CREB and ChREBP oppositely regulate SIRT1 expression in response to energy availability

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Review timeline:

Submission date: 22 January 2011
Editorial Decision: 21 February 2011
Revision received: 20 May 2011
Editorial Decision: 14 June 2011
Revision received: 16 June 2011
Editorial Decision: 17 June 2011
Accepted: 17 June 2011

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 21 February 2011

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

As you will see, while the referees agree that the study is potentially interesting, they also raise a few important concerns, which need to be addressed before the manuscript can be considered for publication in our journal.

Both referees 1 and 2 point out that mutations of CREB and ChREBP binding sites in the SIRT1 promoter should be generated to identify which sites are directly involved in the transcriptional activation of SIRT1. Referee 2 adds that the relative roles of the two described promoter regions need to be determined and referee 3 further indicates that CREB should be knocked down to prove its role in SIRT1 activation. This referee also remarks that SIRT1 protein levels need to be shown throughout the manuscript on high quality western blots. All referees further pinpoint missing controls and insufficient quantifications that need to be included.

Given these evaluations and the constructive referee comments, I would like to give you the opportunity to revise your manuscript, with the understanding that the referee concerns must be fully addressed and their suggestions (as detailed above and in their reports) taken on board. Acceptance of the manuscript will depend on a positive outcome of a second round of review and I should also
remind you that it is EMBO reports 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 included in the next, final version of the manuscript.

I look forward to seeing a revised version of your manuscript when it is ready.

Yours sincerely

Editor
EMBO Reports

REFEREE REPORTS:

Referee #1:

In this study, Noriega and co-workers explore the mechanisms governing SIRT1 expression in response to the metabolic status and demonstrated that SIRT1 levels are transcriptionally regulated by nutrient availability through opposite actions mediated by CREB and ChREBP. These data establish that SIRT1 expression is modulated according to energetic needs. This study is important, very interesting to the field of ChREBP and CREB regulation.

Major comments.

1. Figure 2E. Mutations of the all CREB sites located within 300 and 160 bp of the SIRT1 promoter should be deleted in order to precisely determine which CREB site(s) is/are directly involved in the cAMP-mediated induction of the SIRT1 gene.
2. Figure 2G. It seems odd that an induction of SIRT1 expression remains (although modest) upon ACREB expression. What is the explanation for this? Was all the endogenous CREB titrated upon ACREB expression?
3. Figure 3D. A positive effect of ChREBP/Mlx on the ChoRE of the L-PK gene (for example) should be obtained in parallel as a positive control in this particular experiment. In addition, a rescue experiment of the effect of FSK on the SIRT1 promoter should be performed in a presence of a dominant form of ChREBP.
4. Figure 3E. As a complement of the ChIP experiment performed, the authors should mutate the potential ChoRE/E box presented in Supplemental Figure 4 in order to clearly demonstrate that ChREBP directly binds to this sequence.
5. Figure 4A. ChREBP nuclear localization should be visualized in this particular experiment (either using nuclear extracts and/or immunocytochemistry) under all the conditions presented.

Referee #2:

This manuscript proposes a new regulatory mechanism for SIRT1 expression in response to energy availability. Noriega, et al. investigated the expression level of SIRT1 upon fasting and refeeding. Upon fasting, SIRT1 protein and mRNA level increased in different mouse tissues. Glucagon upregulated SIRT1 protein and mRNA in liver. Using the HepG2 cell line, they also observed that CREB activation/overexpression upregulated SIRT1 mRNA levels. The promoter region required for the CREB effect was identified in the SIRT1 promoter between nt -316 to -160. Upon refeeding, SIRT1 protein and mRNA decreased in liver. SIRT1 expression was upregulated in ChREBP Knock down cells or ChREBP-/- mouse. Acetylation level of PGC-1a was decreased in ChREBP-/- mouse. ChREBP-mediated repression of SIRT1 promoter could be overcome by increasing amounts of CREB. EMSA study showed both CREB and ChREBP bind to a 25 bp SIRT1 promoter region (-146 to -92). CHIP assay showed that during fasting, CREB recruitment to SIRT1 promoter increased while ChREBP binding to the SIRT1 promoter decreased.

-These observations are potentially interesting but assume that all SIRT1 regulation occurs at the transcriptional level. There is growing evidence that SIRT1 expression is also regulated at multiple
other levels. The authors should discuss their data in the context of these other regulatory mechanisms.

- The luciferase assays used to assess SIRT1 promoter activity are presented in a confusing manner. First, the author show that a promoter region localized between nt -316 to -160 is important for CREB to activate SIRT1 transcription. Later in the manuscript, the authors identify another region -146 to -92 as the site where CREB and ChREBP compete to regulate SIRT1 expression. What is the relative role of both sites in SIRT1 transcriptional regulation? Also, luciferase assays are required to confirm that the SIRT1 promoter region corresponding to nt -146 to -92 is really important. Selective mutations that abolish binding of either CREB or ChREBP should be generated and their impact on SIRT1 promoter activity should be assessed.

- Figure 2E, The difference in luciferase activity between D3 and D4 is less than 2 fold-when comparing control and CREB overexpression. However, in fig 2B, the luciferase activity of the 1.2 kb promoter is induced 5 fold upon CREB overexpression. Does this mean that there are other unidentified promoter regions important for the activation? The 1.2 kb promoter construct should be included in figure 2E.

- Fig 3A, Expression level of SIRT1 under fed condition should be included. Does SIRT1 expression level return back to the fed condition?

- Fig 3G, Is PGC-1a also transcriptionally regulated by ChREBP? Are PGC-1a protein levels the same in WT and ChREBP-/- mice.

Referee #3:

Noriega et al. follows the regulation of SIRT1 levels in response to nutrient availability. More specifically the presented study follows the regulation of SIRT1 transcription levels by CREB & ChCREB under normal and nutrient-deprived conditions. This is a very interesting and important study. They demonstrate that when grown with normal media ChCREb binds to the SIRT1 promoter and inhibits its transcription. In contrast, when nutrients were depleted the authors suggest that ChCREB dissociates and CREB induces SIRT1 levels. This study is a continuance of previous publications by Cohen et al. (2004), Nemoto et al. (2004), Kanfi et al. (2008) and Chen et al. (2008). Whereas the first three studies showed an increased in SIRT1 levels under starvation or calorie restriction-CR the last one does not. Still, the previous publications did not agree about the mechanism underlying the increase in SIRT1. Therefore, this study is an important addition to these publications. Nevertheless, the following experiments should be addressed.

1- Along the paper the authors avoid showing SIRT1 protein levels. Moreover, in cases they do show western analysis, the quality is poor. These data should be included in the figures, especially given the controversy regarding the increase in SIRT1 levels upon starvation or CR. Especially, figure2C/D , 3A/F, and 1C should be replaced.

2- Based on our experience, when we performed metabolic assays, there is a large variability between mice. The authors use low number of mice (n=4). We strongly recommend increasing the amount to 6-8 per group.

3- The author should discuss the differences between their findings and Kanfi et al. Could it be tissue/cell line specific?

4- The Authors should use siRNA against CREB in starved cells, in order to prove that CREB is in charge of the increase in SIRT1 levels.

5- It is not clear why the authors follow SIRT1 mRNA levels after 12 hours of starvation and SIRT1 protein levels after 24 hours. Did they follow other time points?

6- The increase in SIRT1 levels in the muscles shown in figure S2 is lower than 20%. Please indicate the p-value for this measurement.
Referee #1: In this study, Noriega and co-workers explore the mechanisms governing SIRT1 expression in response to the metabolic status and demonstrated that SIRT1 levels are transcriptionally regulated by nutrient availability through opposite actions mediated by CREB and ChREBP. These data establish that SIRT1 expression is modulated according to energetic needs. This study is important, very interesting to the field of ChREBP and CREB regulation.

We thank this referee for his/her positive comments and suggestions that have enabled to strengthen the manuscript. A detailed point-by-point answer to his/her comments is provided below.

Major comments.

1. Figure 2E. Mutations of the all CREB sites located within 300 and 160 bp of the SIRT1 promoter should be deleted in order to precisely determine which CREB site(s) is/are directly involved in the cAMP-mediated induction of the SIRT1 gene.

We thank the reviewer for his suggestion. To address this point, we have performed site-directed mutagenesis of the seven putative CREB binding sites. Only mutation of the sites located at -175 and -330 bp resulted in a modest, but significant reduction in the CREB mediated induction of SIRT1 promoter activity. Importantly, the simultaneous mutation of these two sites led to a very marked decrease in the CREB-induced SIRT1 promoter activity. These results illustrate that these two sites are key for the cAMP induction of the SIRT1 promoter. These results are now included in Figure 2F, together with a schematic representation of the SIRT1 promoter to better illustrate the localization of the putative CREB binding sites.

2. Figure 2G. It seems odd that an induction of SIRT1 expression remains (although modest) upon ACREB expression. What is the explanation for this? Was all the endogenous CREB titrated upon ACREB expression?

We thank this reviewer for his/her pertinent concern. The remaining fasting-induction of SIRT1 expression upon ACREB expression is probably due to residual CREB activity, since a residual induction of other well-known CREB targets, such as PGC1α and PEPCK, was also present upon ACREB expression (Fig 1A and 1B in this rebuttal letter).

Figure 1. PGC1α (A) and PEPCK (B) mRNA abundance in liver from fed or 18h fasted mice infected with adenovirus expressing GFP or A-CREB.

3. Figure 3D. A positive effect of ChREBP/Mlx on the ChoRE of the L-PK gene (for example) should be obtained in parallel as a positive control in this particular experiment. In addition, a rescue experiment of the effect of FSK on the SIRT1 promoter should be performed in a presence of a dominant form of ChREBP.
To satisfy the first concern of the reviewer, we provide now luciferase reporter assays demonstrating that ChREBP/Mlx induces significantly the L-PK promoter activity (Figure 3D). This provides a positive control that certifies the quality of our assay.

The reviewer also suggests that dominant negative forms of ChREBP should be used in our experimental conditions. To address this point, we now show how the overexpression of a dominant negative form of Mlx, which blocks the binding of ChREBP to DNA, is enough to prompt an increase in SIRT1 promoter activity (Figure S4B). These results illustrate how the dimer ChREBP/Mlx acts as a repressor of SIRT1 expression in the basal state. Our conclusion is further supported by the fact that an shRNA against ChREBP significantly increases SIRT1 mRNA abundance (Figure 3C) and that a pharmacological inhibition of ChREBP nuclear translocation recovers FSK induction of SIRT1 expression upon high glucose (Figure 4A).

4. Figure 3E. As a complement of the ChIP experiment performed, the authors should mutate the potential ChoRE/E box presented in Supplemental Figure 4 in order to clearly demonstrate that ChREBP directly binds to this sequence.

We thank the reviewer for this important suggestion. In the revised version we now include new results showing that ChREBP mediated repression of SIRT1 promoter activity was lost in a fragment of the SIRT1 promoter devoid of the ChREBP binding site (-160 bp; Figure S4C).

5. Figure 4A. ChREBP nuclear localization should be visualized in this particular experiment (either using nuclear extracts and/or immunocytochemistry) under all the conditions presented.

We have evaluated the nuclear localization of ChREBP as suggested by the reviewer and the results are now shown in Figure S5. Interestingly, FSK stimulation induced ChREBP nuclear export in low and high glucose conditions. However, nuclear levels of ChREBP remained higher upon high glucose. This remaining ChREBP was probably still enough to compete with CREB explaining the lack of induction of SIRT1 mRNA expression by FSK. Finally, stimulation with cantharidic acid decreased significantly the nuclear levels of ChREBP as previously reported.

Referee #2: This manuscript proposes a new regulatory mechanism for SIRT1 expression in response to energy availability. Noriega, et al. investigated the expression level of SIRT1 upon fasting and refeeding. Upon fasting, SIRT1 protein and mRNA level increased in different mouse tissues. Glucagon upregulated SIRT1 protein and mRNA in liver. Using the HepG2 cell line, they also observed that CREB activation/overexpression upregulated SIRT1 mRNA levels. The promoter region required for the CREB effect was identified in the SIRT1 promoter between nt -316 to -160. Upon refeeding, SIRT1 protein and mRNA decreased in liver. SIRT1 expression was upregulated in ChREBP Knock down cells or ChREBP-/- mouse. Acetylation level of PGC-1a was decreased in ChREBP-/- mouse. ChREBP-mediated repression of SIRT1 promoter could be overcome by increasing amounts of CREB. EMSA study showed both CREB and ChREBP bind to a 25 bp SIRT1 promoter region (-146 to -92). CHIP assay showed that during fasting, CREB recruitment to SIRT1 promoter increased while ChREBP binding to the SIRT1 promoter decreased.

We would like to thank this reviewer for his/her kind words and pertinent comments, which have allowed improving the manuscript. A detailed point-by-point answer to his/her comments is provided below.

- These observations are potentially interesting but assume that all SIRT1 regulation occurs at the transcriptional level. There is growing evidence that SIRT1 expression is also regulated at multiple other levels. The authors should discuss their data in the context of these other regulatory mechanisms.
We thank this reviewer for this appropriate suggestion and would like to apologize if we have given the impression that all regulation of SIRT1 occurs at the level of its transcription. We have enhanced the discussion on the regulation of SIRT1 expression and put it in the context of other avenues of regulation of SIRT1 activity (see page 7).

*The luciferase assays used to assess SIRT1 promoter activity are presented in a confusing manner. First, the author show that a promoter region localized between nt -316 to -160 is important for CREB to activate SIRT1 transcription. Later in the manuscript, the authors identify another region -146 to -92 as the site where CREB and ChREBP compete to regulate SIRT1 expression. What is the relative role of both sites in SIRT1 transcriptional regulation? Also, luciferase assays are required to confirm that the SIRT1 promoter region corresponding to nt -146 to -92 is really important. Selective mutations that abolish binding of either CREB or ChREBP should be generated and their impact on SIRT1 promoter activity should be assessed.*

We apologize for a mistake in Figure S3 that may have led to some confusion and have now corrected this. The depicted region for human SIRT1 promoter where the CREB and ChREBP binding sites overlap corresponds to nucleotides -199 to -175 instead to -146 to -92 as previously shown. The region between -199 and -175 was not present in the -160 bp fragment of SIRT1 promoter and is the one necessary for the CREB mediated activation of SIRT1 promoter. We have now included a schematic representation of SIRT1 promoter in Figure 2E to better illustrate the localization of putative CREB binding sites. In addition, we have performed site-directed mutagenesis of each putative CREB binding sites to evaluate its relevance for CREB mediated regulation of SIRT1 promoter activity. The results demonstrate that the sites located at -175 and -330 bp are both necessary for CREB-mediated activation of SIRT1 promoter. These results are now included in the revised Figure 2F.

*Figure 2E, the difference in luciference activity between D3 and D4 is less than 2 fold when comparing control and CREB overexpression. However, in fig 2B, the luciferase activity of the 1.2 kb promoter is induced 5 fold upon CREB overexpression. Does this mean that there are other unidentified promoter regions important for the activation? The 1.2 kb promoter construct should be included in figure 2E.*

We thank this reviewer for this valid remark. We have now included the 1202 bp promoter construct in Figure 2E. We have also added Figure 2F, which shows the results of site-directed mutagenesis performed in each CREB binding site. These data clearly demonstrate that two sites, located at -175 and -330, are necessary for the CREB mediated activation of SIRT1 promoter. However, we can not exclude the participation of other sites since the activity of SIRT1 promoter construct with mutation of the two mentioned sites was not completely lost. Interestingly, D3 and D4 promoter constructs also show higher basal activity. This is in agreement with the results of Che et al., which described that the region comprised between -1202 and -664 bp is involved in HIC1-mediated repression (Che WY Cell 2005).

*Fig 3A, Expression level of SIRT1 under fed condition should be included. Does SIRT1 expression level return back to the fed condition?*

As suggested by the reviewer, we have evaluated SIRT1 expression in liver of mice in fed, fasted and refed conditions. These results show that, upon refeeding, SIRT1 expression returns to the levels found in fed conditions. We have included these results in Figure S4A.

*Fig 3G, Is PGC-1a also transcriptionally regulated by ChREBP? Are PGC-1a protein levels the same in WT and ChREBP-/- mice.*

A Genomatix analysis of the PGC1α promoter shows a putative binding site for ChREBP, which hints towards a possible role of this transcription factor in the regulation of PGC1α expression. However, a thorough evaluation of a possible direct transcriptional regulation of PGC1α by ChREBP is beyond the scope of this manuscript.
Referee #3: Noriega et al. follows the regulation of SIRT1 levels in response to nutrient availability. More specifically the presented study follows the regulation of SIRT1 transcription levels by CREB & ChCREB under normal and nutrient-deprived conditions. This is a very interesting and important study. They demonstrate that when grown with normal media ChCREB binds to the SIRT1 promoter and inhibits its transcription. In contrast, when nutrients were depleted the authors suggest that ChCREB dissociates and CREB induces SIRT1 levels. This study is a continuance of previous publications by Cohen et al. (2004), Nemoto et al. (2004), Kanfi et al. (2008) and Chen et al. (2008). Whereas the first three studies showed an increased in SIRT1 levels under starvation or calorie restriction-CR the last one does not. Still, the previous publications did not agree about the mechanism underlying the increase in SIRT1. Therefore, this study is an important addition to these publications. Nevertheless, the following experiments should be addressed.

We would like to thank this reviewer for his/her positive comments. A detailed point-by-point answer is provided below.

1- Along the paper the authors avoid showing SIRT1 protein levels. Moreover, in cases they do show western analysis, the quality is poor. These data should be included in the figures, especially given the controversy regarding the increase in SIRT1 levels upon starvation or CR. Especially, figure 2C/D, 3A/F, and 1C should be replaced.

We have now improved the quality of our western blots in Figures 1B, 1C, 3A, 3F and also included the quantification of proteins in these blots to underscore the differences in the two main physiological conditions that we have evaluated (fasting and refeeding). We would, however, like to remind the reviewer that our manuscript deals with the differences in SIRT1 expression during fasting (an acute situation with total absence of food), which is distinct from CR (a chronic situation, with regular access to food, albeit in restricted amounts). However, to further highlight the influence of low energy levels on SIRT1 transcription, we include below a time course of glucose deprivation in HepG2 cells that shows a parallel increase of both SIRT1 mRNA and protein levels. We decided, however, not to include these data in the manuscript, as it may bring more questions, given that it involves a cell autonomous effect, unlike the majority of the data in the paper. The increase in SIRT1 mRNA abundance, however, correlates with the increase in p-CREB, supporting the validity of our conclusion that CREB regulates SIRT1 transcription.
Figure 2. SIRT1 protein (A) and mRNA (B) abundance in HepG2 cells deprived of glucose at time indicated (h).

2- Based on our experience, when we performed metabolic assays, there is a large variability between mice. The authors use low number of mice (n=4). We strongly recommend increasing the amount to 6-8 per group.

We completely agree with the reviewer's remark. We have used 6-8 animals per group and/or repeated the experiments twice in case we used a lower number. This way, we feel confident about the robustness of our results. We have added this statement in the material and methods section.

3- The author should discuss the differences between their findings and Kanfi et al. Could it be tissue/cell line specific?

The difference between our findings and those of Kanfi et al are, as pointed out by the reviewer, probably due to the use of different cell lines. Kanfi et al used mainly HEK293A cells, whereas we used HepG2 cells (Figure 2A, 3C, 4A), HeLa and MEF cells (data not shown), and primary mouse hepatocytes (Figure 1D). In our experience, the effects of glucose restriction or deprivation are cell type-specific. In addition, the time of harvesting the cells could be another issue, as Kanfi et al did not control for SIRT1 levels in pre-treated cells.

In the case of the experiments in mice, Kanfi et al only analyze a 24h fast. Using such approach they lose a time-dependent perspective. In Figure S1A we show how SIRT1 mRNA induction peaks between 12 and 18 hrs after the initiation of the fasting period, but is back to normal at 24 hrs. Therefore, while the study of Kanfi et al provides some interesting information, it lacks some crucial experimental details that prove to be key to understand SIRT1 transcriopional regulation.

4- The Authors should use siRNA against CREB in starved cells, in order to prove that CREB is in charge of the increase in SIRT1 levels.

This is a very valid suggestion by the referee. We have performed this experiment in slightly different way by evaluating SIRT1 mRNA abundance upon glucose deprivation in HepG2 cells overexpressing a dominant negative isoform of CREB (ACREB). The results demonstrate that CREB plays a role in the induction of SIRT1 mRNA abundance upon glucose deprivation. We show the results here for the reviewer’s benefit. As discussed with Reviewer #1 (point 2), there is still an induction of SIRT1, probably due to either residual CREB activity or to the existence of complementary mechanisms regulating CREB activity. In this sense, Suwa et al recently reported that the activation of AMPK pathway can also control SIRT1 expression (Suwa M, Metabolism 2011). Preliminary data from our lab confirm those observations and make AMPK a likely candidate to explain the remaining SIRT1 expression in the ACREB-infected cells upon glucose restriction.
Figure 3. SIRT1 mRNA abundance in HepG2 cells infected with adenovirus expressing GFP or ACREB upon glucose deprivation.

5- It is not clear why the authors follow SIRT1 mRNA levels after 12 hours of starvation and SIRT1 protein levels after 24 hours. Did they follow other time points?

We performed a fasting time course that demonstrates that SIRT1 mRNA abundance is gradually increased until 18h (Figure S1). Interestingly, after 24 hours fasting the levels have returned to basal levels. This goes in line with the observation of Rodgers et al., who did not report a difference in SIRT1 mRNA level at 24h fasting. However, both our results and those from Kanfi and Rodgers et al, clearly indicate that the increase in SIRT1 mRNA levels in animal tissues is consolidated at the protein level at 24 hrs of fasting.

6- The increase in SIRT1 levels in the muscles shown in figure S2 is lower than 20%. Please indicate the p-value for this measurement.

We would like to apologize for have omitted this information. We have now clearly stated in the figure legend that we have used a p-value of < 0.05.

2nd Editorial Decision 14 June 2011

Thank you for the submission of your revised manuscript to our offices. We have now received the enclosed reports from the referees that were asked to assess it and I am glad to say that both reports are very positive. However, your manuscript still has minor suggestions that I would like you to incorporate before we can proceed with the official acceptance of your manuscript.

Referee #3 is satisfied with your answer on the differences between your results and those of Kanfi et al., but he/she feels that this issue should be covered in the main text. As you have substantially reduced the character count of your manuscript, a few sentences can be added to the discussion to address this point.

Browsing through the manuscript myself, I have noticed that the description of the statistical analyses performed is not complete. Please describe the statistical method used to calculate statistical significance in all figures, including supplementary data. Also, no description at all of statistical analyses is provided for figure 4.

I look forward to seeing a new revised version of your manuscript as soon as possible.

Yours sincerely

Editor
EMBO Reports

REFEREE REPORTS:

Referee #1:

The authors have adequately addressed the points raised during the time course of the review. Although the authors have not performed point mutation of the ChoRE site within the SIRT1 promoter as suggested, they have now deleted the sequence and the results are now presented in Supplemental Figure 4. This important result should be included in the Manuscript Figures and not as supplemental data.
Referee #3:

Noriega et al. addressed all of our comments except one and I believe that the manuscript can now be published in EMBO Reports. Our only comment that they did not address in the text, was about the differences between their findings and those of Kanfi et al. Nevertheless, they suggest in their response letter that these differences are due to cell line-specific and the time-points at which the samples are collected. Given the complexity of such experiments, I believe that it would be very useful and important to discuss these issues in the manuscript in a few sentences.

2nd Revision - authors’ response 16 June 2011

Answers to Referees’ comments:

Referee #1: The authors have adequately addressed the points raised during the time course of the review. Although the authors have not performed point mutation of the ChoRE site within the SIRT1 promoter as suggested, they have now deleted the sequence and the results are now presented in Supplemental Figure 4. This important result should be included in the Manuscript Figures and not as supplemental data.

We thank the reviewer for his/her comments and suggestion. We have now included supplemental Figure 4C as panel E in Figure 3.

Referee #3: Noriega et al. addressed all of our comments except one and I believe that the manuscript can now be published in EMBO Reports. Our only comment that they did not address in the text, was about the differences between their findings and those of Kanfi et al. Nevertheless, they suggest in their response letter that these differences are due to cell line-specific and the time-points at which the samples are collected. Given the complexity of such experiments, I believe that it would be very useful and important to discuss these issues in the manuscript in a few sentences.

We thank the reviewer for his/her comments and suggestion. We have now included a few sentences on page 5 where we discuss the differences between our findings and those of Kanfi et al.

3rd Editorial Decision 17 June 2011

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports.

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

Yours sincerely,

Editor
EMBO Reports