Anti-proliferative protein Tob negatively regulates CPEB3 target by recruiting Caf1 deadenylase

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Transaction Report:

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

1st Editorial Decision 28 May 2010

Thank you for submitting your manuscript to the EMBO Journal. Your study has now been seen by three referees and their comments to the authors are provided below.

As you can see while the referees find the analysis potentially interesting, they also bring up many different issues that have to be addressed in order to consider publication in the EMBO Journal. One is that the analysis relies too much on overexpression and reporter analysis and that you need to provide better support for the conclusions drawn using loss of function data, looking at endogenous target genes and to provide further support for that the endogenous CPEB3/Tob/Caf1 complex forms. Also, there are a number of technical concerns raised and a better presentation of the some of the data, in particular the western blots, is needed in order to properly evaluate the findings reported. A lot of work is needed to consider publication here, but should you be able to address these criticisms in full, we could consider a revised manuscript. I should remind you that it is EMBO Journal policy to allow a single round of revision only and that, therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript. When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process initiative, please visit our website: http://www.nature.com/emboj/about/process.html

Thank you for the opportunity to consider your work for publication. I look forward to your
In this manuscript, the authors report that the antiproliferative Tob protein interacts directly with the cytoplasmic polyadenylation element-binding proteins CPEB3 and CPEB4. They further map the regions of interaction both on Tob and CPEB3 proteins and they show that Tob bridges interactions between Caf1 deadenylase and CPEB3. To study the functional significance of these interactions, they perform transient transfection experiments with reporters. They show that overexpression of CPEB3 regulates the translation and the stability of reporters encoding target mRNA. By using a tethering approach, they further show that CPEB3 binding to a reporter mRNA activates its deadenylation and that this effect is mediated through Tob and Caf1 proteins.

The physical interaction between Tob and CPEB3/4 proteins reported in this manuscript is novel. This finding is of importance and brings valuable information for future studies of Tob physiological functions. However, some data reported in this manuscript are of poor quality and this diminishes the overall impact of the manuscript.

Specific points:
1 - The northerns shown on figure 5 and 6 are of poor quality. They should be improved. Particularly,
   - From figure 5E, the authors state that expression of CPEB3 alone has no effect on the deadenylation of the mRNA. However, comparison of lanes 13 and 14 with lanes 3 and 4 of figure 5E suggests the opposite that is that CPEB3 has a general effect on deadenylation also not on target mRNA. To rule out this possibility, control mRNAs with mutated MS2-binding sites should be included.
   - Figure 6C shows that in the pulse-chase analysis, even at time 0, the MS2 bs mRNA is deadenylated when coexpressed with MS2-HA-Tob. This could result from a problem in mRNA maturation. This figure is not necessary and can be removed, as it does not bring information regarding CPEB3 function. Indeed, Tob can be linked to mRNAs via its interaction with PABPC1.
2 - Figure 1G suggests that Tob concentration is limiting in Cos7 cells. To strengthen the manuscript, data showing the expression of Tob in T-Rex Hela cells could be included as well as RNAi experiments targeting Tob to further demonstrate its implication in CPEB3 effect on deadenylation. In this vein, it would also be nice to analyze the expression and binding of Tob2 to CPEB3.
3 - The discussion concerning Tob's function in learning and memory page 15 is too long. By contrast a discussion concerning the dual role of Tob in deadenylation (a general role via its interaction with PABPC1 and a specific role via its interaction with CPEB3) is missing. How do the authors reconcile these 2 functions? Also a discussion referring to the function of another deadenylase, PARN, in CPEB-mediated deadenylation is missing.

Minor comments:
4 - As the proteins are expressed everywhere in the cells, the colocalisation data shown in figure 1A are not informative. This panel can be removed.
5 - It would be nice to number the lanes in figures 1A, 1B and 1G as in the figure legends.
6 - In figures 2B and 3B, the length of the wt proteins should be indicated.
7 - In figure 5A legend, it should be mentioned that the precise sites of CPEB3 binding to the GluR2 3'UTR are not known.
8 - In figure 6E, what is lane 21? Also, how do the authors determine the A0 line?
9 - The reference Theis et al 2003 is missing in the references list.
Referee #2 (Remarks to the Author):

In this manuscript Hoshino and co-workers continue their work on Tob-mediated mRNA decay. The authors identify an interesting interaction between Tob and CPEB3 and pursue a number of experiments to determine the interaction domains and identify the functional relevance of the interaction to Caf1-mediated RNA decay via a variety of over-expression and tethering experiments using reporter constructs. Overall I find this to be a potentially very interesting study. I do, however, have several points that should be addressed to both polish the study and provide more convincing data for several key conclusions:

1. An interaction with CPEB4 is presented in Fig. 1D but is not further pursued in the study. Perhaps several experiments can be done to address the functional relevance of this interaction or at least the implications of this additional interaction should be discussed.

2. Fig. 2/3: Were the interactions detected in these pull downs sensitive to RNase? The requirement for CPEB3 RNA binding domains for the interaction with Tob raises the potential issue of RNA bridging.

3. Fig. 4 - While the data on the decrease in reporter protein and RNA expression are well documented, the authors should probably assess RNA decay directly to solidify their conclusion that altered RNA stability is playing a role here.

4. Figs 4D and 5B: A normalization lane of some sort (e.g. actin) should be added to these figures to validate the decrease in total reporter RNA level seen with the GluR2 3' UTR.

5. Fig. 5: There are several issues with the way the data are presented in this set of figures.
   a. 5C - needs an A0 marker lane (e.g. oligo dT RNase H treated sample) to validate the authors interpretation of poly(A) tail length.
   b. 5C - The half life reported on pg. 11 does not seem to correlate with the data that are presented. No apparent change in RNA levels in the (-) lanes occurs until 6 hrs, yet a 2.6hr half life is reported in the text for these samples. Maybe a shorter exposure of the gel would suffice.
   c. 5E - as with Fig. 5C, this panel also requires an A0 marker lane and the T1/2 reported on pg. 11 are not at all obvious from the data that are presented. In fact there appears to be more RNA at 3hrs post shut off than at time 0, yet a 2.5 half life is reported.

6. Fig. 6: The 'partial repression' by the Tob deletion variants is not very clear from the data that are presented. Perhaps a quantitative presentation of the data would address this concern.

Minor Points:

1. Fig. 1G is not discussed in the text until after Fig. 3. Thus I would suggest moving it to ~Fig. 4A (or perhaps a separate Fig.) to improve the flow of the manuscript.

2. Fig. 1E: for the benefit of the general EMBO readership, please clearly indicate in the legend what the asterisks refer to.

3. Fig. 1F/Pg. 7: Since this localization study was done with over-expressed tagged proteins and not endogenous proteins, this limits the significance of the conclusions that can be drawn. Thus the text describing this experiment should be toned down accordingly.

Referee #3 (Remarks to the Author):

The work by Hosoda et all constitutes a timely report linking two relevant topics, the role/mechanism of Tob/caf1 in mRNA-specific deadenylation and the translational regulation by a recently discovered member of the CPEB family of proteins (CPEB3), till now scarcely studied. This work shows that CPEB3 mediates the mRNA specific recruitment of the Tob/caf1 deadenylation complex through the direct interaction with Tob. However, and although the proposed model is certainly appealing and biologically relevant, this work contains a number of technical problems that should be addressed before the validity of the model can be assessed. In addition to the specific points raised below, there are two major general issues that affect the conclusions of the work.

The first one is that there is not a single piece of evidence for the endogenous protein-interactions or target mRNAs regulation. The whole study is based in over-expressed proteins and reporter mRNAs. There is no characterization of whether CPEB3/4 is present in the cells used for the study, and that may change the interpretation of some experiments. Some evidence for the existence of the CPEB3/Tob/Caf1 endogenous complex should be presented. Some evidence of the effect of knocking-down CPEB3 in the deadenylation of endogenous mRNAs would also be required. This
point is even more critical taking into account that overexpressed CPEBs induced severe stress to the cells and do not localize like the endogenous.
The second is that, in general, the western blots are over-contrasted and, in many cases, lanes appear to have been cut and pasted together (in some cases, like fig 1A and 1G, etc this cutting/pasting affects the negative controls). Less contrasted westerns and uncut gels would be required for the reader to judge the results.

Specific points
1- Figure 1F: Given that overexpressed CPEB3 and overexpressed Tob localize in a diffuse manner over the whole cytoplasm is not possible to derive any conclusion as to their co-localization.
2- Figure 3A: Mapping of the CPEB3 domain that interacts with Tob is based on the GST pull down of the 417-685 fragment. But the mobility of this fragment is significantly different in the input and the pull-down. Are the authors sure it is really the 417-685 fragment?
3- If Tob is the limiting factor (page 9/fig 1G), and giving its role in transcription, which is the meaning of the CPEB3-overexpression phenotypes? Is it just squelching Tob from its different endogenous functions? How many folds is CPEB3 overexpressed over the endogenous protein (this would be required to interpret the dose-response of CPEB3 overexpression in reporter mRNA levels and deadenylation)? Depletion of the endogenous CPEB3 would be more informative.
4- Figure 4A-C how are these results different from what was shown in Huang et al. 2006? Do Cos7 cells contain CPEB3? which are the levels of overexpression? Fig 4D-E, which are the differences between the short and long 3'UTRs? Do both contain the CPEB3 binding site?
5- Figures 4, 5 and 6, transfection/transcription/loading controls would be required for the northerns. Ideally, the reporters with the control and regulated 3'UTRs should be cotransfected and differentially identified in the same gel. Figures 5C,E; 6A, E. The differences in the mRNA quantities at time 0, makes difficult to assess possible differences in the degradation rates of the mRNAs.
6- Figure 6A. A control where the dominant negative (Caf1 D161A) is overexpressed in the absence of CPEB3 would be required to address whether the effect is CPEB3 specific or not.
7- Figure 6C, tethering Tob just confirms the already known function of Tob in mRNA deadenylation, does not support any function in CPEB3-mediated mRNA-specific deadenylation.
8- Figure 6E. A control where the dominant negative Tobs (1-160 and 110-218) are overexpressed in the absence of CPEB3 would be required to address whether the effect is CPEB3 specific or not.

Minor points
1- If Orb2 is equivalent to CPEB2, 3 and 4, why limiting the study to CPEB3? Would the results be comparable for CPEB2 and 4, as suggested by the pull-downs from figure 1?
2- The figure legends should be more detailed to properly follow/interpret the depicted results.

1st Revision - authors’ response 11 September 2010

Referee #1:

We thank the reviewer for the constructive comments, valuable suggestions to strengthen our conclusions and positive evaluation of our work.

1) “The northerns shown on figure 5 and 6 are of poor quality. They should be improved.”

According to the reviewer’s suggestion, we have repeated the same experiments in Figs 5 and 6 to improve their quality, and replaced all of the data shown in the Figs 5 and 6 to new ones of high quality. The figures are included as Figs 6 and 7 in our revised manuscript.

“Particularly, - From figure 5E, the authors state that expression of CPEB3 alone has no effect on the deadenylation of the mRNA. However, comparison of lanes 13 and 14 with lanes 3 and 4 of figure 5E suggests the opposite that is that CPEB3 has a general effect on deadenylation also not on target mRNA.”

We think the apparent (non specific) effect of CPEB3 on non-target mRNA was due to the excess amount of CPEB3 used in the experiment. Therefore, we have performed the experiment with
reduced amount of CPEB3 expression vector. The data are shown as Fig. 6F in this new manuscript. In this experimental condition, where CPEB3 alone had no effect on non-target mRNA (compare lanes 1-5 with lanes 11-15), a comparable amount of MS2-CPEB3 (Figure 6G) still had marked effect on deadenylation of the target mRNA (lanes 6-10). These results support our conclusion that CPEB3 is able to accelerate deadenylation of mRNA when tethered to the mRNA, whereas CPEB3 alone has no effect on the deadenylation or decay of non-target mRNA.

“Figure 6C shows that in the pulse-chase analysis, even at time 0, the MS2 bs mRNA is deadenylated when coexpressed with MS2-HA-Tob. This could result from a problem in mRNA maturation. This figure is not necessary and can be removed, as it does not bring information regarding CPEB3 function. Indeed, Tob can be linked to mRNAs via its interaction with PABPC1.”

As it was suggested by the reviewer, we have removed this figure.

2) “Figure 1G suggests that Tob concentration is limiting in Cos7 cells. To strengthen the manuscript, data showing the expression of Tob in T-Rex HeLa cells could be included as well as RNAi experiments targeting Tob to further demonstrate its implication in CPEB3 effect on deadenylation. In this vein, it would also be nice to analyze the expression and binding of Tob2 to CPEB3.”

We appreciate this comment very much. According to the reviewer’s suggestion, we have examined the expression of Tob and Tob2 in T-Rex-HeLa cells by using semi-quantitative RT-PCR and the ability to bind CPEB3 by immunoprecipitation. As shown in Supplementary Figure S3B and S3D, Tob2 is also expressed in T-Rex-HeLa cells and is able to bind CPEB3. Therefore, we have newly performed RNAi experiments with siRNAs targeting both Tob and Tob2. T-Rex-HeLa cells were co-transfected with β-globin reporter gene appended with MS2 binding sites in the 3′UTR, a plasmid expressing MS2-CPEB3 and either Tob/Tob2 siRNA or its control siRNA, and transcripational pulse-chase analysis was performed. The results are added as Supplementary Fig. S3 in our revised manuscript. Tob/Tob2 siRNAs reduced the level of both Tob and Tob2 mRNAs by ~20% and ~30%, respectively (Supplementary Figure S3B). Comparable reduction was also observed in the amount of Tob protein (Supplementary Figure S3C). In a control experiment without MS2-CPEB3, knocking down Tob/Tob2 reduced the rate of deadenylation of the reporter mRNA mainly at the second phase (Supplementary Figure S3A, lanes 11-15). In the presence of MS2-CPEB3, knocking down Tob/Tob2 also led to the suppression of CPEB3-accelerated deadenylation of the reporter mRNA (Supplementary Figure S3A, compare lanes 6-10 with lanes 16-20). These results are consistent with the results obtained with dominant negative mutants of Tob and strengthen our conclusion that Tob mediates CPEB3-accelerated deadenylation.

3) “The discussion concerning Tob’s function in learning and memory page 15 is too long. By contrast a discussion concerning the dual role of Tob in deadenylation (a general role via its interaction with PABPC1 and a specific role via its interaction with CPEB3) is missing. How do the authors reconcile these 2 functions? Also a discussion referring to the function of another deadenylase, PARN, in CPEB3-mediated deadenylation is missing.”

Following the suggestions by the reviewer, we have shortened the discussion concerning Tob’s function in learning and memory on page 15 (old version) and newly added a discussion concerning the dual role of Tob in deadenylation in the Discussion section as follows;

“Tob plays a dual role in mRNA deadenylation by acting as both a general regulator via PABPC1 and a transcript-specific regulator via CPEB3. In this study, β-γAβγ nuclear reporter mRNA with MS2 binding sites in its 3′UTR showed slow and biphasic deadenylation where Pan2-Pan3 and Tob-Cafl-Cm4 function via PABPC1, respectively, in the early and the late phase of the deadenylation (Supplementary Fig. S1A) as previously reported for general mRNAs. While in the presence of MS2-CPEB3, its kinetics of the same reporter mRNA switched to rapid deadenylation where Tob-Cafl functions via CPEB3 (Fig. 6F, 7A and 7B in this revised version). These results suggest that the transcript-specific regulation by Tob via interaction with CPEB3 is dominant over the general regulation via PABPC1.”

We also added a discussion on a possible involvement of PARN in CPEB3-mediated deadenylation as follows;
“In this study, we have identified Caf1 as a responsible deadenylase of CPEB3-mediated deadenylation. However, we cannot rule out the possibility that other deadenylases including PARN might also be involved in the regulation of CPEB3 target mRNAs.”

4) “As the proteins are expressed everywhere in the cells, the colocalisation data shown in figure 1A are not informative. This panel can be removed.”

We believe that the reviewer mean to refer figure 1F rather than 1A, so we have removed the colocalization data in Figure 1F (old version) accordingly.

5) “It would be nice to number the lanes in figures 1A, 1B and 1G as in the figure legends.”

According to the reviewer’s suggestion, we have added lane numbers to Figures 1A, 1B and 1G.

6) “In figures 2B and 3B, the length of the wt proteins should be indicated.”

As it was suggested by the reviewer, we have indicated the length of the wt proteins in Fig. 2B and 3B.

7) “In figure 5A legend, it should be mentioned that the precise sites of CPEB3 binding to the GluR2 3’UTR are not known.”

We have added the following sentences to the Fig. 6A legend (in this revised manuscript).

“CPEB3 binds to multiple regions throughout the length of the GluR2 3’UTR but the precise sites of CPEB3 binding to the 3’UTR are not known.”

8) “In figure 6E, what is lane 21? Also, how do the authors determine the A0 line?”

To show A0 line of β−globin reporter mRNA, we utilized steady-state mRNA as shown in the lane 21 (Fig. 6E old version). We assigned the position of the shortest β-globin reporter mRNA detected on the blot as the A0 line. However, to assign it more precisely, we have newly added lanes of oligo (dT)/RNase H treated sample to Fig. 7B (new version) and also to Figs. 6B, 6C, 6F and 7A as well.

9) “The reference Theis et al 2003 is missing in the references list.”

We have added the reference in the manuscript accordingly.

Referee #2

We thank the reviewer for the constructive comments, valuable suggestions to strengthen our conclusions and positive evaluation of our work.

1) “An interaction with CPEB4 is presented in Fig. 1D but is it not further pursued in the study. Perhaps several experiments can be done to address the functional relevance of this interaction or at least the implications of this additional interaction should be discussed.”

We have now included the following sentences on CPEB4 in the Discussion section.

“In addition, we also found CPEB4 as the binding partner of Tob. CPEB3 and CPEB4 are >95% identical in their RNA-binding domain (RBD), and Tob binds to the highly conserved RBD of CPEB4 (data not shown) as in the case of CPEB3 (Fig. 3). Although functional difference between CPEB3 and CPEB4 is not known, Tob could also regulate CPEB4 target. Thus by identifying targets of CPEB3 and CPEB4 other than GluR2 mRNA, it should be possible to elucidate the regulatory mechanisms underlying other biological processes regulated by Tob.”

2) “Fig. 2/3: Were the interactions detected in these pull downs sensitive to RNase? The requirement for CPEB3 RNA binding domains for the interaction with Tob raises the potential issue of RNA bridging”
All the co-immunoprecipitation experiments showing the interaction between Tob and CPEB3 were performed in the presence of RNase A (Fig. 1A, 1B and 1C). In the presence of RNase A, the efficiencies of the pull downs were reduced, but significant amounts of proteins were reproducibly precipitated. In addition, we also showed that recombinant GST-Tob and MBP-CPEB3, which we purified from E. coli with more than 95% purity (Fig. 1D), bind to each other (Fig. 1E). These results strongly suggest that the binding is direct and not mediated by RNA.

Taking these results into account, we performed the pull down experiments in Fig. 2 and 3 in the absence of RNase A to evaluate the interaction more efficiently.

3) Fig. 4 - While the data on the decrease in reporter protein and RNA expression are well documented, the authors should probably assess RNA decay directly to solidify their conclusion that altered RNA stability is playing a role here.

We appreciate this comment very much. According to the reviewer’s suggestion, we have assessed RNA decay of the CAT reporter in COS-7 cells. COS-7 cells were co-transfected with CAT reporter gene appended with GluR2 3’UTR and CPEB3 expression vector, and the levels of CAT mRNA were examined after transcription was shut-off. The data are shown as Supplementary Figure S1A. The half-life was markedly decreased from 4.8 h to 3.7 h in the presence of CPEB3. Similar result was obtained with β-globin reporter mini gene (BGG (1-39)) appended with GluR2 3’UTR, where the half-life of the β-globin mRNA was reduced from 2.4 h to 1.7 h by CPEB3 (Supplementary Figure S1B). These results confirm that CPEB3 reduces the stability of its target mRNA.

4) “Figs 4D and 5B: A normalization lane of some sort (e.g. actin) should be added to these figures to validate the decrease in total reporter RNA level seen with the GluR2 3’ UTR.”

As suggested by the reviewer, we have added a normalization lane of 28S rRNA to the Figs 5D and 6B and also to Figs 6C, 6F, 7A and 7B (in the revised version) as well.

5) “Fig. 5: There are several issues with the way the data are presented in this set of figures. a. 5C needs an A0 marker lane (e.g. oligo dT RNase H treated sample) to validate the authors interpretation of poly(A) tail length.”

We have introduced an A0 marker lane to Fig. 6C (new version). The result clearly shows that CPEB3 stimulated the decrease in the poly(A) tail length of the mRNA.

“b. 5C - The half life reported on pg. 11 does not seem to correlate with the data that are presented. No apparent change in RNA levels in the (-) lanes occurs until 6 hrs, yet a 2.6hr half life is reported in the text for these samples. Maybe a shorter exposure of the gel would suffice.”

We think that no apparent change in RNA levels in the (-) lanes until 6 hrs observed in Fig. 5C (old version) was due to leak in transcription of the reporter mRNA. In a tet-on system we used in Fig. 5C, transcription was stopped by removing tetracycline from the culture medium, but the transcription was not completely shut-off because of residual tetracycline in cells. We therefore have changed experimental condition to totally wash out tetracycline from the cells. As expected, the leak in transcription was improved and the delay in the early phase of the mRNA decay disappeared. The half-lives re-calculated from the three new independent experiments are 2.4 ± 0.2 h and 1.6 ± 0.2 h in the absence and presence of CPEB3, respectively. We have replaced Fig. 5C to a new one (Fig. 6C in the revised version) and modified the manuscript accordingly.

“c. 5E - as with Fig. 5C, this panel also requires an A0 marker lane and the T1/2 reported on pg. 11 are not at all obvious from the data that are presented. In fact there appears to be more RNA at 3hrs post shut off than at time 0, yet a 2.5 half life is reported.”

We have now included a new data, in which the experimental condition as in Fig. 6C (new version) was applied to avoid leak in transcription. Increased level of the mRNA at 3 hrs post shut off was not observed in this condition and the T1/2 was assessed more precisely. From the three independent experiments, the half-lives of 4.8 ± 0.5 h and 2.2 ± 0.7 h were calculated, respectively,
in the absence (control) and the presence of MS2-CPEB3. We have replaced Fig. 5E to a new data as Fig. 6F. Also, we have added an A₀ marker lane to this figure.

6) “Fig. 6: The 'partial repression' by the Tob deletion variants is not very clear from the data that are presented. Perhaps a quantitative presentation of the data would address this concern.”

We have repeated the same experiments in Fig. 6 (old version) to improve their quality, and replaced all of the data shown in the Fig. 6 to new ones of high quality as Fig. 7. As a result, the half-life of β-globin reporter mRNA (4.6 h) was reduced by CPEB3 (2.7 h), while Tob (1-160) and Tob (110-218) reversed the effect of CPEB3 (to half-lives of 4.9 h and 4.1 h respectively) (please refer to Fig. 7B in our revised manuscript). The values of the mRNA half-lives are also added to the figure legend.

7) “Fig. 1G is not discussed in the text until after Fig. 3. Thus I would suggest moving it to ~Fig. 4A (or perhaps a separate Fig.) to improve the flow of the manuscript.”

According to the reviewer’s suggestion, we moved the data in Fig. 1G to a new Fig. 4.

8) “Fig. 1E: for the benefit of the general EMBO readership, please clearly indicate in the legend what the asterisks refer to.”

We have modified the manuscript accordingly (please see new Fig. 1F).

9) “Fig. 1F/Pg. 7: Since this localization study was done with over-expressed tagged proteins and not endogenous proteins, this limits the significance of the conclusions that can be drawn. Thus the text describing this experiment should be toned down accordingly.”

Other reviewers also mentioned that the data in this figure are not informative, so according to the reviewer’s suggestions we have now removed this figure.

Referee #3

We thank the reviewer for the constructive comments, valuable suggestions to strengthen our conclusions and positive evaluation of our work.

1) “there are two major general issues that affect the conclusions of the work. The first one is that there is not a single piece of evidence for the endogenous protein-interactions or target mRNAs regulation. The whole study is based in over-expressed proteins and reporter mRNAs. There is no characterization of whether CPEB3/4 is present in the cells used for the study, and that may change the interpretation of some experiments. Some evidence for the existence of the CPEB3/Tob/Caf1 endogenous complex should be presented. Some evidence of the effect of knocking-down CPEB3 in the deadenylation of endogenous mRNAs would also be required. This point is even more critical taking into account that overexpressed CPEBs induced severe stress to the cells and do not localize like the endogenous.”

--- whether CPEB3/4 is present in the cells used for the study

CPEB3 was not detected in HeLa cells by Western blotting using rabbit polyclonal antibody raised against human recombinant CPEB3. As shown in Fig. 6D (new version), this antibody recognizes exogenously expressed HA-CPEB3 in HeLa cells very well. In this condition, endogenous CPEB3 was not detected by the antibody. Similar results were obtained using COS-7 cells (Fig. 5B, new version). Since CPEB3 is highly conserved among mammals, we think the inability to detect CPEB3 in COS7 cells is not resulting from species difference. To confirm this, we also examined the expression of CPEB3 mRNA by semi-quantitative RT-PCR. As shown in Supplementary Figure S4C, even CPEB3/4 mRNA was not detected in HeLa cells whereas significant amount of CPEB3/4 mRNA was observed in SK-N-SH1 cells (positive control). Thus it is likely that CPEB3/4 might not be expressed in COS-7 cells and HeLa cells. So this makes it difficult to estimate the levels of overexpression and to perform depletion experiment in these cells. Also, this makes it difficult to show the formation of endogenous CPEB3/Tob/Caf1 complex in HeLa and COS-7 cells.
--- existence of the CPEB3/Tob/Caf1 endogenous complex

Therefore to provide further support for that the endogenous CPEB3/Tob/Caf1 complex forms, we have transiently expressed CPEB3 alone in HeLa and COS-7 cells and CPEB3/Tob/Caf1 complex formation was examined by immunoprecipitation. When HA-CPEB3 was expressed in cells, both Tob and Caf1 were co-precipitated with CPEB3 in both HeLa and COS-7 cells. These results indicate that CPEB3/Tob/Caf1 ternary complex forms in HeLa and COS-7 cells, when CPEB3 is expressed in these cells. The data are added as Supplementary Figure S5.

--- Some evidence of the effect of knocking-down CPEB3 in the deadenylation of endogenous mRNAs would also be required.

We appreciate this comment very much. As described in our manuscript, GluR2 mRNA is the only known target of CPEB3 at present. However, GluR2 as well as CPEB3 appears not to be expressed in cells we used in our study (as described above and Supplementary Fig. S4C). Therefore, we have now performed new experiments with neuroblastoma cells (SK-N-SH), in which GluR2, CPEB3/4, Tob/Tob2 and Caf1 are all expressed (Supplementary Fig. S4C and 4D). The cells are knocked down by siRNAs for CPEB3 and CPEB4, and the effect of depletion of endogenous CPEB3/4 on the level of endogenous GluR2 mRNA was assessed. As is often the case with neural cells, the transfection efficiency is very low (34%) in SK-N-SH cells, and CPEB3 and 4 were depleted respectively by 58% and 64%. In this condition, GluR2 mRNA levels increased by up to 1.6 fold. Furthermore, when Tob1 and Tob2 were knocked down by using the same RNAi strategy, Tob1 and Tob2 mRNAs were also depleted respectively by 63% and 54%, and GluR2 mRNA increased by up to 1.6 fold. These results strengthen our conclusion that Tob and CPEB3/4 negatively regulate the level of GluR2 mRNA. We agree with the reviewer that it is desirable to measure the deadenylation of GluR2 mRNA, however, it was difficult to assess deadenylation and decay rates due to the limitations in transfection efficiency (~30%) in the neural cells as described above. The results are added as Supplementary Fig. S4.

"The second is that, in general, the western blots are over-contrasted and, in many cases, lanes appear to have been cut and pasted together (in some cases, like fig 1A and 1G, etc this cutting/pasting affects the negative controls). Less contrasted westerns and uncut gels would be required for the reader to judge the results."

We have now included new Western/Northern blots, which are less contrasted and without cutting/pasting (Figs. 1A, 1B, 4, 6B, 6C, 6F, 7A, 7B in new version).

2) "Figure 1F: Given that overexpressed CPEB3 and overexpressed Tob localize in a diffuse manner over the whole cytoplasm is not possible to derive any conclusion as to their co-localization."

Other reviewers also mentioned that the data in this figure are not informative, so according to the reviewer’s suggestions we have now removed this figure.

3) "Figure 3A: Mapping of the CPEB3 domain that interacts with Tob is based on the GST pull down of the 417-685 fragment. But the mobility of this fragment is significantly different in the input and the pull-down. Are the authors sure it is really the 417-685 fragment?"

We apologize that the position of the mw marker in the input panel was not in the right place. We have corrected the figure.

4) "If Tob is the limiting factor (page 9/fig 1G), and giving its role in transcription, which is the meaning of the CPEB3-overexpression phenotypes? Is it just squelching Tob from its different endogenous functions? How many folds is CPEB3 overexpressed over the endogenous protein (this would be required to interpret the dose-response of CPEB3 overexpression in reporter mRNA levels and deadenylation)? Depletion of the endogenous CPEB3 would be more informative."

We have already described in (1). Please see above.

5) "Figure 4A-C how are these results different from what was shown in Huang et al. 2006?"

Huang et al. reported that CPEB3 negatively regulates GluR2 gene expression by using primary cultured hippocampal neuron and endogenous GluR2 mRNA. This is consistent with our results.
using COS7/HeLa cells and reporter mRNA appended with GluR2 3'UTR. They concluded translation of GluR2 mRNA is controlled by CPEB3, however, our results in Fig. 5 (in this revised manuscript) shows that the steady-state level of both the reporter protein and its mRNA were reduced to ~30% and ~40%, respectively, by CPEB3. These results indicate that CPEB3 regulates the expression of its target transcript not only at translation as suggested by Huang et al. but also at mRNA stability. In this revised manuscript, we also added new data with SK-N-SH cells as described above. The fact that knocking down CPEB3 increased the level of GluR2 mRNA further strengthened our conclusion that CPEB3 regulates its target mRNA stability.

"Do Cos7 cells contain CPEB3? which are the levels of overexpression?"

We have already described in (1).

"Fig 4D-E, which are the differences between the short and long 3'UTRs? Do both contain the CPEB3 binding site?"

It has been reported that the 3' end of the GluR2 transcripts are alternatively processed to form a long and short 3'UTR. As reported by Huang et al. 2006, CPEB3 binds to multiple regions throughout the length of the 3'UTR, and thus both forms of GluR2 mRNA are thought to contain the CPEB3 binding site, although the precise site of CPEB3 binding are not known. Consistent with this, CPEB3 reduced the level of both short and long forms of GluR2 reporter mRNA as shown in Figs 5D, 5E, 6B and 6C.

"Figures 4, 5 and 6, transfection/transcription/loading controls would be required for the northerns. Ideally, the reporters with the control and regulated 3'UTRs should be cotransfected and differentially identified in the same gel. Figures 5C-E; 6A, E The differences in the mRNA quantities at time 0, makes difficult to assess possible differences in the degradation rates of the mRNAs."

As suggested by the reviewer, we have added a normalization lane of 28S rRNA to the Figs. 4, 5 and 6. We agree with the reviewer that cotransfection of the reporters with the control and regulated 3'UTRs followed by differential identification in the same gel is ideal, but due to time limitations, we used the above method at this time.

"Figure 6A. A control where the dominant negative (Caf1 D161A) is overexpressed in the absence of CPEB3 would be required to address whether the effect is CPEB3 specific or not."

According to the reviewer’s suggestion, we have performed the same experiment as Figure 6A (old version) in the absence of CPEB3. The data are added as Supplementary Fig. S2A. The β-globin reporter mRNA showed slow and biphasic decay kinetics, where a slow and synchronous deadenylation occurred in the first phase (0-4 h) and a fast and less-synchronous deadenylation with a concomitant decay of the mRNA body occurred in the late phase (>4h). Dominant negative mutants Pan2 D1083A and Caf1 D161A reduced the rate of deadenylation at the first and the second phases, respectively. The results are consistent with our previous report. Thus in the absence of CPEB3, the β-globin reporter mRNA exhibited a deadenylation and decay kinetics similar to the general mRNA, while tethering CPEB3 to the mRNA caused a shift to a rapid deadenylation and decay kinetics. Dominant negative effect of Caf1 D161A is not specific to the CPEB3-mediated deadenylation, and Caf1 D161A also affects general mRNA deadenylation as expected. Caf1 forms a complex with Tob to regulate deadenylation of both general and specific mRNA via interaction with PABPC1 and CPEB3, respectively. The discussion concerning the dual role of Tob-Caf1 in deadenylation has been included in the Discussion section.

"Figure 6C, tethering Tob just confirms the already known function of Tob in mRNA deadenylation, does not support any function in CPEB3-mediated mRNA-specific deadenylation."

We have now removed this figure accordingly.

"Figure 6E. A control where the dominant negative Tobs (1-160 and 110-218) are overexpressed in the absence of CPEB3 would be required to address whether the effect is CPEB3 specific or not."
According to the reviewer’s suggestion, we have performed the same experiment as Figure 6B (new version) in the absence of CPEB3. The data are added as Supplementary Fig. S2B. Tob deletion mutant Tob (110-218), which contains PABPC1-binding sites (both primary and secondary PAM2 motifs) but not Caf1-binding region (BTG domain), reduced the rate of deadenylation of β-globin reporter mRNA mainly at the second phase. This result is consistent with the previous report with dominant negative Tob F139A/F274A, showing that Tob functions in the second phase of deadenylation of general mRNA (Funakoshi et al, 2007). In contrast, Tob (1-160), which has Caf1-binding region and secondary PAM2 motif did not affect the rate of deadenylation. Since the secondary PAM2 motif binds weakly but significantly to PABPC1, Tob (1-160) can bind both Caf1 and PABPC1, and is expected to mediate the binding between Caf1 and PABPC1 (and not to function as a dominant negative mutant for the decay of general mRNA). However, as described in the manuscript, both Tob (1-160), which contains Caf1-binding region but not CPEB3-binding region, and Tob (110-218), which contains CPEB3-binding region but not Caf1-binding region function as dominant negative mutants for CPEB3-mediated deadenylation and decay. Thus, dominant negative effect of Tob (1-160) but not Tob (110-218) is specific to the CPEB3-mediated deadenylation.

Tob forms a complex with Caf1 to regulate deadenylation of both general and specific mRNA via interaction with PABPC1 and CPEB3, respectively. The discussion concerning the dual role of Tob-Caf1 in deadenylation has been included in the Discussion section.

10) “If Orb2 is equivalent to CPEB2, 3 and 4, why limiting the study to CPEB3? Would the results be comparable for CPEB2 and 4, as suggested by the pull-downs from figure 1?”

CPEB2-4 are known as human orthologs of the Drosophila orb2 and thus are very similar to each other. Especially, CPEB2, CPEB3 and CPEB4 are >95% identical in their RNA-binding domain (RBD) and actually bind the same SELEX sequence with high affinity. However, functional difference between these proteins is not known at present. In this study, we found that Tob binds to the highly conserved RBDs of CPEB3 (Fig. 3) and CPEB4 (results comparable to Fig. 3; data not shown) with similar affinity. Thus, we thought CPEB2-4 could redundantly function with Tob and it was difficult to show functional difference between these proteins. Therefore we used CPEB3 as the representative of CPEB2-4.

11) “The figure legends should be more detailed to properly follow/interpret the depicted results.”

We have modified the manuscript accordingly.
REFEREE REPORTS

Referee #1 (Remarks to the Author):

In this second version of their manuscript, the authors have clearly taken into account earlier reviewers' comments and performed several additional experiments to strengthen their manuscript. Thus, the quality of the results presented in the Western and Northern blots has significantly improved and the data are now more convincing. They have also performed depletion experiments to demonstrate the function of endogenous proteins.

Overall, the manuscript is of interest to a wide readership. Nevertheless, a few minor points still need to be addressed before publication:

1. Pages 13 and 14, the authors now discuss that primary and secondary PAM2 motifs present in Tob proteins are able to trigger PABPC1 binding. This is not very clear and in contradiction with what is presented in figure 2. This should be clarified.

2. As the ratio between signal and background is very low, the Northern of figure S1A is not convincing. However, it is possible to remove this figure as similar results are obtained from figure S1B.

3. One cannot rely satisfactorily on semi-quantitative RT-PCR to quantify the results of the siRNA experiments. This is especially true for figure S4. Could the authors present at least real-time quantitative RT-PCR data instead?

4. Page 16, line 9, the authors write: "for the first time". They should not forget for their discussion about the biological function of Tob that members of this family of proteins have already been shown to interact with factors involved in cell signaling and transcription, such as the Smad factors.

Referee #2 (Remarks to the Author):

The revised manuscript is improved but unfortunately has not in my opinion adequately addressed all of the concerns raised in the previous reviews. Issues that remain include:

1. Fig. S1 A/B - the half life differences that are reported are relatively small and no error bars are included to indicate the reproducibility of the data obtained. Therefore I do not find this evidence convincing as presented.

2. Fig. S3 - It is unclear to me how the authors can conclude from these data that Tob/Tob2 siRNA is 'significantly suppressing deadenylation rates'. The data that are presented in the figure are smears that are very difficult to interpret and no quantification has been preformed to determine significance or reproducibility.

3. Fig. S4 - These represent very important data in study since it is now clear that the authors other work was done in cells that are naturally missing one or more of the factors involved in the regulatory model that is proposed (drawing appropriate concern over the endogenous relevance of the model). Given the broad effects of Tob in cells, I believe that it is imperative that the authors perform mRNA half life analysis in these knockdowns and add a control for a non-CPEB/Tob target mRNA. The current data presented in the Fig. are a good first step but not sufficient in my opinion to fully address the previous critiques.

4. This may be a bit picky but since the model presented suggests a ternary protein complex involving a deadenylase across a poly(A) tail, RNAse A treatment (which cleaves only at pyrimidines) may not be sufficient to rule out RNA bridging since it would not degrade the poly(A) tail.

Referee #3 (Remarks to the Author):

The revised version the manuscript by Hosoda et al. has been significantly improved over the previous version and the majority of the points raised by the reviewers have been satisfactorily addressed with new experiments.

There are a couple of aspects, however, where I do not necessarily agree with the answers provided by the authors.

- 1- To provide evidence or the existence of the CPEB3/Tob/Caf1 endogenous complex the authors use transiently expressed CPEB3 in Hela and cos-7 cells, which do not express endogenous CPEB3.
Ectopically expressed CPEB3 cannot prove the existence of an endogenous complex. Why do not use SK-N-SH cells, where all the endogenous proteins and target mRNAs are expressed (Fig S4), to prove that this complex exist under normal conditions and is not the result of the cellular stress caused by overexpressing these proteins?

- 2- Specific point 5: "Figures 4,5 and 6 require transfection/transcription/loading controls" The use of 28S rRNA provides a loading control but it does not normalize for transfection and transcriptional variations.
- 3- Regarding the specific point 3 from reviewer 1 "Also a discussion referring to the function of another deadenylase, PARN, in CPEB-mediated deadenylation is missing" It would be nice to expand the discussion beyond the single sentence included in the revised version. More over taking into account that Huang et al reported that CPEB1 also contains "classical" CPEs and is bound by CPEB1.

Overall, and despite some limitations derived from the technical difficulties of working with neuronal cells (The only ones where all three CPEB3/Tob/Caf1 seems to be expressed), the new version of this work provides sufficient experimental evidence to support the existence of a new mRNA-specific deadenylation and decay pathway mediated by the CPEB3/Tob/caf1 complex.

2nd Revision - authors’ response 10 December 2010

Referee #1:

1) “Pages 13 and 14, the authors now discuss that primary and secondary PAM2 motifs present in Tob proteins are able to trigger PABPC1 binding. This is not very clear and in contradiction with what is presented in figure 2. This should be clarified.”

We have modified the manuscript accordingly (see page 8, 14 and Figure 2B legend).

2) “As the ratio between signal and background is very low, the Northern of figure S1A is not convincing. However, it is possible to remove this figure as similar results are obtained from figure S1B.”

As it was suggested by the reviewer, we have removed this Figure. Instead, we have newly included the half-life data as Figure 6E, which was derived from the results in Figure 6C. We have repeated the experiments in Figure 6C and added error bars to indicate the reproducibility. The results shown in Figure 6E (new version) are representative of three independent experiments. This time we have performed the experiments with exogenously expressed Flag-GST-CAT as a transfection/transcription/loading control. The data clearly indicate that the half-life of the reporter mRNA is significantly reduced from 2.4 h to 1.6 h (short form) and 2.6 h to 1.9 h (long form) by the presence of CPEB3.

3) “One cannot rely satisfactorily on semi-quantitative RT-PCR to quantify the results of the siRNA experiments. This is especially true for figure S4. Could the authors present at least real-time quantitative RT-PCR data instead? ”

Yes. We have performed real-time quantitative RT-PCR to quantify the results of the siRNA experiments. The data are shown as Figure 9A and 9B. This time we have performed half-life analysis in these knockdowns and newly added the result of GAPDH as a control for a non-CPEB/Tob target mRNA. As shown in Figure 9A, the half-life of GluR2 mRNA but not GAPDH mRNA (control) was significantly increased by 2.0-2.5 fold by knocking down either CPEB3/4 or Tob/Tob2. These results strengthen our previous conclusion that Tob/Tob2 as well as CPEB3/4 regulate GluR2 mRNA in neuroblastoma cells.

4) “Page 16, line 9, the authors write: "for the first time". They should not forget for their discussion about the biological function of Tob that members of this family of proteins have already been shown to interact with factors involved in cell signaling and transcription, such as the Smad factors.”
We meant that the factor CPEB3 identified in our study is the first example to show that Tob is involved in the regulation of specific transcript rather than general mRNA. However, since it is misleading to the readers, as described by the reviewer, we have removed the phrase “for the first time”.

Referee #2:

1) “Fig. S1 A/B - the half life differences that are reported are relatively small and no error bars are included to indicate the reproducibility of the data obtained. Therefore I do not find this evidence convincing as presented.”

Other reviewers also mentioned that the data in Figure S1A (old version) are not convincing. We have removed this Figure accordingly. Instead, we have newly included the half-life data as Figure 6E, which was derived from the results in Figure 6C. We have repeated the experiments in Figure 6C and added error bars to indicate the reproducibility. The results shown in Figure 6E (new version) are representative of three independent experiments. This time we have performed the experiments with exogenously expressed Flag-GST-CAT as a transfection/transcription/loading control. The data clearly indicate that the half-life of the reporter mRNA is significantly reduced from 2.4 h to 1.6 h (short form) and 2.6 h to 1.9 h (long form) by the presence of CPEB3.

2) “Fig. S3 - It is unclear to me how the authors can conclude from these data that Tob/Tob2 siRNA is ‘significantly suppressing deadenylation rates’. The data that are presented in the figure are smears that are very difficult to interpret and no quantification has been performed to determine significance or reproducibility.”

According to the reviewer’s suggestion, we have repeated the same experiments in Figure S3A-C (old version) to improve their quality, and replaced all of the data shown in the Figs to new ones of high quality. This time we have performed the experiments with exogenously expressed Flag-EGFP as a transfection/transcription/loading control (Figure S2A), and quantification has been performed to determine significance or reproducibility (Figure S2B). As shown in Figure S2A, in a control experiment without MS2-CPEB3, knocking down Tob/Tob2 reduced the rate of deadenylation of the reporter mRNA mainly at the second phase (compare lanes 3-7 with 13-17). In the presence of MS2-CPEB3, CPEB3-accelerated deadenylation of the reporter mRNA is significantly suppressed by knocking down Tob/Tob2 compared to a control experiment with luciferase siRNA (compare lanes 8-12 with lanes 18-22) to a level nearly comparable to that observed without CPEB3 (compare lanes 18-22 with 13-17). These results are consistent with the results obtained with dominant negative mutants of Tob (Figure S1B). Also, mRNA half-lives analyzed by quantifying the levels of mRNA in Figure S2A shows that knocking down Tob/Tob2 repressed CPEB3-accelerated decay rate (Figure S2B, compare closed triangle with open triangle) to a level nearly comparable to that observed without CPEB3 (open circle). These results strengthen our previous conclusion that CPEB3-accelerated deadenylation and decay is mediated by Tob/Tob2.

3) “Fig. S4 - These represent very important data in study since it is now clear that the authors other work was done in cells that are naturally missing one or more of the factors involved in the regulatory model that is proposed (drawing appropriate concern over the endogenous relevance of the model). Given the broad effects of Tob in cells, I believe that it is imperative that the authors perform mRNA half life analysis in these knockdowns and add a control for a non-CPEB/Tob target mRNA. The current data presented in the Fig. are a good first step but not sufficient in my opinion to fully address the previous critiques.”

According to the reviewers suggestion, we have performed half-life analysis in these knockdowns and newly included the result of GAPDH as a control for a non-CPEB/Tob target mRNA. This time we have performed real-time quantitative RT-PCR to quantify the results of the siRNA experiments. The data are shown as Figure 9A and 9B. As is often the case with neural cells, the transfection efficiency is low (~60%) in SK-N-SH cells and both Tob and Tob2 mRNAs were reduced by Tob/Tob2 siRNAs respectively to 45% and 46% compared to the control siRNA in three independent experiments (Figure 9B). In this condition, the half-life of
the GluR2 mRNA but not GAPDH mRNA (control) was significantly increased by 2.0 fold (Figure 9A). Similar results were obtained with CPEB3/4 siRNAs (Figures 9A and B). From these results we conclude that Tob/Tob2 as well as CPEB3/4 regulate GluR2 mRNA expression in neuroblastoma cells.

4) "This may be a bit picky but since the model presented suggests a ternary protein complex involving a deadenylase across a poly(A) tail, RNase A treatment (which cleaves only at pyrimidines) may not be sufficient to rule out RNA bridging since it would not degrade the poly(A) tail."

Following the suggestion by the reviewer, we have newly performed co-immunoprecipitation experiment with RNase I (which cleaves 3' to all bases) instead of RNase A. When lysate of SK-N-SH cells was immunoprecipitated with anti-CPEB3 antibody, the precipitated fraction contained Caf1 as well as Tob. This result, taken together with the results shown in Figure 1-4 and S3 (new version), strengthens our conclusion that the CPEB3/Tob/Caf1 ternary complex forms in cells without RNA bridging. The result is included as Figure 9E.

Referee #3:

1) "To provide evidence or the existence of the CPEB3/Tob/Caf1 endogenous complex the authors use transiently expressed CPEB3 in Hela and cos-7 cells, which do not express endogenous CPEB3. Ectopically expressed CPEB3 cannot prove the existence of an endogenous complex. Why do not use SK-N-SH cells, where all the endogenous proteins and target mRNAs are expressed (Fig S4), to prove that this complex exist under normal conditions and is not the result of the cellular stress caused by overexpressing these proteins?"

According to the reviewer’s suggestion, we have provided new evidence on the existence of the endogenous CPEB3/Tob/Caf1 complex by using SK-N-SH cells. When lysate of SK-N-SH cells was immunoprecipitated with anti-CPEB3 antibody, the precipitated fraction contained Caf1 as well as Tob. This result, taken together with the results shown in Figure 1-4 and S3 (new version), strengthens our conclusion that the CPEB3/Tob/Caf1 ternary complex forms in cells. The result is added as Figure 9E.

2) "Specific point 5: "Figures 4, 5 and 6 require transfection/transcription/loading controls" The use of 28S rRNA provides a loading control but it does not normalize for transfection and transcriptional variations."

We believe that the reviewer meant to refer to Figures 5, 6 and 7 rather than 4, 5 and 6, so we have performed all of these experiments with exogenously expressed Flag-EGFP, Flag-GST-CAT or Flag-CAT as transfection/transcription/loading controls, and replaced all of the data shown in the Figure 5, 6 and 7 (old version) to new ones (Figure 5, 6 7 and 8). We also replaced all of the data in Figure S2 and S3 (old version) as well by performing the experiments with transfection/transcription/loading controls. The results are included as Figure S1 and S2 (new version).

3) "Regarding the specific point 3 from reviewer 1 "Also a discussion referring to the function of another deadenylase, PARN, in CPEB-mediated deadenylation is missing" It would be nice to expand the discussion beyond the single sentence included in the revised version. More over taking into account that Huang et al reported that CPEB1 also contains "classical" CPEs and is bound by CPEB1."

We have included the following sentences in the “Discussion” section.

In this study, we have identified Caf1 as the deadenylase responsible for CPEB3-mediated deadenylation. Dominant negative mutant of Caf1 (Caf1 D161A) but not that of Pan2 (Pan2 D1083) repressed CPEB3-accelerated deadenylation almost completely to a level comparable to that observed in the absence of CPEB3 (compare lanes 13-17 in Figure 8A with lanes 6-10 in Figure S1A). However, we cannot totally rule out the possibility that other deadenylases might also be involved in the regulation of CPEB3 target mRNAs. In contrast, CPEB is known to
bind directly to PARN deadenylase and regulate deadenylation of its target mRNA that contains CPEs in processes including germ cell development. In spite of the sequence similarity between CPEB and CPEB3, both proteins differ in their binding specificities for RNA and also in their requirement of CPSF and AAUAAA cis element for translation activation. Therefore it is interesting to assume that CPEB and CPEB3 differentially utilize the two deadenylases for the regulation of the target mRNAs.

Thank you for submitting your revised manuscript to the EMBO Journal. Your study has now been seen by the original referees # 2 and 3. As you can see below, both referees are pleased with the introduced changes and support publication in the EMBO Journal. Referee #2 has some remaining concerns regarding the interpretation of some of the data, but also indicates that these can be addressed with appropriate text changes. I would ask you to address the remaining concerns in final revision, please note that no new data is needed. I also noted that for some of the figures (Fig1E, Fig2A and S2E) it appears as if the lanes have been sliced together without proper indication such as a thin white or black line. Given this, I would kindly ask you if you could also send me the original scans for the figures in question when you submit your revised version and to clearly mark on the figures where splicing has taken place, assuming that the lanes come from the same gel. Please note that this is our standard procedure in such cases. Please also carefully check the other figures.

When you send us your revision, please include a cover letter with an itemised list of all changes made, or your rebuttal, in response to comments from review. When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process initiative, please visit our website: http://www.nature.com/emboj/about/process.html

I look forward to reading the final version.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS

Referee #2 (Remarks to the Author):

Overall, I find this version of the manuscript to be very significantly improved over previous versions. I commend the authors for the inclusion of improved data to support their conclusions. My only lingering concern (which I believe can be readily addressed with writing changes) is the author's interpretation of biphasic decay rates in several figures and the determination of a deadenylation rate change of 12-60 nts/hr in Fig. 7B. These conclusions are drawn from data that are extremely difficult in my opinion to accurately quantify due to the smeary nature of the bands. Unless the authors can provide clear quantitation to support these conclusions, I see no need to over-interpret these experimental data and infer biphasic kinetics or 'to-the-nucleotide' deadenylation rates.

Referee #3 (Remarks to the Author):

The revised version of the manuscript by Hosoda et al has appropriately addressed all the experimental and conceptual points raised in the previous two revisions and therefore I consider this work now suitable for publication.
Referee #2:

“My only lingering concern (which I believe can be readily addressed with writing changes) is the author’s interpretation of biphasic decay rates in several figures and the determination of a deadenylation rate change of 12-60 nts/hr in Fig. 7B. These conclusions are drawn from data that are extremely difficult in my opinion to accurately quantify due to the smeary nature of the bands. Unless the authors can provide clear quantitation to support these conclusions, I see no need to over-interpret these experimental data and infer biphasic kinetics or ‘to-the-nucleotide’ deadenylation rates.”

According to the reviewer’s suggestion, we have modified the manuscript.

(1) biphasic deadenylation kinetics:

In a previous paper, Yamashita et al. (Shyu lab) defined the biphasic nature of the mRNA deadenylation. According to their definition, the first phase is characterized by (i) Pan2 dependency, (ii) lack of the decay of mRNA body, (iii) poly(A) size ranging from 110-200 nt and (iv) synchronous nature of the deadenylation, while the second phase is characterized by (i) Caf1 dependency, (ii) the rapid decay of mRNA body, (iii) poly(A) size of < 110 nt and (iv) less synchronous nature of the deadenylation. Based on these criteria, we assigned the boundary between the first and second phases to around 3 h in our system using β-globin reporter (BGG(1-39)-MS2bs mRNA) and T-Rex HeLa cells as follows. The data in Supplementary Figure S1A were quantified, and the deadenylation and decay kinetics of the β-globin reporter were shown as Figure S1C. During 0-2 h, (i) the deadenylation of the β-globin transcript was slowed by a dominant negative mutant of Pan2 (Pan2 D1083A) (Fig. S1C, middle), (ii) the mRNA body underwent no decay (Fig. S1C, left), and (iii) the poly(A) tail was shortened to around 120 nt (Fig. S1C, middle). After 4 h, (i) the deadenylation was markedly slowed by a dominant negative mutant of Caf1 (Caf1 D161A) (Fig. S1C, middle), (ii) the mRNA body underwent rapid decay (Fig. S1C, left), (iii) the poly(A) tail was shortened below 100 nt (Fig. S1C, middle). Although the criteria (iv) is less clear due to the smeary nature of the bands (as pointed out by the reviewer), these results clearly indicate that the β-globin reporter mRNA exhibits biphasic decay kinetics typical for general mRNA.

We also provided other quantified data as Supplementary Figure S1C (right) and Figure S2F, where the second phase of the deadenylation is specifically slowed by the dominant negative mutant of Tob (Tob (110-218) and siRNA-mediated knocking down of Tob, respectively. These results also support our conclusion that the β-globin reporter mRNA exhibits biphasic decay kinetics.

We have modified the manuscript accordingly (marked in pages 12, 14, 17 and 44-47).

(2) deadenylation rate change:

According to the reviewer’s suggestion, we have removed the value of the rate change (page 11).

Additional Correspondence

Thank you for submitting your revised manuscript to the EMBO Journal. I have now had a chance to take a look at the revised manuscript and I am happy with the introduced changes. Thank you also for sending along the original scans for figure 1,2 and S2E. It all looks fine. There is one last thing and that is that I would like to ask do. Would you mark in fig 1E, 2A and S2E with a black line or white space where the cuts have been made? I also would like you to indicate in the particular figure legends that the samples came from the same gels. It just makes everything more clear. You can send me the modified fig1, 2, S2E and the modified manuscript text by email and we will then upload it for you. Once we get the files, we will accept the manuscript.

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