) and 5 figures plus 5 supplementary figures, which should directly relate to their corresponding main figure.

- Shortening of the manuscript text may be made easier by combining the Results and Discussion section, which may eliminate some redundancy that is inevitable when discussing the same experiments twice. Parts of the materials and methods can further be moved to the Supplementary Information, however, please note that the materials and methods essential for the understanding of the experiments described in the main manuscript file must remain in the main text.

While I am thus sorry to say that we cannot take further steps towards publication in The EMBO Journal, I do hope that you will take the chance to submit your revised manuscript to EMBO Reports. Please feel free to contact either myself or my colleague Esther Schnapp at EMBO Reports with any questions in this matter.

Thank you for giving us the chance to consider your work.

Editor
The EMBO Journal

REFEREE REPORTS:

Referee #1 (Former ref#2):

In this study, the authors examine the impact of the transcription factor FBI-1 on the activity of

Sam68 involved in the regulation of Bcl-X alternative splicing and consequently in the regulation of apoptosis. This antagonistic effect of FBI-1 on Sam68 is exerted by direct interaction of the factors, and could lead to a perturbation of chromatin acetylation and/or RNAPII interaction. Modulation of Bcl-X alternative splicing correlates with apoptosis in several cell types, depending on the amount of FBI-1 and Sam68 [remark1].
The interaction between the 2 proteins depends on the C-ter half (278-584) of FBI-1 and on aa 400 to 420 of Sam68. The Sam68 mutant V229F is described as being defective in its RNA-binding activity (and exerting a dominant negative effect on the splicing activity of endogenous Sam68). The del400-420 mutant of Sam68 can't interact with FBI-1. FBI-1 antagonizes the Sam68 functions in regulation of apoptosis and in the regulation of Bcl-X AS. Overexpression of FBI-1 inhibits the interaction of Sam68 with the Bcl-X mRNA as shown by CLIP [remark 6]. However, FBI-1 doesn't impair interaction of Sam68 with RNA in general (as shown by the polyU pull-down), with hnRNPA1, with itself (homodimerization), nor interferes with Sam68 phosphorylation by the Fyn kinase [remark 7]. These observations suggest that FBI undertakes its antagonistic effect on Sam68 co-transcriptionaly during the synthesis of the Bcl-X transcripts. This possibility is also suggested by the coIP experiment showing that interaction of FBI with the RNAPII depends on Sam68 (Fig6A), and by the ChIP experiment showing that the presence of FBI on the Bcl-X exon2 is decreased after knock-down of Sam68 (Fig6B). Moreover the interaction of Sam68 to RNAPII is increased after the FBI knock-down (Fig7G) and the interaction of Sam68 to BclX transcripts detected by CLIP is decreased after overexpression of FBI (Fig7E) [remark 5]
In addition, because FBI-1 was already described to associate with HDACs (and Fig7A), the authors explore whether FBI could affect the acetylation of chromatin of the Bcl-X locus. Here, ChIP experiments suggest that acetylated histones do increase on BclX exon2 after FBI knock-down (Fig7B) [remark 3]. Inhibition by a drug of HDAC activity mimics the effect of knocking-down FBI-1 on the splicing of the endogenous BclX (Fig7C), increases the BclX transcript-binding of Sam68 and impaires the FBI antagonistic effect on the RNA binding activity of Sam68 (Fig 7E). [remark 2]
Moreover, the RNAPII phosphorylations on Ser2 and Ser 5 are also increased on exon2 after FBI-1 knock-down (Fig7F) [remark 4]
Finally, the last figures show that a big part of the RNAPII-Sam68 interaction can be modulated by the FBI-1 (7F) and also by the HDAC inhibitor (7G). [remark 8]

My concerns are:

[remark1] To reconcile the beginning of the article on the apoptotic effect of FBI with the cotranscriptional regulation of BclX splicing, the authors need to show that Sam68/FBI-dependent apoptosis is mediated by the BclX AS. In its present form, the data does not prove that the studied process is a direct cause of apoptosis. For example, does the inhibition of HDAC in PC3/lnPCa cells induce apoptosis in a Sam68-dependent manner? More directly and may be more convincingly, can the apoptosis induced by the FBI/Sam68 imbalance in prostate cells be prevented by forcing the expression of anti-apoptotic Bcl-X form (by just an Bcl-XL expression vector)?

[remark 2] Because the effect of FBI on the Bcl-X splicing is argued to take place cotranscriptionnally through histone acetylation, the inhibition of HDAC activity should impair the effect of FBI overexpression on the splicing not only of the minigene (Fig7D), but also of the endogenous BclX gene pre-mRNA. This effect on the endogenous pre-mRNA should be shown.

[remark 3] : In Fig 7B, the authors treat their cells with DRB and proceed to the ChIP after the release of the RNAPII. The authors should explain in the text such a protocol was chosen and show the data for the time point t0 (before the removal of the DRB). Possibly, the best solution may be to redo the ChIP experiment without DRB treatment?

[remark 4] To argue that the action of FBI on RNAPII phosphorylation is "local", the authors must show that RNAPII is not increased elsewhere by for example providing a PCR of the distal intron 2 (where acetylation is unchanged in Fig 7B) or of another control locus. The data would also be strengthen if the authors showed the level of total RNAPII (4H8 antibody for example). This would allow to estimate accumulation of the RNAPII at exon2 and could suggest that the RNAPII may be faster in absence of FBI-1. Then, the authors could correlate the presence of the RNAPII with the histone acetylation (which increase in these condition Fig7B) as shown by the team of Alberto Kornblihtt.

[remark 5] Authors must show that a Sam68 target gene other than BclX is well occupied by Sam68 but unaffected by FBI-1; This would allow to determine whether the effect of FBI is truly "local" (on the BclX gene) - i.e. cotranscriptional - or whether FBI has a general effect on Sam68 RNA binding activity wherever it is.

[remark 6] Authors must show a negative control of their CLIP : i.e. a part of the Bcl-X transcript where Sam68 is not bound or an unrelated transcript such as GAPDH, cyclophilin.... They must indicate the part of the mRNA detected by the RT-qPCR in fig5, 7E and Sfig6.

[remark 7] the experiment presented in Fig S5B should be addressed with the full length FBI and not only with the CT mutant.

[remark 8] The co-immunoprecipitation presented in Fig 7G/H is very nice, but in my mind, it suggests a very general effect of HDAC activity on FBI, Sam68, and RNAPII interactions (not just a local co-transcriptional effect). It is indeed difficult to believe that local histone acetylation at Bcl-X exon 2 changes the interaction between RNAPII and an important pool of Sam68 (Fig7 G and H) via a change in the CTD phosphorylation as suggested in the discussion. A local effect could eventually be better explored by a ChIP and reChIP experiment. Alternatively, the authors may want to discuss the possibility that one of the partners of the complex may be acetylated (so, sensitive to LBH). There is a precedent to this as Sam68 can be acetylated by CBP resulting in increased RNA binding activity (Babic et al 2004 Oncogene 23(21):3781-9).

Referee #2 (Former ref #1):

Bielli et al. have identified the splicing factor SAM68 as a novel interactor of the transcription factor FBI-1 and validated this observation by mutation analysis and pull down experiments. Expression of FBI-1 inhibited the previously demonstrated pro-apoptotic effect of SAM68. Both proteins are overexpressed in prostate cancer cells and depletion of FBI-1 resulted in increased apoptosis suggesting that the elevated levels of FBI-1 prevented the pro-apoptotic activity of SAM68 in cancer cells. Since the apoptotic effect of SAM68 is known to be mediated via its regulatory role in BCL-X alternative splicing, the authors tested whether FBI-1 affects alternative BCL-X splicing. Expression of a SAM68 isoform which exerts dominant negative effects on BCL-X splicing was able to counteract the FBI-1 effect suggesting that the protein acts on splicing activity. In support, FBI-1 knockdown promoted the pro-apoptotic BCL-Xs. Using an established BCL-X minigene system, they show that FBI-1 favored BCL-Xl in a SAM68 dependent manner and required FBI-1/SAM68 interaction. In cancer cells, expression of SAM68 only had an effect on splicing in benign BPH1 cells which contain lower levels of FBI-1 and knockdown of FBI-1 in cancer prostate cancer cells restored SAM68-dependent BCL-X splicing. CLIP experiments show that expression of FBI-1 reduce binding of endogenous SAM68 to BCL-X mRNA. Co-IP experiments indicate interaction of FBI-1 with RNA pol II in a SAM68-dependent manner and ChIP experiments demonstrate its association with the length of the BCL-X transcription unit in a SAM68-dependent manner. Finally, the authors show that knockdown of FBI-1 leads to increased BCL-X acetylation. Inhibition of HDACs mimics the effect of FBI-1 knockdown enhanced binding of SAM68- to BCL-X. Based on these data the authors propose a model in which FBI-1 sequesters SAM68 via physical interaction from the RNA and thus promotes BCL-Xl splicing.

This is a nicely detailed characterization of the FBI-1/SAM68 interplay. The proposed model is interesting and novel and supported by several key pieces of evidence. The link, albeit somewhat tentative, to the situation in cancer cells is similarly of interest.

There are a few points that should be addressed:

The weakest aspect of the study is the role of acetylation and HDACs in this mechanism. The authors propose that HDACs act as linkers between the acetylation and FBI-1 and in that way establish the physical interaction with chromatin (fig. 8). This is not very plausible and no data to directly demonstrate such a role of HDACs is presented. A prediction from this model is that HDACs are similarly in the FBI-1/POLII/SAM68 complex. Is that true? The demonstrated

interaction of FBI-1 with HDACs does not address this as this may be a separate, not-transcription association population of molecules. The linker role of HDAC seems somewhat implausible since HDACs presumably only very transiently interact with chromatin during the time they modify it. It is hard to imagine that they could mediate interactions that would result in sequestration of FBI-1 and SAM68. This part of the model should either be toned down or additional data should be provided.

Related to the above point, the interplay between FBI-I and acetylation was not clear to me. Why does knockdown of FBI-1 increase acetylation levels (fig. 7)?
The authors use a BCL-X minigene to assess the effect of HDAC inhibitors on splicing. This is only a valid experiment if the minigene is chromatinized. The authors should show that it is.

Referee #3 (Former ref #3):

The manuscript by Bielli et al. presents evidence for a role of FBI-1 in the splicing of Bcl-X that arises from its interaction with Sam68. FBI-1 was found in a screen to interact with Sam68, although it is not clear from a comparison of Fig 1D and Supp Fig 1D whether a significant porportion of endogenous Sam68 is bound even when FBI-1 is over-expressed. Plasmid over-expression assays showed that FBI-1 antagonized the triggering of apoptosis by Sam68. Interestingly, depletion of endogenous FBI-1 left the cells more vulnerable to transfection with smaller masses of Sam68 plasmid (Figure 2 C, Figure 3, Supp Fig 3). However, there is no evidence that FBI-1 counteracts apoptosis induced by other means. FBI-1 depletion was found to affect endogenous Bcl-X splicing (Figures 4A,B). Disappointingly, the authors shifted to a Bcl-X minigene assay, even though they did not use Bcl-X mutants. This showed that FBI-1 overexpression affected Bcl-X splicing unless Sam68 was depleted, whereas the effect of Sam68 ovrexpression did not require but was augmented by FBI-1 depletion (Figs 4 and Supp Fig 4). The authors concluded that FBI-1 affected Bcl-X splicing via interactions with Sam68. FBI-1 was shown to reduce the interaction of Sam68 with Bcl-X RNA based on crosslinking-immunoprecpitation, but not to affect Sam68 affinity purification on poly(U) sepharose. Given that poly(U) is not the optimal substrate for Sam68 and may not bind to the same site as a specific substrate, it was curious to infer that the difference was connected to transcription. Co-precipitation experiments showed that FBI-1 barely associated with RNAPII after Sam68 depletion (Figure 6A). Figure 6B showed that depletion of Sam68 reduced the association of FBI-1 with Bcl-X exon 2, although the association of FBI-1 with other exons was unaffected. Finally, Figure 7 suggests that FBI-1 suppresses exon 2 histone acetylation (H4/H3) but that if acetylation is increased then FBI-1 no longer affects splicing or Sam68 binding, and that acetylation leads to increased association of Sam68 with RNAPII and decreased association of FBI-1.
The manuscript contains an impressive volume of data, much of it very clean, and in general I think the study makes a worthwhile contribution in that it demonstrates that FBI-1 affects the actions of Sam68. It is not possible, from the results shown, to devise a clear molecular model. In this regard, I think Figure 8 is not really very helpful. I think it would be helpful if the authors modified the text or figure legends to clarify the points I list below.

1. In Figure 4A and 4B, splicing of the endogenous Bcl-X gene in HEK293T cells is measured. I cannot see any Xs isoform in the control in Fig 4A, whereas the qPCR analysis in 4B suggests that the ratio of Xs/Xl in such cells is 1. At first sight, this is scarcely credible. I imagine that some normalization has been done. It is important for the credibility of the work that the real value is given first, and that it is then stated that this value has been set to 1. In other figures where the splicing ratios are indicated, it should be made absolutley clear whether there has been normalization and, if so, based on which sample.

2. There are other apparent contradictions. Figure 1D and supplementary 1D give quite different indications about the proportion of Sam68 associated with FBI-1. This is also worth a comment.

3. The t-tests in Figs 4D and F are inappropriate. The important comparison is between the second and fourth bars in each chart, and the p-value for this comparison should be given (as in Figure Supp 4E, for example).

4. It should be made clear when qPCR was used to measure the ratios of the isoforms.

5. The conditions for co-immunoprecipitation and GST pull-down assays are not given in detail in the methods, but instead one is referred to Paronetto et al., 2007. That paper does not state that the inclsion of nucleases was done routinely. It is very important for many of the conclusions reached in this manuscript that ribonuclease or DNase were used to treat the lysates, to prevent bridging by nucleic acids when protein interactions are being tested. This is an important issue in the field, and I am sure the authors are aware of this. There should be a clear statement in the legend to each figure stating that this was the case (Figs 1D, 6, 7A, G, H, and Supp Figs 1D, 2F and 5).

6. Figure 6A does look a bit doubtful. Was the experiment replicated?

7. Figure 6B appear to show only a small difference, across all three regions tested, in the level of FBI-1 associated with the Bcl-X gene after depletion of Sam68. The authors connect this to the result in Figure 6A. However, 6A appears to show an almost complete loss of RNAPII-associated FBI-1. It seems likely that the link to RNAPII (direct or DNA/RNA-bridged?) is more susceptible than is the link to the gene.

8. The discussion is not very helpful. The authors refer to facts that they have established, usually at the start of every paragraph, without referring to the figure showing the critical data. This is bad practice. Readers need to be able to find the figure and the key lanes etc. as they read the discussion so that they can decide whether they accept the conclusion or not.

Moreover, there is almost no discussion of the quality or reliability of the data. For example, I would have expected some discussion of the validity of minigene/overepression methods, given that the Bcl-X minigene shows much higher relative levels of expression of Bcl-Xs than the endogenous gene and that the concentrations of proteins are presumably much higher than the endogenous levels. Is this likely to affect the probability of seeing interactions?

Transfer to EMBO reports - authors' response to revision comments 09 December 2013

We are pleased to submit to your attention the revised manuscript entitled: "**The transcription factor FBI-1 modulates SAM68-mediated BCL-X alternative splicing and apoptosis**", by Pamela Bielli, Roberta Busà, Savino Di Stasi, Manuel J. Munoz, Flavia Botti, Alberto R. Kornblihtt and Claudio Sette, for possible publication in *EMBO Reports*.

We have now addressed all the points you mentioned in our last email (Remark 6 of Referee 1 and Remarks 1-5 of Referee 3). A point-to-point rebuttal letter to these remarks is attached below. In addition, as you mentioned in previous emails, we have now also added the information on the number of experiments performed for each figure panel, when appropriate, and added the scale bars where they were missing.

Regarding your request of the 1-2 sentences summary, we propose:

"Alternative splicing is tightly coupled to transcription in the majority of human genes, but the mechanisms connecting these processes are only partially known. This study unveils a direct role

for the oncogenic transcription factor FBI-1 in the regulation of alternative splicing through interaction with a splicing regulator".

The 2-3 bullet points for the key findings are:

1. the transcription factor FBI-1 directly interacts with the splicing regulator SAM68;
2. FBI-1 impairs binding of SAM68 to the BCL-X mRNA, thus modulating splicing outcome and favouring cell survival;
3. FBI-1 modulates BCL-X splicing by a mechanism that involves histone deacetylase activity.

With the hope that our manuscript will now be considered suitable for publication in *EMBO Reports*, we wish to submit it to your attention.

Looking forward to hearing from you.

Response to Referees:

Referee #1 (Former ref#2):

"[remark 6] Authors must show a negative control of their CLIP : i.e. a part of the Bcl-X transcript where Sam68 is not bound or an unrelated transcript such as GAPDH, cyclophilin.... They must indicate the part of the mRNA detected by the RT-qPCR in fig5, 7E and Sfig6."

As suggested by the reviewer, we now show as negative control of the CLIP a distal region of the intron 2 where SAM68 does not bind to the BCL-X pre-mRNA. We also add a scheme, in the new Figure 5 and Supplementary Figure 5, of the regions amplified by qRT-PCR.

Referee #3 (Former ref #3):

Remark 1. In Figure 4A and 4B, splicing of the endogenous Bcl-X gene in HEK293T cells is measured. I cannot see any Xs isoform in the control in Fig 4A, whereas the qPCR analysis in 4B suggests that the ratio of Xs/Xl in such cells is 1. At first sight, this is scarcely credible. I imagine that some normalization has been done. It is important for the credibility of the work that the real value is given first, and that it is then stated that this value has been set to 1. In other figures where the splicing ratios are indicated, it should be made absolutley clear whether there has been normalization and, if so, based on which sample."

This issue was already partially addressed in the first version of the EMBO Reports manuscript we submitted, in which we removed the quantification of the ratio by densitometry in Figure 3A (old Figure 4A in the EMBO Journal version to which the Reviewer refer). Indeed, since the pro-apoptotic short variant is expressed at very low level compared to the long isoform, it is not visible in conventional PCR of control cells using cycle number that does not saturate the signal of the

BCL-XL variant. Nevertheless, qualitatively it is obvious that knockdown of FBI-1 increases expression of the short pro-apoptotic BCL-Xs variant (see Figure 3A). To obtain quantitative data of the effect of FBI-1, we then designed an exon-junction primer and a quantitative real time PCR (qPCR) approach. By using this approach we found that the expression level of the long isoform is about 50 time more expressed compare to the short isoform (data not shown). Notably, in cells depleted for FBI-1 this ratio is reduced to 40 time. In the "splicing assay and RT-PCR analyses" section of supplemental material we have now explained in detail how the ratio between the two isoforms have been calculated by qPCR in new Figure 3B. Briefly, the qPCR analysis of BCL-X variant was calculated by the delta-delta Ct (cycle threshold) method. ΔCt was calculated as the difference between CtBCL-Xs and CtBCL-XL (ΔCt = CtBCL-Xs- CtBCL-XL). ΔΔCt was then calculated as the difference between ΔCt of si-FBI-1 samples and ΔCt of control samples (si-CTRL) (ΔΔCt = ΔCtsi-FBI- ΔCtsi-CTRL). Fold variation (F) of each samples compared to the control was then estimated as follow: F= 2(-ΔΔCt).
Moreover, as suggested by the Referee, we now specify in the figure legends when normalization have been performed, as in new figure 3C.

Remark 2. There are other apparent contradictions. Figure 1D and supplementary 1D give quite different indications about the proportion of Sam68 associated with FBI-1. This is also worth a comment."

The aim of these experiments was to confirm the interaction between FBI-1 and SAM68 by different experimental strategies (ie mammalian Two-hybrid, GST-pulldown experiments, co-immunoprecipitation experiments). We did not perform experiments focused to quantify the proportion of SAM68 associated with FBI-1. In particular, the two experiments mentioned by the Referee cannot be directly compared, because in Figure 1D we co-immunoprecipitated the endogenous proteins whereas in Figure S1D Flag-FBI-1 was overexpressed by transfection. Under this latter condition, it is likely that a portion of FBI-1 occupies sites in the cells that are not normally occupied by the endogenous protein and this might lower the efficiency of association with the endogenous SAM68. Indeed, our results suggest that the interaction is more efficient between the endogenous proteins, which is more relevant from a physiological point of view than the association between recombinant proteins. Specific experiments to determine the relative affinity for SAM68 of endogenous versus recombinant FBI-1 are beyond the scope of the present manuscript.

Remark 3. The t-tests in Figs 4D and F are inappropriate. The important comparison is between the second and fourth bars in each chart, and the p-value for this comparison should be given (as in Figure Supp 4E, for example).

As suggested by the Referee we have now performed statistical tests between the second and fourth bars in the new Figure 3D and F (old Figure 4D and F). The p value of Student's t-test are now reported in all the Figure legends, when appropriate.

Remark 4. It should be made clear when qPCR was used to measure the ratios of the isoforms"

We performed qRT PCR to measure the ratio of BCL-X isoforms only in new Figure 3B (old Figure 4B). We modified the text of the Figure legends to avoid confusion with conventional RT-PCR.

Remark 5. The conditions for co-immunoprecipitation and GST pull-down assays are not given in detail in the methods, but instead one is referred to Paronetto et al., 2007. That paper does not state that the inclsion of nucleases was done routinely. It is very important for many of the conclusions reached in this manuscript that ribonuclease or DNase were used to treat the lysates, to prevent bridging by nucleic acids when protein interactions are being tested. This is an important issue in the field, and I am sure the authors are aware of this. There should be a clear statement in the legend to each figure stating that this was the case (Figs 1D, 6, 7A, G, H, and Supp Figs 1D, 2F and 5).

We routinely add DNase and RNase in the GST-pulldown and co-immunoprecipitation analyses. We now specify this point in the Material and Methods section

1st Editorial Decision 12 December 2013

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

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

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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.

