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Long non-coding RNA *gadd7* interacts with TDP-43 and regulates *Cdk6* mRNA decay

Xuefeng Liu, Dan Li, Weimin Zhang, Mingzhou Guo and Qimin Zhan

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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 04 May 2012

Thank you for submitting your manuscript for consideration by the EMBO Journal. It has now been seen by three referees whose comments are shown below.

As you will see from the reports all three reviewers are generally positive about the findings reported in your paper; however they do raise important concerns that need to be addressed further. One recurring issue in all reports is the specificity of the reported protein-RNA interactions. The referees suggest a number of feasible experiments to clarify this issue, but the suggestion of genome-wide mapping of TDP43 binding sites is not an absolute requirement from our side as it would change the scope of the paper.
Please do not hesitate to contact me if you have questions related to the referee process and the requests made by the referees.

Given the referees' positive recommendations, we offer you the opportunity to submit a revised version of the manuscript, addressing the comments of all three reviewers. I should add that it is EMBO Journal policy to allow only a single round of revision, and acceptance or rejection of your manuscript will therefore depend on the completeness of your responses in this revised version.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Peer-Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website:

http://www.nature.com/emboj/about/process.html

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed.

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

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS:

Referee #1:

The paper by Liu et al. reports that the non-coding RNA, gadd7, is induced following UV-damage and other stressors, and is linked to cell cycle progression at the G1/S checkpoint. Specifically the authors demonstrate that knockdown of gadd7 with siRNA reduces the accumulation of cells in the G1 phase following UV damage. This is followed up with a well-controlled series of experiments to identify the putative mechanistic details. Using complementing RIP and biotinylated RNA pulldowns they show that gadd7 interacts with TDP-43 through specific regions of the RNA transcript, and that in response to UV damage TDP-43 expression is unchanged, but binds the increased gadd7. Finally the authors propose the gadd7 lncRNA acts as a decoy for TDP-43 to reduce Cdk6 occupancy, the effect of which is to regulate the expression of Cdk6. However, this conclusion is mainly based on the Fig. 5c, where double siRNA transfection of Gadd7 and TDP-43 is used to support the argument that Gadd7 acts via TDP-43 to regulate degradation of Cdk6 mRNA. It is clear in this result that Gadd7 and TDP-43 have opposite effects on Cdk6. However, in the double knockdown, Cdk6 levels are an intermediate between single knockdowns - so it is plausible that the two factors regulate Cdk6 by independent mechanisms. The conclusion therefore requires support of further experiments, as detailed below.

1. Changes in gadd7 expression using RT-PCR are modest and difficult to interpret in figures 1A and 1C. Given that the authors use qPCR of gadd7 and GAPDH later in the manuscript, figure 1 would strongly benefit from a more quantitative analysis with qPCR.
2. If gadd7 acts as decoy, then its effect should not be specific for Cdk6, but would also affect other RNAs bound by TDP-43. Therefore, it is surprising that gadd7b doesn't have an effect on the CFTR splicing regulation by TDP-43. One could argue that gadd7 only sequesters TDP-43 from 3' UTRs. To address this, another 3'UTR bound by TDP-43 needs to be assessed. For instance, TDP-43 binds to its own 3'UTR to autoregulate its own expression. Does gadd7 interfere with the binding of TDP-43 to its own 3' UTR? If yes, since TDP-43 is known to autoregulate its own expression, it's surprising that the expression of TDP-43 doesn't change upon gadd7 expression. If gadd7 doesn't affect TDP-43 binding to its own 3' UTR, then the decoy hypothesis proposed by the authors is unlikely to be true.
3. Fig. 6b is the only evidence supporting the statement that "Gadd7 sequesters TDP-43 from cyclin-dependent kinase 6 (Cdk6) mRNA". Since RIP allows indirect interactions, the result is open to many interpretations. It is necessary that RIP is done also with several other targets of TDP-43, such as its own mRNA. Alternatively, it would be even more convincing if a genome-wide analysis using CLIP was used, which would also show more comprehensively if Gadd7 acts as a decoy to remove TDP-43 from its other mRNA targets.
4. At present, it is not possible to say whether the effects of Gadd7 result from regulation of transcription, transcript stability or other effects. In order to claim that release of TDP-43 from the Cdk6 3'UTR mediates the effect of Gadd7, it is necessary to make a luciferase construct containing the Cdk6 3' UTR, and demonstrate that Gadd7 regulates this construct in a manner that depends on

the TDP-43 binding site.

Minor comments:

1. Supplementary figure 5C has no statistics.
2. The paper would benefit from further proof-reading due to several errors. However, on the whole it is well written.
3. Page 3 - When describing lincRNA-p21, the sentence in its present form does not make sense and should be modified to "LincRNA-p21, a lncRNA downstream of p53,..."
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Referee #2:

The authors study the regulation of cell cycle G1/S checkpoint by the long non-coding growth arrest and DNA damage-inducible transcript 7 (gadd7). They first confirm and extend the notion that gadd7 is induced by genotoxic stress and growth arresting conditions, and that gadd7 contributes to G1/S checkpoint control. Using biotinylated gadd7 RNA as an affinity probe, they discover heterogenous nuclear ribonucleoproteins as interactor proteins. Among these, TDP-43 is selected and validated in a convincing manner. Importantly, it is found that gadd7 apparently competes with TDP-43 binding to Cdk6 mRNA, previously identified independently as a TDP-43 target. Although the direction of TDP-43 effects in the CHO-K1 cells used here are inverse to the original findings in HeLa cells (likely reflecting cell type specificity), the present data are most concise and functionally consistent. The manuscript is written very well, the results are clearly displayed and comprehensible, and make a clear point for this interesting and innovative report.

The authors should consider the following suggestions to improve the paper:
1. Although I agree with the argument that some TDP-43 mediated mRNA effects are remarkably variable among different cell types (the authors must comprehensively discuss from the published TDP-43 screens where Cdk6 alterations were measured and where not), it is still a matter of concern how relevant the observed findings are. In the present context, it would be desirable to check the gadd7 - TDP-43 - Cdk6 pathway in appropriate cell cycle and/or cancer models.
2. Some more epistatic experiments would strengthen the conclusions. Does Cdk6 (and TDP-43) transfection overcome the G1/S checkpoint failure after gadd7 RNAi?
3. Along these lines, how specific/relevant is the gadd7 - Cdk6 connection? TDP-43 affects thousands of transcripts. The authors do provide the CFTR ex9 splice assay as a general negative specificity control, but could there not be other, cell cycle genes involved in the present process?
4. Comparative competition experiments as in Fig. 3D should be done also with Cdk6 RNA. Measure affinities and confirm that gadd7 RNA binds TDP-43 more strongly than Cdk6 RNA.
5. Please spell out the full sequences around the (UG) repeats in Fig. 3F.

Minor Corrections:
6. Fig. 1A annotation gadd45 (not gaddd45)
7. Page 5, line 18: delete "clearly" - the effects in Fig. 2B and C are not so strong (Fig. 2B lacks statistics)
8. Fig. 3A and Fig. 6E are dispensable; methodology and effect are clear enough

Referee #3:

This manuscript describes a potentially interesting study that addresses a mechanism for the CHO-specific lncRNA gadd7 in modulating the activity of TDP-43 in regulating the stability of the Cdk6 mRNA. However in its present form, I find several aspects of the study to be rather preliminary and lacking the requisite data to provide strong support for the models that are put forth. My main concerns include:

1. Fig. 2: The negative effects of gadd7 on cell growth are not well-supported in my opinion. Panel

B shows a relatively small effect on cell number, particularly when the data in panel C indicate that the control cells have failed to recover from the stress of the lipofectamine based transfection protocol used (only ~40% viable based on the MTT assay). This large amount of cell death in the control cells brings into question any conclusions on cell growth drawn from these comparative data. Less toxic transfection conditions need to be established to provide a more meaningful comparison.
2. Fig. 2D/E: I would recommend that the supplementary data be moved into the main text and these representative traces switched into the supplementary data. The supplementary data make the point better since they also allow the reader to assess the reproducibility of the experiment.
3. Fig. 3: Two points: (a) The authors need to better establish the binding specificity of TDP-43 for gadd7 RNA. In panel D, the use of tRNA as a control competitor RNA is not optimal due to its unique structural content. An ssRNA competitor would be much more informative. (b) the authors infer from these large deletion RNAs that the tgtgtg repeats (which should be UGUGU since its RNA) are involved in the binding. It would be very easy to demonstrate the importance of these repeats by making the appropriate deletions/mutations in the RNA. Establishing the specificity of TDP-43 and the underlying reasons for it are important conclusions that provide impact for the study.
4. Fig. 3/Table S1 and elsewhere: The data indicate that gadd7 associates with a variety of major RNA binding proteins in addition to TDP-43. Therefore in the gadd7 KD experiments (e.g. Fig. 6), without additional controls how can the authors be sure that the effects that are observed are occurring through TDP-43 effects and not any of the other RBPs that are associated with the lncRNA? The study needs to be more comprehensive in its analysis of the effects of the gadd7 RNA on RBPs in order to ensure that the conclusions drawn are appropriate.
5. The differences observed on the relationship of TDP-43 to the cdk6 mRNA here versus the Ayala et al 2008 study published in PNAS are surprising. I would strongly recommend that the authors follow up their idea of cell-type specific differences to try to come up with a more in depth explanation of this troubling discrepancy.
6. Fig. 4: Could the increase in RNP complexes under UV treatment conditions simply be due to an increased stabilization of existing complexes by UV cross-linking (thus they are more readily co-IPPT'd) rather than an increase in the binding as concluded by the authors?
7. Fig. 6: It is very surprising that the extremely large change in stability of the Cdk6 mRNA (panel D) is not reflected by a dramatic overall increase in cdk6 mRNA abundance in Panel C (its abundance is only up ~10-15%). Why the discrepancy in these data?
8. Other points:
a. The figure legends/methods are a bit unclear in spots. In particular, due error bars in all cases represent independent experiments or the sampling error in individual samples?
b. The grammar/writing in several places of the manuscript could use some polishing.

1st Revision - Authors' Response 03 August 2012

Referee #1:

The paper by Liu et al. reports that the non-coding RNA, gadd7, is induced following UV-damage and other stressors, and is linked to cell cycle progression at the G1/S checkpoint. Specifically the authors demonstrate that knockdown of gadd7 with siRNA reduces the accumulation of cells in the G1 phase following UV damage. This is followed up with a well-controlled series of experiments to identify the putative mechanistic details. Using complementing RIP and biotinylated RNA pull downs they show that gadd7 interacts with TDP-43 through specific regions of the RNA transcript, and that in response to UV damage TDP-43 expression is unchanged, but binds the increased gadd7. Finally the authors propose the gadd7 lncRNA acts as a decoy for TDP-43 to reduce Cdk6 occupancy, the effect of which is to regulate the expression of Cdk6. However, this conclusion is mainly based on the Fig. 5c, where double siRNA transfection of Gadd7 and TDP-43 is used to support the argument that Gadd7 acts via TDP-43 to regulate degradation of Cdk6 mRNA. It is clear in this result that Gadd7 and TDP-43 have opposite effects on Cdk6. However, in the double knockdown, Cdk6 levels are an intermediate between single knockdowns - so it is plausible that the two factors regulate Cdk6 by independent mechanisms. The conclusion therefore requires support of further experiments, as detailed below.

1. Changes in gadd7 expression using RT-PCR are modest and difficult to interpret in figures 1A and 1C. Given that the authors use qPCR of gadd7 and GAPDH later in the manuscript, figure 1 would strongly benefit from a more quantitative analysis with qPCR.

Responses: We appreciate the reviewer's suggestion. The induction of *gadd7* by DNA damage and growth arrest signals has been re-analysed with qPCR, and similar results were observed as shown in the revised Figure 1. The former Figure 1 detected by RT-PCR has been moved to supplementary data (Supplementary Figure S1).

2. If gadd7 acts as decoy, then its effect should not be specific for Cdk6, but would also affect other RNAs bound by TDP-43. Therefore, it is surprising that gadd7 doesn't have an effect on the CFTR splicing regulation by TDP-43. One could argue that gadd7 only sequesters TDP-43 from 3' UTRs. To address this, another 3'UTR bound by TDP-43 needs to be assessed. For instance, TDP-43 binds to its own 3'UTR to auto regulate its own expression. Does gadd7 interfere with the binding of TDP-43 to its own 3' UTR? If yes, since TDP-43 is known to auto regulate its own expression, it's surprising that the expression of TDP-43 doesn't change upon gadd7 expression. If gadd7 doesn't affect TDP-43 binding to its own 3' UTR, then the decoy hypothesis proposed by the authors is unlikely to be true.

Responses: The reviewer is right. We indeed found that overexpression of TDP-43 resulted in reduction of endogenous TDP-43 protein, accompanied by accumulation of shorter products as reported by Polymenidou et al in nature neuroscience in 2011. To take the reviewer's advice, in the revised manuscript, we examined the effect of *gadd7* on the binding of TDP-43 to its own 3'UTR. However, we found that depletion of *gadd7* did not influence the binding as shown in the revised Figure 6D. This result correlates with the finding that the expression of TDP-43 is not changed upon depletion of *gadd7*. There may be several explanations accounting for the unchanged binding and expression. Firstly, the binding site in TDP-43 3'UTR is not the canonical UG-repeat that is known to be the preferred target of TDP-43. Second, the TDP-43-binding site is located in an alternatively spliced intron in the 3'UTR of the TDP-43 pre-mRNA. Thus, *gadd7* may preferentially sequester TDP-43 from 3'UTR with UG repeats in the exon but not in the intron.

3. Fig. 6b is the only evidence supporting the statement that "Gadd7 sequesters TDP-43 from cyclin-dependent kinase 6 (Cdk6) mRNA". Since RIP allows indirect interactions, the result is open to many interpretations. It is necessary that RIP is done also with several other targets of TDP-43, such as its own mRNA. Alternatively, it would be even more convincing if a genome-wide analysis using CLIP was used, which would also show more comprehensively if Gadd7 acts as a decoy to remove TDP-43 from its other mRNA targets.

Responses: This is an important comment. Since genome-wide analysis using CLIP may need too much time, RIP has been done to detect several other 3′UTR targets of TDP-43 in the revised manuscript, such as *TDP-43*, *MEF2D*, *CSNK1A1*, *CSNK2A1*, *Grn*, *SLC1A2* and *hNFL*. In addition, we also analysed the expression of these targets on *gadd7* loss. As expected, *gadd7* could remove TDP-43 from most of these targets and regulate their expression. However, due to its very low expression in CHO-K1 cells, hNFL was hardly detected in RIP assay. Thus, the results for *TDP-43*, *MEF2D*, *CSNK1A1*, *CSNK2A1*, *Grn* and *SLC1A2* have been added in the revised Figure 6D and 6E.

Furthermore, we have also analysed the inhibitory effect of *gadd7* on the interaction of TDP-43 and *Cdk6* mRNA using competition experiment in the revised manuscript. As shown in revised Figure 6C, we observed that non-biotinylated *gadd7* RNA significantly inhibited TDP-43 association with *Cdk6* mRNA in a dose-dependent manner, while *gadd7* mutant RNA with mutated TDP-43 binding sites failed to interfere with the binding even at high dose.

4. At present, it is not possible to say whether the effects of Gadd7 result from regulation of transcription, transcript stability or other effects. In order to claim that release of TDP-43 from the Cdk6 3'UTR mediates the effect of Gadd7, it is necessary to make a luciferase construct containing the Cdk6 3' UTR, and demonstrate that Gadd7 regulates this construct in a manner that depends on the TDP-43 binding site.

Responses: We appreciate the reviewer's suggestion. These experiments have been done in the revised manuscript. As shown in revised Figure 7B, wild type *gadd7*, but not mutant, could reduce

the relative luciferase activity of reporter containing the *Cdk6* 3'UTR downstream of firefly luciferase gene.

Minor comments

1. Supplementary figure 5C has no statistics.

Responses: To take the reviewer's advice, statistics has been provided in the revised Supplementary Figure S6C (the former Supplementary Figure S5C).

2. The paper would benefit from further proof-reading due to several errors. However, on the whole it is well written.

Responses: We really appreciate the reviewer's advice. In the revised manuscript, we have conducted a thorough editing work to improve the quality of paper.

3. Page 3 - When describing lincRNA-p21, the sentence in its present form does not make sense and should be modified to "LincRNA-p21, a lncRNA downstream of p53,..."

Responses: We appreciate this comment. The sentence has been revised in the revised version of the manuscript.

4. The start of the first paragraph on 4 requires re-wording in places.

Responses: Thanks for the comment. The sentence has been revised.

5. Page 10 - "Consistent with the observation that silence of TDP-43..." - should be silencing

Responses: It has been revised according to the reviewer's suggestion.

6. Page 12 - The last sentence of the results requires re-wording

Responses: The sentence has been re-written according to the reviewer's suggestion.

7. Page 15 - Half-time should be half-life

Responses: The half-time has been changed to half-life in the revised manuscript.

Referee #2:

The authors study the regulation of cell cycle G1/S checkpoint by the long non-coding growth arrest and DNA damage-inducible transcript 7 (gadd7). They first confirm and extend the notion that gadd7 is induced by genotoxic stress and growth arresting conditions, and that gadd7 contributes to G1/S checkpoint control. Using biotinylated gadd7 RNA as an affinity probe, they discover heterogenous nuclear ribonucleoproteins as interactor proteins. Among these, TDP-43 is selected and validated in a convincing manner. Importantly, it is found that gadd7 apparently competes with TDP-43 binding to Cdk6 mRNA, previously identified independently as a TDP-43 target. Although the direction of TDP-43 effects in the CHO-K1 cells used here are inverse to the original findings in HeLa cells (likely reflecting cell type specificity), the present data are most concise and functionally consistent. The manuscript is written very well, the results are clearly displayed and comprehensible, and make a clear point for this interesting and innovative report.

The authors should consider the following suggestions to improve the paper:

1. Although I agree with the argument that some TDP-43 mediated mRNA effects are remarkably variable among different cell types (the authors must comprehensively discuss from the published TDP-43 screens where Cdk6 alterations were measured and where not), it is still a matter of

concern how relevant the observed findings are. In the present context, it would be desirable to check the gadd7 - TDP-43 - Cdk6 pathway in appropriate cell cycle and/or cancer models.

Responses: We appreciate the reviewer's comments on these issues. Although several papers have reported that TDP-43 could bind to *Cdk6* as measured by RIP-seq or iCLIP-seq, the expression of Cdk6 regulated by TDP-43 was not well shown in these papers. To our knowledge, to date, the expression of Cdk6 appears only to be well measured by Ayala et al in 2008 PNAS. We have added this information in the revised manuscript.

Since *gadd7* was only detected in the hamster cells, we are unable to exert functional work in other cancer models. Indeed, we have demonstrated that the *gadd7* - TDP-43 - Cdk6 pathway mainly functions in G1 phase. To make the results clear, we analysed the G1/S checkpoint upon simultaneous knockdown of *gadd7* with TDP-43 or Cdk6. We found that simultaneous knockdown of *gadd7* with TDP-43 or Cdk6 substantially rescued the defective G1/S checkpoint by *gadd7* loss. These new data are shown in revised Figure 5E and 5F.

2. Some more epistatic experiments would strengthen the conclusions. Does Cdk6 (and TDP-43) transfection overcome the G1/S checkpoint failure after gadd7 RNAi?

Responses: Because the expression of Cdk6 was increased after *gadd7* knockdown, we then analysed the effect of *gadd7* on the G1/S checkpoint after Cdk6 or TDP-43 depletion and found that depletion of TDP-43 or Cdk6 rescued the G1/S checkpoint failure upon *gadd7* loss. These results have been provided in the revised Figure 5E and 5F.

3. Along these lines, how specific/relevant is the gadd7 - Cdk6 connection? TDP-43 affects thousands of transcripts. The authors do provide the CFTR ex9 splice assay as a general negative specificity control, but could there not be other, cell cycle genes involved in the present process?

Responses: We really thank for the reviewer's question. Indeed, recent papers have identified large sets of putative TDP-43 RNA targets through RIP-seq or CLIP-seq. However, only small parts of these targets have been further validated, and among these targets, *Cdk6* seems to be the only transcript that is involved in G1/S transition. Furthermore, we also detected the binding of TDP-43 to other G1 phase-related transcripts. However, no significant association was observed between TDP-43 and these transcripts. The results have been provided in the revised Figure 6A.

4. Comparative competition experiments as in Fig. 3D should be done also with Cdk6 RNA. Measure affinities and confirm that gadd7 RNA binds TDP-43 more strongly than Cdk6 RNA.

Responses: Thanks for the reviewer's helpful suggestions. We have taken this advice and conducted such competition experiments in the revised manuscript. However, as shown in the revised Figure 6C, the binding affinity of TDP-43 with *gadd7* RNA is lower compared with *Cdk6* RNA in competition experiments, although the binding of TDP-43 to *gadd7* RNA is stronger than *Cdk6* RNA in RIP assay.

The reason for this discrepancy may be due to the differences between these two kinds of experiments. In biotin RNA pull-down assay, the biotin-labelled transcripts were pulled down by streptavidin beads, and their associated cellular proteins were subjected to Western blotting. The signal of Western blotting reflects the quantity of protein associated with biotin-labelled RNAs. *Cdk6* 3′UTR may capture more TDP-43 than *gadd7* RNA in competition experiments, as *Cdk6* 3′UTR is much longer than *gadd7* RNA and contains more UG or GU repeats. However, in RIP assay, the antibody against TDP-43 was captured by protein A/G beads, and co-precipitated RNAs were then analysed by RT-PCR. The UG or GU repeats in *gadd7* is much denser than in *Cdk6* 3′UTR. Hence, TDP-43 molecular may bind to *gadd7* more strongly than *Cdk6* RNA in RIP assay. Furthermore, based on the induction of *gadd7* upon genotoxic and nongenotoxic stresses, *in vivo*, the binding of TDP-43 to *gadd7* should be stronger than *Cdk6* and sequester TDP-43 from *Cdk6* mRNA following DNA damage.

5. Please spell out the full sequences around the (UG) repeats in Fig. 3F.

Responses: We have taken the reviewer's advice and added the sequences around the (UG) repeats in revised Fig. 3E.

Minor Corrections:

6. Fig. 1A annotation gadd45 (not gaddd45)

Responses: We are sorry about this mistake, and have corrected it in the revised version.

7. Page 5, line 18: delete "clearly" - the effects in Fig. 2B and C are not so strong (Fig. 2B lacks statistics)

Responses: Thanks for this suggestion. The word "clearly" has been deleted. The statistics has been provided in the revised Fig. 2B.

8. Fig. 3A and Fig. 6E are dispensable; methodology and effect are clear enough

Responses: We have deleted the Fig. 3A. But we retain the Fig. 6E (the revised Figure 7C) as this model may make the conclusion clear at a glance.

Referee #3:

This manuscript describes a potentially interesting study that addresses a mechanism for the CHO-specific lncRNA gadd7 in modulating the activity of TDP-43 in regulating the stability of the Cdk6 mRNA. However in its present form, I find several aspects of the study to be rather preliminary and lacking the requisite data to provide strong support for the models that are put forth. My main concerns include:

1. Fig. 2: The negative effects of gadd7 on cell growth are not well-supported in my opinion. Panel B shows a relatively small effect on cell number, particularly when the data in panel C indicate that the control cells have failed to recover from the stress of the lipofectamine based transfection protocol used (only ~40% viable based on the MTT assay). This large amount of cell death in the control cells brings into question any conclusions on cell growth drawn from these comparative data. Less toxic transfection conditions need to be established to provide a more meaningful comparison.

Responses: We appreciate the reviewer's comments. In our studies, we repeated these assays many times and found that once *gadd7* was knocked down, the cells would grow faster than control, although the effects of *gadd7* depletion are not so strong in Fig. 2B and 2C. One explanation accounting for the small effect might be that basal CHO-K1 cells already grow very quickly (although it was cultured with 5% serum) so that it is hard to make the cell grow much faster upon depletion of *gadd7*.

In addition, we did not see significant cell death of CHO-K1 caused by transfection using lipofectamine. The value in the Fig. 2C represents the MTT absorbance in 570 nm. This way may not deliver a clear presentation. In the revised Figure 2B and 2C, the growth curve and MTT absorbance were re-analysed as relative cell number and viability compared with negative control, respectively.

2. Fig. 2D/E: I would recommend that the supplementary data be moved into the main text and these representative traces switched into the supplementary data. The supplementary data make the point better since they also allow the reader to assess the reproducibility of the experiment.

Responses: We appreciate the reviewer's advice. To take this suggestion, we have moved the supplementary data into the main text (the revised Figure 2D and 2E), and moved the representative results to the supplementary information (the revised Supplementary Figure S2).

3. Fig. 3: Two points: (a) The authors need to better establish the binding specificity of TDP-43 for gadd7 RNA. In panel D, the use of tRNA as a control competitor RNA is not optimal due to its

unique structural content. An ssRNA competitor would be much more informative. (b) the authors infer from these large deletion RNAs that the tgtgtg repeats (which should be UGUGU since its RNA) are involved in the binding. It would be very easy to demonstrate the importance of these repeats by making the appropriate deletions/mutations in the RNA. Establishing the specificity of TDP-43 and the underlying reasons for it are important conclusions that provide impact for the study.

Responses: We appreciate the reviewer's suggestions. In the revised manuscript, these points are well taken:
(a) We used the *GAPDH* RNA as the control competitor in the revised Fig. 3C and Fig. 6C.
(b) The UG or GU repeats have been mutated and the interaction between these mutants and TDP-43 was analysed. As expected, the association between *gadd7* and TDP-43 was almost completely abolished once all UG and GU repeats were mutated as shown in the revised Figure 3F.

4. Fig. 3/Table S1 and elsewhere: The data indicate that gadd7 associates with a variety of major RNA binding proteins in addition to TDP-43. Therefore in the gadd7 KD experiments (e.g. Fig. 6), without additional controls how can the authors be sure that the effects that are observed are occurring through TDP-43 effects and not any of the other RBPs that are associated with the lncRNA? The study needs to be more comprehensive in its analysis of the effects of the gadd7 RNA on RBPs in order to ensure that the conclusions drawn are appropriate.

Responses: We thank the reviewer for this excellent question. *gadd7* could interact with several proteins, the majorities of which were the heterogeneous nuclear ribonucleoproteins (hnRNPs). It is reported that C-terminal region of TDP-43 is capable of binding directly to several proteins of the hnRNP family including hnRNP A1/A2/B1, which is critical for the function of TDP-43. Thus, we also studied the relationship between *gadd7* and other hnRNPs, such as hnRNP A2/B1. The preliminary results showed that hnRNP A2/B1 seemed to work together with TDP-43 to co-regulate Cdk6 expression, and *gadd7* competitively interacted with these two proteins. However, in this paper, we focused on the relationship between *gadd7* and TDP-43, as comprehensive study of the hnRNPs might make the research work complex and beyond the scope of this submission. In the future, more comprehensive study will be done to analyse how *gadd7* affects hnRNPs complex.

5. The differences observed on the relationship of TDP-43 to the cdk6 mRNA here versus the Ayala et al 2008 study published in PNAS are surprising. I would strongly recommend that the authors follow up their idea of cell-type specific differences to try to come up with a more in depth explanation of this troubling discrepancy.

Responses: The reviewer is right. We appreciate this question.
Regarding the differences observed on the relationship of TDP-43 to the *Cdk6* mRNA in our study versus the Ayala et al 2008 study published in PNAS, we noticed that the cell line used in PNAS is HeLa cell line. As we known, HeLa is HPV positive cancer cell line derived from human cervical cancer cells. However, CHO-K1 is an immortalized cell line derived from Chinese hamster ovary. It means that both the species and backgrounds of these two cell lines are totally different, which may be the possible causes for this discrepancy. As reported by Ayala et al in PNAS, the loss of TDP-43 has no effect on Cdk6 protein levels in chicken cells (DF-1), since chicken *Cdk6* lacks TG repeats completely. Furthermore, although all of human, rat, mouse and hamster *Cdk6* have the TG repeats, the distribution and mounts of TG repeats are quite different. In addition, the different sources and features of these two cell lines may lead to other different factors involved in this process. Therefore, the mechanism by which TDP-43 regulates the Cdk6 may be different, and then lead to various outcomes. As suggested by the reviewer, we have added these opinions in the discussion section of the revised manuscript.

6. Fig. 4: Could the increase in RNP complexes under UV treatment conditions simply be due to an increased stabilization of existing complexes by UV cross-linking (thus they are more readily co-IPPT'd) rather than an increase in the binding as concluded by the authors?

Responses: UV irradiation used in our assay is quite different from canonical UV cross-linking. In our assay, exponentially growing CHO-K1 cells were irradiated with 40 J/m^2 UV radiation. After cells exposure to UV irradiation, fresh medium was added and the cells were cultured for 4 h till harvest. However, in UV cross-linking assay, cells are usually irradiated with much higher dose of

UV irradiation (e.g. 150 J/m2) for once or several times, and harvested immediately without additional culture. In addition, lysates of cells or tissue can also be cross-liked *in vitro*. Therefore, based on the induction of *gadd7* following UV irradiation and the differences between our UV irradiation and UV cross-linking, the increased association between *gadd7* and TDP-43 following UV irradiation should be due to the increased expression of *gadd7* rather than increased stabilization of complexes. Furthermore, the binding of TDP-43 to other RNA targets, such as *GAPDH* or *Cdk6*, did not increase following UV irradiation, which also excludes the possibility that increased interaction between *gadd7* and TDP-43 is the result of increased stabilization of complexes.

7. Fig. 6: It is very surprising that the extremely large change in stability of the Cdk6 mRNA (panel D) is not reflected by a dramatic overall increase in cdk6 mRNA abundance in Panel C (its abundance is only up ~10-15%). Why the discrepancy in these data?

Responses: Thanks for this nice question. The expression of Cdk6 is regulated by *gadd7* through post-transcription rather than transcription level. mRNA levels are the result of an regulated balance between de novo transcription and mRNA decay. Hence, after transcription is blocked by actinomycin D, the effect of *gadd7* on Cdk6 expression will be amplified as interference from newly-synthesized RNA is abolished.

8. Other points:

a. The figure legends/methods are a bit unclear in spots. In particular, due error bars in all cases represent independent experiments or the sampling error in individual samples?

Responses: We thank the reviewer for the insightful suggestions. We made much efforts to improve the figure legends and methods. In the present version, we indicated the number of experiments and the types of replicates in the revised figure legends.

b. The grammar/writing in several places of the manuscript could use some polishing.

Responses: To take the reviewer's advice, we have conducted a thorough editing work in the revised manuscript in order to prove the quality of the paper.

2nd Editorial Decision 15 August 2012

Thank you for submitting a revised version of your manuscript to the EMBO Journal. It has now been seen by two of the original referees whose comments are shown below. As you will see while referee #2 finds that the revised version has addressed the issues raised by the referees, referee #1 finds that additional experiments are required to fully support the model that gadd7 works through TDP43 sequestration. In particular, the referee argues that the broader consequence of gadd7 expression for TDP43 function remains unclear and questions the normalization procedure and stringency employed in the RIP experiments in fig 6D.

Due to this discrepancy between the referees, I consulted with referee #2 and discussed your manuscript with my colleagues in the editorial team. As a result we have reached the conclusion that since your study focuses on the role of gadd7 as a regulator of Cdk6 and does not aim to address all TDP43 cellular targets, we will not insist that you perform the additional splicing experiments suggested by referee #1. However, you should address the issue by discussing the mechanistic implications of the different behavior of various RNAs upon depletion of gadd7. You should also comment on why some TDP43 targets may escape regulation by gadd7 and how the high TDP43 binding affinity for Cdk6 relative to gadd7 can be accommodated in the sequestration model.

With regard to the RIP experiments in fig. 6D, the enrichment of target genes in the TDP43-IP as a result of gadd7 depletion is an important argument in support of the TDP43 sequestration model. I therefore have to ask you to provide RIP-data based on a high stringency cross-linking protocol as requested by referee #1 and with a clear normalization for transcript levels to ensure that the observed enrichment reflects functional complex formation and not an indirect effect of altered expression of TDP43 target genes upon depletion of gadd7.

In addition, there are a few remarks regarding the submission of a revised version that I would like to make. We now generally encourage the publication of source data, particularly for electrophoretic gels and blots, with the aim of making primary data more accessible and transparent to the reader. Would you be willing to provide files comprising the original, uncropped and unprocessed scans of all gels used in the figures? We would need 1 file per figure (which can be a composite of source data from several panels) in jpg, gif or PDF format, uploaded as "Source data files". The gels should be labelled with the appropriate figure/panel number, and should have molecular weight markers; further annotation would clearly be useful but is not essential. These files will be published online with the article as a supplementary "Source Data". Please let me know if you have any questions about this policy.

Thank you again for giving us the chance to consider your manuscript for The EMBO Journal, I look forward to your revision.

Yours sincerely,

Editor
The EMBO Journal.

REFEREE REPORTS:

Referee #1:

The authors have performed a number of additional experiments to address the requests of referees. Unfortunately, the results of these experiments do not provide a solid support for the gadd7 sequestration model.

Initially the authors proposed that gadd7 specifically regulates Cdk6 (but not CFTR). Now the authors also assessed other mRNAs bound by TDP-43, and found same changes in these mRNAs as in Cdk6. If gadd7 sequesters TDP-43 from all its RNA targets, as the authors now suggest, why is the splicing function of TDP-43 in CFTR intact? Is the CFTR just a special case, or are other exons also intact? Since dozens of validated exons are known, this would be easy to test. The authors should test more regulated exons - for instance, the alternative exon in MEF2D could be analysed.

Only a few validated mRNA targets of TDP-43 at the level of mRNA stability are known. TDP-43 mRNA is the best studied example, and the authors now find no change in expression of TDP-43 mRNA (nor any change in RNA binding), which is perplexing. In contrast, most of the mRNAs that bind TDP-43, such as for instance MEF2D, were not changed at the level of mRNA stability in the past studies. For example, TDP-43 was shown to regulate alternative splicing, and not mRNA stability of MEF2D. Expression changes in these mRNAs are therefore unlikely to involve a direct activity of TDP-43.

Fig. 6C shows that the in vitro affinity of TDP-43 to Cdk6 is larger than to gadd7, which raises concerns about the gadd7 sequestration model. Moreover, the new Fig. 6D and 6E show that the changes in TDP-43 binding (6D) reflect the changes in expression (6E). This indicates that the changes observed by RIP assay may be an artifact of changes in expression of these mRNAs. This could be due to inappropriate normalization of the binding data. I did not find any description of RIP normalization in the manuscript. Did the authors normalize the RIP data? Moreover, to avoid the re-association of mRNAs in RIP, the RIP data would be more convincing if the authors UV crosslinked the cells before performing RIP (as in Fig 5C here http://genomebiology.com/2012/13/3/R17/figure/F5).

Referee #3:

Overall I find the revised manuscript to be much improved and responsive to the comments raised in the previous round of review. The study significantly advances our understanding of the role for a

lincRNA from both a biological and mechanistic perspective. Its timely and should have significant impact in the field.

2nd Revision - Authors' Response 06 September 2012

Response to Editor:

Thank you for submitting a revised version of your manuscript to the EMBO Journal……. However, you should address the issue by discussing the mechanistic implications of the different behaviour of various RNAs upon depletion of gadd7. You should also comment on why some TDP43 targets may escape regulation by gadd7 and how the high TDP43 binding affinity for Cdk6 relative to gadd7 can be accommodated in the sequestration model.

Responses: We highly appreciate your encouraging suggestion. In the revised version of the manuscript, we have commented on these issues in the discussion section and addressed all the concerns raised by the reviewer point by point in this response letter to the reviewer (see below).

With regard to the RIP experiments in fig. 6D, the enrichment of target genes in the TDP43-IP as a result of gadd7 depletion is an important argument in support of the TDP43 sequestration model. I therefore have to ask you to provide RIP-data based on a high stringency cross-linking protocol as requested by referee #1 and with a clear normalization for transcript levels to ensure that the observed enrichment reflects functional complex formation and not an indirect effect of altered expression of TDP43 target genes upon depletion of gadd7.

Responses: According to your advice, we have performed the RIP assay in UV cross-linked cells and normalized the binding data to transcript levels (input) (see Supplementary Figure S7). Similar to native RIP assay in Figure 6D, UV cross-linking RIP showed that depletion of *gadd7* was sufficient to increase the binding of TDP-43 to most of these 3'UTR targets.

In addition, there are a few remarks regarding the submission of a revised version that I would like to make. We now generally encourage the publication of source data, particularly for electrophoretic gels and blots, with the aim of making primary data more accessible and transparent to the reader. Would you be willing to provide files comprising the original, uncropped and unprocessed scans of all gels used in the figures? We would need 1 file per figure (which can be a composite of source data from several panels) in jpg, gif or PDF format, uploaded as "Source data files". The gels should be labelled with the appropriate figure/panel number, and should have molecular weight markers; further annotation would clearly be useful but is not essential. These files will be published online with the article as a supplementary "Source Data". Please let me know if you have any questions about this policy.

Responses: Yes, we are delighted to do this.

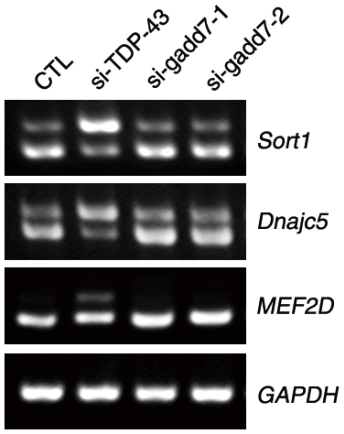
Response to reviewers:

Referee #1:

The authors have performed a number of additional experiments to address the requests of referees. Unfortunately, the results of these experiments do not provide a solid support for the gadd7 sequestration model.

Initially the authors proposed that gadd7 specifically regulates Cdk6 (but not CFTR). Now the authors also assessed other mRNAs bound by TDP-43, and found same changes in these mRNAs as in Cdk6. If gadd7 sequesters TDP-43 from all its RNA targets, as the authors now suggest, why is the splicing function of TDP-43 in CFTR intact? Is the CFTR just a special case, or are other exons also intact? Since dozens of validated exons are known, this would be easy to test. The authors should test more regulated exons - for instance, the alternative exon in MEF2D could be analysed.

Responses: We appreciate the reviewer's comment and suggestion on these issues. As we known, TDP-43 has thousands of putative RNA targets with its binding sites in various regions, including intron, exon, 3′UTR, 5′UTR and ncRNAs. Furthermore, although the canonical TDP-43 binding site is UG-rich motif, TDP-43 could also bind to non-UG repeat sequences. Therefore, based on the complexity of TDP-43 targets, *gadd7* appears not to sequester TDP-43 from all its RNA targets and then regulate their functions. As suggested by the reviewer, the alternative splicing function of *gadd7* in other several targets (*MEF2D*, *Sort1* and *Dnajc5*) was also analysed (Figure attached below). We find that *gadd7* has no function in alternative splicing of these genes as in CFTR. In addition, *gadd7* seems not to sequester TDP-43 from all its 3'UTR targets, such as its own 3'UTR and *SLC1A2*, indicating that *gadd7*-mediated sequestering function can be RNA specific and some other factors may be involved in this process. Thus, we conclude that *gadd7* is able to sequester TDP-43 from at least some of its 3'UTR targets but not all its RNA targets.



Effects of TDP-43 and *gadd7* depletion on alternative splicing of *MEF2D*, *Sort1* and *Dnajc5*.

The indicated siRNAs targeting TDP-43 and *gadd7* were transfected into CHO-K1 cells. 48 h after transfection, total RNA was extracted, and splicing pattern was examined by semi-quantitative RT-PCR. *GAPDH* was used as internal reference. CTL: control siRNA.

Only a few validated mRNA targets of TDP-43 at the level of mRNA stability are known. TDP-43 mRNA is the best studied example, and the authors now find no change in expression of TDP-43 mRNA (nor any change in RNA binding), which is perplexing. In contrast, most of the mRNAs that bind TDP-43, such as for instance MEF2D, were not changed at the level of mRNA stability in the past studies. For example, TDP-43 was shown to regulate alternative splicing, and not mRNA stability of MEF2D. Expression changes in these mRNAs are therefore unlikely to involve a direct activity of TDP-43.

Responses: We are really grateful to the reviewer's question. Indeed, TDP-43 could auto regulate its own protein level, but it needs to note that the mechanism is very special. The TDP-43-binding site is located in the alternatively spliced intron 7 on the 3'UTR of its own mRNA and is not a UG-

repeat but a 34-nucleotide sequence. TDP-43 auto regulates its own protein level by directly binding and enhancing splicing of intron 7, thereby triggering degradation (Avendano-Vazquez et al, 2012; Polymenidou et al, 2011). Thus, splicing of intron 7 is a critical event for TDP-43 auto regulation. In our cell-based assay, we also found that overexpression of TDP-43 resulted in reduction of endogenous TDP-43 protein, accompanied by accumulation of shorter products as reported by Polymenidou et al. However, *gadd7* has no effect on the expression patter of TDP-43. As discussed above, *gadd7* appears not to regulate the alternative splicing, thus it is reasonable to explain that *gadd7* has no effect on the expression of TDP-43.

With regard to *MEF2D*, TDP-43-binding sites are present not only in intron but also in 3'UTR of mRNA, which indicates that TDP-43 can remain associated with the *MEF2D* mRNA after splicing is finished and thereby likely regulate additional aspects of RNA processing, such as turnover and stability. Indeed, in addition to its effect on RNA splicing, TDP-43 also influences lots of mRNA expression.

Fig. 6C shows that the in vitro affinity of TDP-43 to Cdk6 is larger than to gadd7, which raises concerns about the gadd7 sequestration model. Moreover, the new Fig. 6D and 6E show that the changes in TDP-43 binding (6D) reflect the changes in expression (6E). This indicates that the changes observed by RIP assay may be an artifact of changes in expression of these mRNAs. This could be due to inappropriate normalization of the binding data. I did not find any description of RIP normalization in the manuscript. Did the authors normalize the RIP data? Moreover, to avoid the re-association of mRNAs in RIP, the RIP data would be more convincing if the authors UV cross linked the cells before performing RIP (as in Fig 5C here http://genomebiology.com/2012/13/3/R17/figure/F5).

Responses: We really appreciate the reviewer's comment and advice. Although the *in vitro* affinity of TDP-43 to *Cdk6* is larger than to *gadd7*, the *in vivo* binding of TDP-43 to *Cdk6* RNA is weaker than to *gadd7* RNA in RIP assay. The reason for this discrepancy may be due to the differences between these two kinds of experiments as discussed before. Briefly, since *Cdk6* 3′UTR is much longer than *gadd7* RNA and contains more UG or GU repeats, *Cdk6* 3′UTR may capture more TDP-43 than *gadd7* RNA in biotin RNA pull-down assay. However, based on the more dense and centralized UG or GU repeats in *gadd7*, TDP-43 molecular may bind to *gadd7* more strongly than *Cdk6* RNA in RIP assay. Furthermore, RNA secondary structures probably mediate the association between TDP-43 and these targets. The secondary structures of biotin-labelled RNA transcripts produced *in vitro* may be different from *in vivo* transcripts. RIP assay is an *in vivo* method, which may more accurately reflect the *in vivo* binding affinity than biotin RNA pull-down assay. More importantly, following UV irradiation, *gadd7* is substantially induced and more *gadd7* binds to TDP-43, thus leading to sequestration of TDP-43 from *Cdk6* RNA and then down regulation of *Cdk6* expression.

We are sorry about the less clear description for the legend of Figure 6. The RIP data were normalized to corresponding IgG with same input and treatment as TDP-43 antibody. We have added this information in the legend in the revised version of the manuscript. Furthermore, as suggested by the reviewer, we have done RIP assay in UV cross-linked cell extracts and normalized the binding data to transcript levels (input) to avoid interference from changed expression and re-association of mRNAs in native RIP. Similar to native RIP assay, enrichment to TDP-43 was observed for most of RNA targets (including *Grn*, the binding of which was reduced in the native RIP) in UV cross-linking RIP assay when *gadd7* was depleted (see revised Supplementary Figure S7)

Referee #3:

No issues to address

References

Avendano-Vazquez SE, Dhir A, Bembich S, Buratti E, Proudfoot N, Baralle FE (2012) Autoregulation of TDP-43 mRNA levels involves interplay between transcription, splicing, and alternative polyA site selection. *Genes Dev* 26: 1679-1684

Polymenidou M, Lagier-Tourenne C, Hutt KR, Huelga SC, Moran J, Liang TY, Ling SC, Sun E, Wancewicz E, Mazur C, Kordasiewicz H, Sedaghat Y, Donohue JP, Shiue L, Bennett CF, Yeo GW, Cleveland DW (2011) Long pre-mRNA depletion and RNA missplicing contribute to neuronal vulnerability from loss of TDP-43. *Nat Neurosci* 14: 459-468

3rd Editorial Decision 17 September 2012

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

As you will see the referee finds that the requested experiments have been sufficiently addressed. However, s/he still requires you to include the new splicing experiments in a main figure and asks that you rephrase the results and discussion to present sequestration as one of several possible explanations for the differential effect of gadd7 expression on TDP-43 targets. With these additional changes implemented in the manuscript s/he would support publication.

Given this positive recommendation from the referee we invite you to submit a revised version of your manuscript including these final changes. In addition, I would like to remind you to upload source data for all figures as mentioned in my previous email.

Thank you again for giving us the chance to consider your manuscript, I look forward to your revision.

Yours sincerely,

Editor
The EMBO Journal.

REFEREE REPORT:

Referee #1:

The authors have made a significant effort to perform additional experiments. The authors now convincingly show that gadd7 affects the interactions between TDP-43 and some of its mRNA targets. They also show that splicing function of TDP-43 remains intact, which is important for the manuscript. It is therefore crucial that the latest results with splicing changes of MEF2D, Sort1 and Dnajc5 are added into one of the main figures of the manuscript.

So far, the past studies where a protein was sequestered showed loss of interaction with all of its RNA targets. The authors provide no data to explain how TDP-43 could be sequestered from some RNAs, but not others. They also provide no imaging data to confirm that TDP-43 is sequestered. Therefore, the authors can not assume sequestration, and instead in the abstract and results they should just describe what they find: gadd7 affects the interactions between TDP-43 and some of its mRNA targets. In the discussion, they can mention sequestration as one possible model, but they should also discuss other models, such as indirect effects of cell stress, changes in other RBPs, etc.

3nd Revision - Authors' Response 21 September 2012

Response to Editor:

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

As you will see the referee finds that the requested experiments have been sufficiently addressed. However, s/he still requires you to include the new splicing experiments in a main figure and asks that you rephrase the results and discussion to present sequestration as one of several possible explanations for the differential effect of gadd7 expression on TDP-43 targets. With these additional changes implemented in the manuscript s/he would support publication.

Responses: Thank you very much for your suggestion. In the revised version of the manuscript, the new splicing experiments were added to Figure 5 according to your advice. Additionally, the result and discussion about the differential effect of *gadd7* on TDP-43 targets were greatly revised. In the revised manuscript, we have discussed the several possible models of *gadd7* regulation on TDP-43 and its target mRNAs.

Given this positive recommendation from the referee we invite you to submit a revised version of your manuscript including these final changes. In addition, I would like to remind you to upload source data for all figures as mentioned in my previous email.

Responses: Thank you very much for your reminding. All source data for figures were uploaded according to your suggestion.

Response to reviewers:

Referee:

The authors have made a significant effort to perform additional experiments. The authors now convincingly show that gadd7 affects the interactions between TDP-43 and some of its mRNA targets. They also show that splicing function of TDP-43 remains intact, which is important for the manuscript. It is therefore crucial that the latest results with splicing changes of MEF2D, Sort1 and Dnajc5 are added into one of the main figures of the manuscript.

Responses: We greatly appreciate the reviewer's suggestion. In the revised version of the manuscript, we have added the splicing results of *MEF2D*, *Sort1* and *Dnajc5* into Figure 5.

So far, the past studies where a protein was sequestered showed loss of interaction with all of its RNA targets. The authors provide no data to explain how TDP-43 could be sequestered from some RNAs, but not others. They also provide no imaging data to confirm that TDP-43 is sequestered. Therefore, the authors can not assume sequestration, and instead in the abstract and results they should just describe what they find: gadd7 affects the interactions between TDP-43 and some of its mRNA targets. In the discussion, they can mention sequestration as one possible model, but they should also discuss other models, such as indirect effects of cell stress, changes in other RBPs, etc.

Responses: We appreciate the reviewer's advice. In the revised manuscript, we have revised the abstract and results, and discussed several possible models of *gadd7* regulation on TDP-43 and its target mRNAs instead of just describing the sequestration as the regulatory model according to the reviewer's suggestion. The mechanism discussed in the revised manuscript is shown below.

One of the possible mechanisms by which *gadd7* affects the interaction between TDP-43 and its RNA targets is that *gadd7* directly binds to and sequesters TDP-43 from its 3'UTR targets. Furthermore, other indirect effects may also exist. TDP-43 has extensive interactions with RNA binding proteins (Freibaum et al, 2010; Sephton et al, 2011). The changed expression of these RNA binding proteins or interaction with TDP-43 upon *gadd7* depletion may also affect the binding of TDP-43 to its targets. Moreover, under stress, TDP-43 is localized to stress granules (SGs) (Colombrita et al, 2009; Freibaum et al, 2010; McDonald et al, 2011) which are dynamic triage

centres for mRNA storage, decay or re-initiation during stress conditions (Anderson & Kedersha, 2008), thus the localization of TDP-43 to SGs may likely contribute to the changed interaction with its RNA targets upon *gadd7* depletion.