THE EMBO JOURNAL

Manuscript EMBO-2009-70662

Bipartite Functions of the CREB Coactivators Selectively Direct Alternative Splicing or Transcriptional Activation

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Review timeline:

Submission date:	17 February 2009
Editorial Decision:	11 March 2009
Revision received:	08 June 2009
Editorial Decision:	23 June 2009
Revision received:	06 July 2009
Accepted:	07 July 2009

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 11 March 2009

Thank you for submitting your manuscript for consideration by The EMBO Journal. It has now been seen by four reviewers, whose comments are attached below. As you will see, all referees find your study generally of interest and are thus in principle supportive of its publication. Nevertheless, their reports also raise a number of more substantive issues, concerning mainly the physiological significance of the reported TORC coactivator function in alternative splicing, as well as the depth of mechanistic insights into how TORCs may achieve such regulation.

In light of the overall interest expressed by the referees, I would thus like to invite you to prepare a revised version of the manuscript, taking into account the various points brought up by the reviewers. I should point out, however, that for such a revision to be successful, it will be essential to address the major concern of referees 1 and 2 with regard to the physiological significance, by at least showing the ability of endogenous (i.e. not overexpressed) TORCs to influence the splicing of endogenous CREB target genes. In addition, there are also a number of editorial issues that should be dealt with before sending us a revised version: this includes an extended introduction providing also background on TORC activators (keeping in mind the broad readership of our journal), streamlining of the results section (e.g. by skipping non-essential parts such as the not-shown TORC2 interactome analysis, page 5), and adjusting the bibliography format according to EMBO J style guidelines. Furthermore, it appears that in some of the gels (e.g. Figure 4B) separate lanes may have been cut and assembled into one panel (presumably from different parts of the same gel?) - this would at least have to be clearly indicated using a separating line and an explanation in the respective figure legends, as well as through the provision of an original data scan as a supplement for the editors (please also see the additional information on digital image processing below and in our authors' instructions).

Should you be able to satisfactorily deal with these main issues, we should be happy to consider a revised manuscript for publication. Please be reminded however that it is EMBO Journal policy to allow a single round of major revision only, and that it is therefore essential to diligently answer to all the points raised at this stage if you wish the manuscript ultimately to be accepted. In any case, please do not hesitate to get back to us should you need feedback on any issue regarding your revision.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely,

Editor
The EMBO Journal

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REFEREE REPORTS:

Referee #1 (Remarks to the Author):

In the paper the authors describe a new role for the TORC co-activators in which overexpression of the proteins can alter splice site selection and enhance splicing of genes containing a functional CRE site. The paper demonstrates that overexpression of TORCs alterned splicing of several minigenes, including luciferase reporters driven by either the CRE inducible EVX1 and CDX4 promoters, which contain an ED1 , an HIV minigene, as well as the Nr4a2 gene. The authors show convincing evidence that the activity of the TORCs on splicing appears to be separate from that mediating transcription based on several observations. First, a region of the TORC coding region is dispensable for transcriptional activation, and second, reporters driven by TATA-less promoters, which are only weakly induced by TORCs, seem similarly affected at the level of splicing by TORCs. Overall the paper is well written and convincing. However, the paper does not present evidence that the enhancement of splicing by TORCs or the exon skipping by TORCs is physiologically relevant. All the data is obtained via overexpression of TORCs. The paper would be greatly enhanced if the authors could demonstrate that any splicing event of an endogenous gene was effected by physiologic levels of TORC proteins. The obvious experiment is to determine if splicing of a CRE-regulated gene is altered by activation of TORCs. Several papers have shown that CRE-mediated gene expression can be blocked in a variety of cell types by inhibition of TORCs through either RNAi or dominant negative proteins. It seems essential to show that the alternate splicing observed after overexpression of TORC proteins can also be observed after physiologic induction of endogenous TORC activity through increased cAMP or caclium signalling. If such an example of splice site regulation could be demonstrated, I would certainly recommend publication in EMBO.

Other comments that should be addressed are listed below.
Synergy between PKA and TORCs and transcriptional activation are claimed. As there is no data with TORC only in figure 2 A and S5, I cannot see how this is claimed and the data are not convincing as the values are ~2 fold different from that in Figure 1A with TORC alone. Thus the additivity could be experimental variability. The authors are probably right but to judge true synergy (or even additivity) TORC only data needs to be obtained and shown within the same experiment as the TORC plus PKA experiments.

Similarly, the authors claim synergy of TORC and PKA with splicing. Again, figures 2B and 2C suffer from having no TORC only data. In this case, comparison to Figure 1 would suggest significant synergy, but having the data within one experiment would allow the authors to say there is synergy with some qualitative or quantitative confidence.

The authors do a careful comparison of the effect of different level of transcripts on splicing to demonstrate that the effect of TORCs are not due to dilution of splicing factors. In Figure 3 they titrate in increasing levels of a minigene without a CRE/TORC response element, and show that

simply raising the level of reporter mRNA in the cell has no effect on splice site selection. However, it appears from the RTPCR data that the absolute level of expression from the non-TORC responsive LTR vector is lower than the TORC-driven promoter. If there is a threshold of expression needed to get enhanced splicing, the conclusion of this experiment could be wrong and that there is indeed a limiting factor which regulates splicing that is diluted out upon very high levels of transcript produced by TORC activation. Here it would be worthwhile to again use a promoter of comparable strength as a control to show that TORCs directly effect splicing rather than effect some crucial level of expression or ratio of expression to plasmid DNA template from the transient transfection experiments.

The authors also show effects of TORC overexpression on an endogenous gene., NR4A2. The authors show that torc overexpression via adenoviral vectors causes a 2 fold shift in Exon 3 and Exon7 splicing compared to a GFP vector or results obtained in a non-neuronal cell (a hepatocyte). This data is hard to interpret, as there are different levels of Nr4a2 transcript in the two cell lines, different levels of induction by TORCs and likely different level of torc proteins in the two cell lines. A more convincing way to ask if TORCs effect splicing would be to induce an endogenous TORC protein via a physiologic stimuli (increasing cAMP for example) and show that splicing ratios are changed in a TORC dependent manner due to endogenous levels of TORC protein.

Data in Figure 5 suggests increased transcription activation is not required for splicing effects. However in this experiment the change in splicing is modest, only 2 fold versus 20 fold for the EVX torc iducible vector shown in Figure 1. Is two fold significant or is this within the level of noise in terms of transcriptional induction by TORCs on the CDC27 promoter. Is there synergy on the CDC37 splicing reporter with PKA?

Further studies with the GAL4 TORCs on TATA+ and TATA- vectors suggested that similar levels of splicing could be induced by TORCs in spite of the observation that induction of expression by GAL4Torc on the non-tata containing promoter driven vector was much weaker than that of the TATA containing promoter. However, again, there still was greater than 10-fold (at least) induction of the non-tata vector in figure S10 and the question, again making it difficult to separate out the effects of TORCs on transcription from those on regulation of splicing. It would be worth noting these caveats in the text. However, other data in the paper, does strongly support a bifuncitonal role of the TORC proteins, particularly demonstrating that TORC deletion mutants or MAML fusion proteins can retain strong transcriptional activation but are defective in altering splice site selection.

Referee #2 (Remarks to the Author):

This study explores the role of TORC coactivators in modulating the alternative splicing of transcripts produced from CREB responsive genes. cAMP and calcium normally control TORC nuclear localization and its ability to coactivate CREB targets. But how TORC affects gene expression has been uncertain in part because it has no known enzymatic activities. It is clear from testing a variety of artificial reporter plasmids in HEK293T cells that TORC overexpression can alter the splicing pattern for some but not all constructs. cAMP-responsive alternative splicing was not tested, but protein kinase-A overexpression alone had a modest (two-fold) effect. The TORC-responsive splicing of one endogenous gene (Nr4a2) was examined in hepatocytes and PC12 cells, and the result suggests that this function of TORC is tissue specific. Overall, the manuscript describes an interesting phenomenon and provides novel insight into the mechanistic roles of TORC as a transcriptional co-regulator, although the physiological significance of TORC-responsive alternative splicing remains uncertain.

Major points:

1. Demonstrating that the alternative splicing of endogenous CREB target genes is affected by 1) elevated levels of cAMP or calcium, and 2) knockout or knockdown of endogenous TORC, would significantly strengthen the study.

2. Another concern is that the transient transfection assays to measure alternative splicing were performed using HEK293T cells. The response of genes to TORC overexpression may be abnormal because these cells express E1A and SV40 large T antigen that bind to the other major coactivators

of CREB, CBP and p300. E1A can have a major effect on CBP/p300, relocalizing them in the genome and causing global changes in gene expression and histone acetylation. (Ferrari et al. Epigenetic reprogramming by adenovirus e1a, Science 2008. Horwitz et al. Adenovirus small e1a alters global patterns of histone modification, Science, 2008).

3. RNAi knockdown of overexpressed TORC isn't terribly informative (Figs. 4C, S8).

Minor point:

4. There is some confusion in the literature because TORC can also refer to Target of Rapamycin Complex. Recently, it appears that the official gene name CRTC is being used in studies about the coactivator, so it would helpful to include that in the title or abstract.

Referee #3 (Remarks to the Author):

The manuscript by Amelio et al. presents a series of experiments with transfected minigenes and endogenous genes to assess the dual roles of the TORC transcriptional co-activators in transcription and alternative splicing. Although the roles of co-activators and promoter sequences on the coupling of transcription with alternative splicing have been described before (Aubouef et al; Cramer et al.) the whole subject of coupling still needs additional research like the one provided in this manuscript to gain insights into its generality and particularities. The authors seem to be aware of this and are very meticulous in recognizing the published literature in the field. The main novelty in this manuscript is the finding that TORC's transcription and splicing activities can be experimentally split and show cell-specificity. In general, the experiments are convincing and the drawn conclusions are accurate. The use of both exon skipping and 3' splice site selection models of alternative splicing strengthen the validity of the results. I have nothing to add to the experiments presented. However I would like to see the following theoretical aspects treated in the Discussion, before acceptance.

1. It seems clear that one can obtain alternative splicing activity in the absence of transcriptional activation. However, binding to promoter is needed for the splicing activity. The authors must discuss and give their opinion on the possible mechanisms to explain why this is so. PGC-1 is an example. Do TORCs act in the same way? Furthermore, the authors should discuss whether they think that the need for promoter binding is still an example of coupling. Otherwise the reader might have the impression that in the absence of transcriptional activity TORC acts as a typical splicing regulatory protein when this is not strictly the case.

2. A closely related subject to discuss is what the authors understand for transcriptional activity. An increase in luciferase activity or of mRNA measured by qPCR certainly reflects transcriptional activation. However, since transcription involves both initiation and elongation and internal elongation speeds are known to regulate alternative splicing, the possibility that promoter-bound TORC influences elongation without affecting total mRNA levels should be taken into account.

Minor points
- I understand that the CDC37 promoter used in Fig. 5 is a TATA-less promoter that contains a CRE site. Is it so? Please clarify.

- The model in Fig. 8 should be omitted. The extranuclear events are vaguely linked to the presented results. The intranuclear events are too sketchy.

Referee #4 (Remarks to the Author):

Regulation of transcription and splicing are two processes that are now recognized to often be tightly coupled. In most studies thus far, this involves transcription factors inducing changes in splicing as a direct consequence of their effect on transcription. In contrast, the authors here provide compelling evidence that the TORC co-activators can induce changes in splicing and transcription, but that these effects are not directly linked.
First the authors show that two splicing minigenes, when expressed from CRE-containing promoters

show TORC-dependent changes in both transcription and splicing. The possibility that changes in splicing are an indirect effect of saturating amounts of transcript is ruled out, and sensitivity of both effects to a dominant negative CREB also supports and active role of TORC in these effects. Interestingly, in one case they observe an cell-specific example in which TORC activates transcription but not splicing, suggesting these are mechanistically uncoupled. Further evidence for uncoupling comes from tethering experiments in which TORC induces changes in splicing without significantly altering transcription. Finally a set of deletion mutants in TORC inhibit splicing effects of TORC without altering transcriptional activity.
Together, these studies provide an intriguing model for a protein that can separately regulate transcription and splicing. While this is interesting on it's own, further mechanistic understanding would strengthen the study. In particular there are several immediate questions raised by the data:
1) What in the splicing process is being enhanced or suppressed by TORC? While the use of two splicing minigenes in the study supports the notion that the effect of TORC is general, the fact that in one case TORC induces exon skipping and in the other TORC enhances overall splicing leave some confusion. Could other exons be tested in the ED1 minigene to look for some pattern to the TORC effect? Also, does the TORC have to associate within a promoter to alter splicing or does it also work if tethered internally to the minigene?
2) Is there some hint how the proline-sequence might interact with the splicing machinery? Can one just tether the 334-434 domain to a promoter and achieve splicing regulation? Are splicing changes observed in the absence of the TA domain - i.e. mutants that are deleted for the activation domain but retain the rest of the protein?
3) Is there any explanation for why some of the effects on splicing are ~5 fold while others are statistically significant but only 1.5-2 fold? Does this mean a component of the effect is independent of transcription but an additional "boost" may arise as a secondary consequence of transcription?

1st Revision - authors' response 08 June 2009

Referee #1 (Remarks to the Author):

In the paper the authors describe a new role for the TORC co-activators in which overexpression of the proteins can alter splice site selection and enhance splicing of genes containing a functional CRE site. The paper demonstrates that overexpression of TORCs altered splicing of several minigenes, including luciferase reporters driven by either the CRE inducible EVX1 and CDX4 promoters, which contain an ED1, an HIV minigene, as well as the Nr4a2 gene. The authors show convincing evidence that the activity of the TORCs on splicing appears to be separate from that mediating transcription based on several observations. First, a region of the TORC coding region is dispensable for transcriptional activation, and second, reporters driven by TATA-less promoters, which are only weakly induced by TORCs, seem similarly affected at the level of splicing by TORCs. Overall the paper is well written and convincing. However, the paper does not present evidence that the enhancement of splicing by TORCs or the exon skipping by TORCs is physiologically relevant. All the data is obtained via overexpression of TORCs. The paper would be greatly enhanced if the authors could demonstrate that any splicing event of an endogenous gene was affected by physiologic levels of TORC proteins. The obvious experiment is to determine if splicing of a CRE-regulated gene is altered by activation of TORCs. Several papers have shown that CRE-mediated gene expression can be blocked in a variety of cell types by inhibition of TORCs through either RNAi or dominant negative proteins. It seems essential to show that the alternate splicing observed after overexpression of TORC proteins can also be observed after physiologic induction of endogenous TORC activity through increased cAMP or calcium signaling. If such an example of splice site regulation could be demonstrated, I would certainly recommend publication in EMBO.

We are in complete agreement with the Reviewer that the paper would be greatly enhanced by demonstrating splicing of an endogenous gene via physiologic levels of TORC (CRTC) proteins. Moreover, the message we would like the readers to takehome from this manuscript is that cAMP signaling by way of the CRTC coactivators can alter the relative amounts of splice products in addition to robustly upregulating

transcriptional activation. The timing of this manuscript is especially important due to the large numbers of ongoing investigations looking at the roles of these dynamic proteins in metabolism and neurobiology. Our hope is that investigators will examine alternative splicing in addition to transcriptional activation when identifying tissue-specific CRTC target promoters. Therefore, we have addressed this concern in what is now Figure 4B by adding an additional experiment to prove that external stimuli that increase intracellular cAMP and calcium levels can mediate alternative splicing of the endogenous Nr4a2 gene (Figure 4B), a characterized CRTC2-responsive gene.

In addition to the modification of Figure 4, the following text has been added to the results: "Physiologic cues that regulate cAMP or both cAMP and calcium signaling pathways have been shown to converge on the CRTCs in non-excitable and excitable cells to regulate CRE-responsive genes (Screaton et al, 2004). Therefore, we analyzed RNA isolated from primary rat hepatocytes or neuronally differentiated PC12 (ND-PC12) cells that were treated with KCl or forskolin (FSK) (Figure 4B). Primary hepatocytes treated with KCl or FSK did not display significant changes in Nr4a2 exon 3 alternative splice site selection despite FSK-induced transcription. In contrast, both KCl-mediated depolarization and FSK-induction treatments promote alternative splice site selection in Nr4a2 exon 3 within ND-PC12 cells, and costimulation with both KCl and FSK further potentiates this shift in alternative splicing."

Other comments that should be addressed are listed below.
Synergy between PKA and TORCs and transcriptional activation are claimed. As there is no data with TORC only in figure 2 A and S5, I cannot see how this is claimed and the data are not convincing as the values are ~2 fold different from that in Figure 1A with TORC alone. Thus the additivity could be experimental variability. The authors are probably right but to judge true synergy (or even additivity) TORC only data needs to be obtained and shown within the same experiment as the TORC plus PKA experiments. Similarly, the authors claim synergy of TORC and PKA with splicing. Again, figures 2B and 2C suffer from having no TORC only data. In this case, comparison to Figure 1 would suggest significant synergy, but having the data within one experiment would allow the authors to say there is synergy with some qualitative or quantitative confidence.

We failed to provide a sound rational or conclusion for our experiments involving PKA. We have added additional text in the introduction to assist the readers with the rational and have also clarified the conclusions in the results section. To summarize the introduction regarding transcriptional activation, PKA performs two roles in response to elevations in cAMP. PKA phosphorylates CREB at serine 133 and indirectly causes the dephosphorylation of CRTC. Dephosphorylation of CRTC allows it to be released by 14:3:3 proteins translocate to the nucleus and binding with CREB at the CRE. We anticipated that if PKA was simply facilitating CRTC translocation very little additional splicing activity would be observed while overexpressing CRTCs. However, there is quite a substantial increase in the splicing suggesting that PKA has an effect in addition to regulating the subcellular localization of CRTC. We are unsure how PKA facilitates CRTC-mediated splicing, but it is likely having an additional role. A report in 2003 by Doug Black's laboratory demonstrated that PKA could directly phosphorylate polypyrimidine tract-binding protein (PTB); however, the effect of PKA stimulation on PTB function was not clear. That manuscript goes on to suggest that there are likely other splicing proteins that are PKA targets and we concur with that notion. Our data would suggest that CRTC is a prerequisite for PKA-mediated function. We have stricken the term synergy and replaced it with robustly. Moreover, we have amended the text to indicate that PKA is having an effect on splicing that is independent of CRTC. To hopefully provide some clarification to the future readers we have added the following statement to the discussion, "Moreover, PKA may contribute to TORC-dependent alternative splicing through phosphorylation of components within the recruited splicing complex (Xie et al., 2003)."

The authors do a careful comparison of the effect of different level of transcripts on splicing to

demonstrate that the effect of TORCs are not due to dilution of splicing factors. In Figure 3 they titrate in increasing levels of a minigene without a CRE/TORC response element, and show that simply raising the level of reporter mRNA in the cell has no effect on splice site selection. However, it appears from the RTPCR data that the absolute level of expression from the non-TORC responsive LTR vector is lower than the TORC-driven promoter. If there is a threshold of expression needed to get enhanced splicing, the conclusion of this experiment could be wrong and that there is indeed a limiting factor which regulates splicing that is diluted out upon very high levels of transcript produced by TORC activation. Here it would be worthwhile to again use a promoter of comparable strength as a control to show that TORCs directly effect splicing rather than effect some crucial level of expression or ratio of expression to plasmid DNA template from the transient transfection experiments.

We apologize for the confusion regarding this figure, but each blot was exposed for different times to obtain a figure where all bands were visible without saturation of the film. The absolute levels of expression were considerably higher from the non-TORC responsive LTR promoter compared to the EVX1 promoter. Due to the discrepancies in exposure time we demonstrated the relative strength of each promoter as determined by qPCR in supplemental figure 7 panel A.

The authors also show effects of TORC overexpression on an endogenous gene, NR4A2. The authors show that torc overexpression via adenoviral vectors causes a 2 fold shift in Exon 3 and Exon7 splicing compared to a GFP vector or results obtained in a non-neuronal cell (a hepatocyte). This data is hard to interpret, as there are different levels of Nr4a2 transcript in the two cell lines, different levels of induction by TORCs and likely different level of torc proteins in the two cell lines. A more convincing way to ask if TORCs effect splicing would be to induce an endogenous TORC protein via a physiologic stimuli (increasing cAMP for example) and show that splicing ratios are changed in a TORC dependent manner due to endogenous levels of TORC protein.

We are in complete agreement with the Reviewer that the paper would be greatly enhanced by demonstrating splicing of an endogenous gene via physiologic levels of TORC (CRTC) proteins. To address this concern, which was shared by Reviewer #1, we have added an additional experiment to prove that stimuli affecting cAMP and calcium signaling can mediate alternative splicing of the endogenous Nr4a2 gene (Figure 4B).

In addition to the modification of Figure 4, the following text has been to the results, "Physiologic cues that regulate cAMP or both cAMP and calcium signaling pathways have been shown to converge on the CRTCs in non-excitable and excitable cells to regulate CRE-responsive genes (Screaton et al, 2004). Therefore, we analyzed RNA isolated from primary rat hepatocytes or neuronally differentiated PC12 (ND-PC12) cells that were treated with KCl or forskolin (FSK) (Figure 4B). Primary hepatocytes treated with KCl or FSK did not display significant changes in Nr4a2 exon 3 alternative splice site selection despite FSK-induced transcription. In contrast, both KCl-mediated depolarization and FSK-induction treatments promote alternative splice site selection in Nr4a2 exon 3 within ND-PC12 cells, and costimulation with both KCl and FSK further potentiates this shift in alternative splicing."

Data in Figure 5 suggests increased transcription activation is not required for splicing effects. However in this experiment the change in splicing is modest, only 2 fold versus 20 fold for the EVX torc inducible vector shown in Figure 1. Is two fold significant or is this within the level of noise in terms of transcriptional induction by TORCs on the CDC27 promoter. Is there synergy on the CDC37 splicing reporter with PKA?

The reviewer brings up an important point regarding the CDC37 promoter that we failed to highlight. The CDC37 promoter is a CREB Response Element (CRE)-containing TATA-less promoter that possesses a basal level of transcriptional activity. However, it is important to note that this basal transcription remains unaltered by TORCs. Furthermore, this lack of transcriptional induction in the presence of TORCs has been

previously documented (Conkright et al. Mol. Cell 2003). Therefore, the observed 2-fold induction of EDI skipping is significant as there is no transcriptional induction. Furthermore, PKA also promotes EDI skipping independent of transcriptional induction and PKA plus TORCs further potentiates this EDI skipping affect. These data have been added to Figure 5 along with clarification to the Results section.

Further studies with the GAL4 TORCs on TATA+ and TATA- vectors suggested that similar levels of splicing could be induced by TORCs in spite of the observation that induction of expression by GAL4Torc on the non-tata containing promoter driven vector was much weaker than that of the TATA containing promoter. However, again, there still was greater than 10-fold (at least) induction of the non-tata vector in figure S10 and the question, again making it difficult to separate out the effects of TORCs on transcription from those on regulation of splicing. It would be worth noting these caveats in the text. However, other data in the paper, does strongly support a bifuncitonal role of the TORC proteins, particularly demonstrating that TORC deletion mutants or MAML fusion proteins can retain strong transcriptional activation but are defective in altering splice site selection.

The Reviewer makes a valid point regarding our GAL4 data as there is clearly an induction of the non-TATA vector in figure s10. However, the data does clearly demonstrate that there is not a linear relationship between transcription and splicing. Therefore, in agreement with the reviewer, we have noted these caveats in the text by changing the following conclusion, "Collectively, these data demonstrate that the ability of the TORC coactivators to regulate pre-mRNA processing is not solely coupled with increases in transcriptional activity." to, "Collectively, these data demonstrate that the ability of the CRTC coactivators to regulate pre-mRNA processing is not a linear relationship with increases in transcriptional activity suggesting these events may not be mechanistically linked." We are unsure, but speculate that the ability of TORC to induce some transcriptional activity might be the result of what could be a weak TATA box present in the GAL4 INR promoter (Emami et al., 1995).

Referee #2 (Remarks to the Author):

This study explores the role of TORC coactivators in modulating the alternative splicing of transcripts produced from CREB responsive genes. cAMP and calcium normally control TORC nuclear localization and its ability to coactivate CREB targets. But how TORC affects gene expression has been uncertain in part because it has no known enzymatic activities. It is clear from testing a variety of artificial reporter plasmids in HEK293T cells that TORC overexpression can alter the splicing pattern for some but not all constructs. cAMP-responsive alternative splicing was not tested, but protein kinase-A overexpression alone had a modest (two-fold) effect. The TORC-responsive splicing of one endogenous gene (Nr4a2) was examined in hepatocytes and PC12 cells, and the result suggests that this function of TORC is tissue specific. Overall, the manuscript describes an interesting phenomenon and provides novel insight into the mechanistic roles of TORC as a transcriptional co-regulator, although the physiological significance of TORC-responsive alternative splicing remains uncertain.

Major points:
1. Demonstrating that the alternative splicing of endogenous CREB target genes is affected by 1) elevated levels of cAMP or calcium, and 2) knockout or knockdown of endogenous TORC, would significantly strengthen the study.

We are in complete agreement with the Reviewer that the paper would be greatly enhanced by demonstrating splicing of an endogenous gene via physiologic levels of CRTC proteins. To address this concern, which was shared by Reviewer #1, we have added an additional experiments to prove that stimuli affecting cAMP and calcium signaling can mediate alternative splicing of the endogenous Nr4a2 gene (Figure 4B).

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Similar to previous findings, knockdown of CRTC ablates virtually all induction of gene expression from cAMP-responsive targets. This is likely due to the fact that CRTCs are rate-limiting components of the cAMP signaling pathway.

2. Another concern is that the transient transfection assays to measure alternative splicing were performed using HEK293T cells. The response of genes to TORC overexpression may be abnormal because these cells express E1A and SV40 large T antigen that bind to the other major coactivators of CREB, CBP and p300. E1A can have a major effect on CBP/p300, relocalizing them in the genome and causing global changes in gene expression and histone acetylation. (Ferrari et al. Epigenetic reprogramming by adenovirus e1a, Science 2008. Horwitz et al. Adenovirus small e1a alters global patterns of histone modification, Science, 2008).

We thank the reviewer for this important point, which we failed to adequately address in the original manuscript. Several additional cell lines that do not contain SV40 large Tantigen or adenovirus E1A were tested using the EVX1 ED I splicing minigene reporter and these data are reported Supplemental Figure S3B. We also added the following text amendment to the results section, "The affects of CRTC on alternative splicing were also observed in cell lines that do not express SV40 large T antigen or adenovirus E1A (Supplemental Figure S3B)." Interestingly, on the same promoter/minigene configuration the robustness of CRTC1 to alter exon inclusion varied significantly depending on the cell line tested.
In addition, data presented in supplement figure S10 A-F, demonstrates that neither CBP overexpression or a mutation in CREB (S133A) that block CBP/p300 recruitment alter CRTC-mediated splice site selection.

3. RNAi knockdown of overexpressed TORC isn't terribly informative (Figs. 4C, S8).

We agree with the Reviewer and the data in Figure 4C has been moved to Supplemental Figure 8.

Minor point:

4. There is some confusion in the literature because TORC can also refer to Target of Rapamycin Complex. Recently, it appears that the official gene name CRTC is being used in studies about the coactivator, so it would helpful to include that in the title or abstract.

We concur with the reviewer's suggestion have decided to begin using the gene symbol for this publication and all subsequent publications. Please note that we have changed the manuscript title and text to reflect the official gene symbol for TORCs (CREB Regulated Transcription Coactivator; CRTC).

Referee #3 (Remarks to the Author):
The manuscript by Amelio et al. presents a series of experiments with transfected minigenes and endogenous genes to assess the dual roles of the TORC transcriptional co-activators in transcription and alternative splicing. Although the roles of co-activators and promoter sequences

on the coupling of transcription with alternative splicing have been described before (Aubouef et al; Cramer et al.) the whole subject of coupling still needs additional research like the one provided in this manuscript to gain insights into its generality and particularities. The authors seem to be aware of this and are very meticulous in recognizing the published literature in the field. The main novelty in this manuscript is the finding that TORC's transcription and splicing activities can be experimentally split and show cellspecificity. In general, the experiments are convincing and the drawn conclusions are accurate. The use of both exon skipping and 3' splice site selection models of alternative splicing strengthen the validity of the results. I have nothing to add to the experiments presented. However I would like to see the following theoretical aspects treated in the Discussion, before acceptance.

1. It seems clear that one can obtain alternative splicing activity in the absence of transcriptional activation. However, binding to promoter is needed for the splicing activity. The authors must discuss and give their opinion on the possible mechanisms to explain why this is so. PGC-1 is an example. Do TORCs act in the same way? Furthermore, the authors should discuss whether they think that the need for promoter binding is still an example of coupling. Otherwise the reader might have the impression that in the absence of transcriptional activity TORC acts as a typical splicing regulatory protein when this is not strictly the case.

In contrast to PGC-1, the CRTC coactivators lack any conserved, definable RNAbinding domain; therefore, we hypothesize that CRTCs unlike PGC-1 provide a scaffold for the assembly of a larger complex of proteins that may directly bind the transcript. In addition to directly binding RNA, PGC-1 has been shown to vacate promoter occupancy and process with the polymerase. We have very preliminary data that suggesting that the CRTC2 coactivators may function in a similar manner, but unfortunately it just far too preliminary for us to feel comfortable proposing this mechanism.
Regarding coupling transcription and the impression that in the absence of transcriptional activity the CRTC coactivators may function as a typical splicing protein, the reviewer raises very valid point. Although we do not believe this to be true, the reviewer is correct that we did not adequately address transcriptional activity in the discussion. We made the following amendment to the discussion, "Thus CRTCs function as integrators of extracellular signals to influence transcript diversity by acting either as a conduit between components of the transcription and splicing machineries or as autonomous regulators of each process (Figure 8). However, while TORCs can selectively activate splicing in some promoter contexts without activating transcription, these promoters possess basal transcriptional activity; therefore, the effects of the TORCs on pre-mRNA splicing could still formally be considered co-transcriptional despite the absence of coactivator-induced transcription."

2. A closely related subject to discuss is what the authors understand for transcriptional activity. An increase in luciferase activity or of mRNA measured by qPCR certainly reflects transcriptional activation. However, since transcription involves both initiation and elongation and internal elongation speeds are known to regulate alternative splicing, the possibility that promoter-bound TORC influences elongation without affecting total mRNA levels should be taken into account.

The reviewers point is well taken. Our simplistic measurements of quantitative PCR and luciferase do not take into account for effects that CRTC coactivators may have on elongation rates. Perhaps subsequent follow-up studies utilizing RNA polymerase with mutations in the c-terminal domain will allow us to access if the CRTC coactivators ability to alter splice site selection is dependent on elongation rates. However, at this time what we would really like to accomplish is to disseminate our findings to the many investigators that are investigating the biological role of the CRTC2.

Minor points
- I understand that the CDC37 promoter used in Fig. 5 is a TATA-less promoter that contains a CRE site. Is it so? Please clarify.

We thank the Reviewer for noting this and apologize for the confusion. We have changed the manuscript text to clarify that the CDC37 promoter is indeed a TATA-less

CRE-containing promoter.

The model in Fig. 8 should be omitted. The extranuclear events are vaguely linked to the presented results. The intranuclear events are too sketchy.

Given the additional data presented herein regarding the alternative splicing mediated by physiologic cues, we feel that this model figure provides a concise overview of the study findings. However, we are indifferent as to whether the figure remains in the manuscript and will defer to the editor on whether to include or exclude this model.

Referee #4 (Remarks to the Author):

Regulation of transcription and splicing are two processes that are now recognized to often be tightly coupled. In most studies thus far, this involves transcription factors inducing changes in splicing as a direct consequence of their effect on transcription. In contrast, the authors here provide compelling evidence that the TORC co-activators can induce changes in splicing and transcription, but that these effects are not directly linked. First the authors show that two splicing minigenes, when expressed from CRE-containing promoters show TORC-dependent changes in both transcription and splicing. The possibility that changes in splicing are an indirect effect of saturating amounts of transcript is ruled out, and sensitivity of both effects to a dominant negative CREB also supports and active role of TORC in these effects. Interestingly, in one case they observe an cell-specific example in which TORC activates transcription but not splicing, suggesting these are mechanistically uncoupled. Further evidence for uncoupling comes from tethering experiments in which TORC induces changes in splicing without significantly altering transcription. Finally a set of deletion mutants in TORC inhibit splicing effects of TORC without altering transcriptional activity. Together, these studies provide an intriguing model for a protein that can separately regulate transcription and splicing. While this is interesting on it's own, further mechanistic understanding would strengthen the study.

In particular there are several immediate questions raised by the data:

1) What in the splicing process is being enhanced or suppressed by TORC? While the use of two splicing minigenes in the study supports the notion that the effect of TORC is general, the fact that in one case TORC induces exon skipping and in the other TORC enhances overall splicing leave some confusion. Could other exons be tested in the ED1 minigene to look for some pattern to the TORC effect? Also, does the TORC have to associate within a promoter to alter splicing or does it also work if tethered internally to the minigene?

Our data indicates that CRTC2 must be directed to the promoter by some mechanism, whether by CREB or as a GAL4 fusion. We have not done an extensive survey to determine if TORC associated with regions outside of the promoter can still alter splicing. Several careful studies have previously demonstrated that functional CRE element are found proximal to the transcriptional initiation site and changing the location can have devastating effects on transcriptional regulation. We would speculate that similar results would be observed for splicing; however, with our limited resources we have not pursued these studies.

2) Is there some hint how the proline-sequence might interact with the splicing machinery? Can one just tether the 334-434 domain to a promoter and achieve splicing regulation? Are splicing changes observed in the absence of the TA domain - i.e. mutants that are deleted for the activation domain but retain the rest of the protein?

Within the proline rich region, there are a few motifs that are conserved between all three family members and provide a hint of what might be the critical docking sites. We have initiated a study to systematically examine this region by alanine scanning. We will then creation of point mutants by site directed mutagenesis to determine the critical residues. By defining the critical residues and creating point mutants we are confident we will be able to identify specifically the component of the

splicing machinery that interacts directly with CRTC2 by LC-MS/MS. These studies are quite involved and we hope to have the results ready for review by this upcoming winter or spring to submit for review.

3) Is there any explanation for why some of the effects on splicing are ~5 fold while others are statistically significant but only 1.5-2 fold? Does this mean a component of the effect is independent of transcription but an additional "boost" may arise as a secondary consequence of transcription?

We have little insight as to why the relative robustness on splice varies between promoters. We see a similar phenomenon on gene transcription in primary hepatocytes where CRTC2 will induce the Agxt1 gene 39.6 fold, PGC-1 11.8 fold and the Cpt1a 4.0 fold (data unpublished). These genes are very important for metabolic function in the liver but induced at different levels. Moreover, there are significantly more relative transcripts of Nr4A2 in hepatocytes compared to ND-PC12 cells after induction with cAMP and/or CRTC2, yet no boost in splicing occurs in this cell type.

2nd Editorial Decision 23 June 2009

Thank you for submitting your revised manuscript. It has now been seen once more by the original referee 2, and I am happy to inform you that s/he considers the major concerns satisfactorily addressed, especially the main issue of splicing regulation of an endogenous target through physiological stimuli acting through CRTC2 that both referee 1 and 2 had raised. We should therefore be able to proceed with publication of your study after a few remaining minor issues have been additionally addressed (see referee 2's comments below). With regard to the outstanding editorial issues: first, I think it would be best to include the original blots you provided in the covering letter as part of the regular supplementary material, only briefly denoting that these are the original data connected to Fig 4C, and putting a brief note to that effect also into the legend for Fig 4C. Concerning the model in Fig 8, I have no problem with leaving it in the paper for the purpose of a broader view, even though I agree that this type of figure may appear more typical for a (mini-) review on the subject. Finally, in the reference list there are still several occasions of journal names not properly abbreviated, and on one instance (Lander et al) the author list would need to be truncated by 'et al' after the 20th name.

I would thus like to ask you to change the manuscript accordingly in a last round of revision, and to return the paper to us as soon as possible. Once we will have received this final version, we should then be able to swiftly proceed with the acceptance of your paper.

I am looking forward to receiving your final version.

With best regards,

Editor
The EMBO Journal

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REFEREE REPORTS:

Referee #2 (Remarks to the Author):

The authors have adequately addressed my major concerns.

Fig. 4B now shows that adenovirus CRTC2 shRNA causes about a four-fold reduction in Nr4a2 expression and reduces by about one half the ratio of the two splice mRNA variants in ND-PC12 cells. As the authors note this suggests that CRTC2 contributes to alternative splicing but other splicing factors may also respond to cAMP and calcium signaling.

A western blot showing that endogenous CRTC2 protein is reduced by the shRNA in ND-PC12 cells would be helpful.

Sometimes it isn't clear in the manuscript when CRTC is being used as a generic term or in reference to a specific member of the family.

2nd Revision - authors' response 06 July 2009

Please find enclosed our revised manuscript "Independent Bipartite Functions of the CREB Coactivators Direct Alternative Splicing and Transcriptional Activation" for consideration for publication in EMBO J.

We have performed the requested western blot demonstrating that the CRTC2 shRNA molecule in ND-PC12 cells reduces endogenous CRTC2 protein and present this data in Supplemental Figure S8A. In compliance with your request, we have also provided the original data scans for the data presented in Figure 4C and present this data in Supplemental Figure S8B. Moreover, we have addressed the editorial issues concerning the bibliography format as you requested.

