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A large-scale functional screen identifies Nova1 and Ncoa3 as regulators of neuronal miRNA function

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(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.)

Editor: Anne Nielsen

1st Editorial Decision 14 November 2014

Thank you for submitting your manuscripts to The EMBO journal. I have now read them both and also discussed the reported findings with other members of the editorial team. However, I am afraid the outcome of these discussions is that we cannot offer to pursue publication of these manuscripts as two separate studies at the current stage.

I understand that you would aim to describe the two hits from the screen in separate studies, but in our view neither manuscript currently offers sufficient advance and mechanistic insight to be a strong candidate for publication on its own in The EMBO Journal. However, we would be interested in a combined manuscript that highlights the strength of your screen by describing these two new factors with distinct roles in stimulating miRNA function in neurons. I realize that you already have a lot of data in both of the separate manuscripts, but as you also point out in the discussions there are also a number of open questions remaining at this stage - especially with regard to mechanism and specificity - that would have to be addressed further for the separate studies to provide the level of insight and conclusiveness required for publication here.

If you (and the first authors of either study) would be interested in submitting the two manuscripts to us as a fused study, I'd be happy to be involved in the discussion of how best to structure the figures (possibly allowing parallel data for the functional role of Ncoa3 and Nova1 to be presented together).

Please feel free to contact me with any questions to this and thank you again for giving us the opportunity to consider this work. I am sorry that we cannot be more positive about the manuscripts as separate stories but I do hope you will be interested in submitting them to us as a fused manuscript.

Resubmission 24 November 2014

2nd Editorial Decision 09 January 2015

Thank you for submitting your manuscript for consideration by the EMBO Journal and my apologies for the slight delay in communicating our decision to you, brought on by the recent holidays. We have now heard back from all three referees and you will find their comments included below.

As you will see from the reports, all referees express interest in the findings reported in your manuscript, but they do also raise a number of concerns that will have to be addressed in detail before they can support publication of the manuscript.

I would particularly ask you to focus your efforts on the following points:
-> please provide further mechanistic insight on the role for Nova1 in modulating miRISC function (ref #1 and #3)
-> please comment on the timing and efficiency of knock-down experiments (ref #2 and #3). I realize that part of this problem results from the merger of the two original manuscripts but I would still ask you to comment on the points and include additional experimental data, if necessary.
-> please also expand the analysis of transcriptional control by Ncoa3, especially with regard to miRNA specificity as pointed out by ref#1. I would also ask you to comment/clarify the lack of effect seen upon Drosha KD that puzzles all three refs.

In addition, to these more general points, the refs ask you to address a number of minor concerns regarding data presentation and to comment on a few possible inconsistencies. Ref #3 also asks for the inclusion of a positive control for the initial screen, but in our view this point lies outside the scope of a revision. You will see that both refs #2 and #3 remain rather unconvinced by the Ncoa3 localisation data in fig 5 and you may consider removing that data altogether.

Given the referees' overall positive recommendations, I would like to invite you 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 of your manuscript will therefore depend on the completeness of your responses in this revised version.

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

REFEREE COMMENTS

Referee #1:

In this manuscript 'A large-scale functional screen identifies Nova1 and Ncoa3 as regulators of neuronal miRNA function' Störchel and colleagues performed an siRNA-mediated loss-of-function screen in mouse primary cortical neurons to identify novel factors that can impact the miRNA repression of a luciferase reporter gene containing miR-134 binding sites in its 3' untranslated region (3' UTR). This approach and subsequent validation identified the RNA-binding protein Nova1 as well as the transcriptional co-activator Ncoa3 as proteins that might promote miRNA function. Follow-up experiments uncovered that Nova1 physically associates with Argonaute 2 (Ago2) in the cell cytoplasm to somehow promote miRISC activity, and supporting experiments show that Nova 1

can suppress reporter gene expression when tethered to the Luciferase 3' UTR. Ncoa3 was found to function upstream of Ago2 possibly by a direct transcriptional activation of Ago2 expression, to regulate dendrite complexity.

The strategy and assays used in this study are generally considered appropriate and the identification of factors required for the miRNA pathway in neurons is an interesting topic that could in principle be appropriate for publication in EMBO J. However the mechanistic aspect of this work is only minimally developed and there are some apparent discrepancies that need to be addressed.

Major concerns:

1) Although the authors show that Nova1 can associate with Ago2 in co-IP experiments, the role of Nova1 in the miRNA pathway is obscure throughout the manuscript even though they concluded and discussed that Nova1 regulates miRISC activity. What possible role might Nova1 have? Considering the tethering data (Figure 5B) it is possible that Ago2 recruits Nova1 to target 3' UTRs. Does Nova1 associate with target mRNA? Is this possible recruitment miRNA/Ago2 dependent? This could be addressed by Nova1 pull-down and q.RT-PCR. Does ectopic Nova1 expression enhance miRISC activity in non-neuronal cells? Additional insight into the role of Nova1 in miRISC regulation is needed.

2) Similarly, since the authors conclude that Ncoa3 might be required for Ago2 expression it seems preliminary (based on the presented data) to suggest that this is by the direct transcriptional regulation of Ago2 expression. The inclusion of Ncoa3 chromatin IPs and functional assays using luciferase reporters containing Ago2 promoter/regulatory sequences combined with Ncoa3 knockdown are considered necessary to make this conclusion that Ncoa3 directly regulates Ago2 expression. Also, since not all 3' UTRs tested were responsive to Ncoa3 depletion, how can the apparent target site specificity be explained is Ago2 is required for miRISC function? Why are only some sites responsive to Ncoa3 depletion? What determines the observed specificity?

3) If miRNAs are important in this context (as would be concluded from the experiments with Nova1 or Ncoa3 knockdown in this manuscript) then how do the authors explain the lack of any phenotype with Drosha knockdown (Figure 6D-F)? This seems at odds with the conclusion of this study and especially relevant given the presented data that Ago2 expression can rescue Ncoa3 knockdown phenotypes.

Other concerns:
1) Why did the authors use a pan-Nova antibody for the Western blot in Figure 2E? There are so many bands detected with this antibody that the evidence for any specific interaction between Ago2 and Nova1 is unconvincing. It would be reassuring to include a cleaner blot using the antibody that specifically recognizes Nova1 (i.e. the same antibody used for the IHC in Figure 2D).

2) Figure 4A, 4B: The authors mention that Limk1 protein level is significantly elevated with knockdown of Nova1 and Ncoa3, but the data supporting this is also unconvincing. Cleaner Western blots are required. Why are two Limk1 bands detected in Figure 4A whereas a single band is shown in Figure 4B? Also, isn't the authors' conclusion that both Nova1 and Ncoa3 depletion can lead to elevated Link1 inconsistent with the limk1-luc assay (in Figure 3E)?

3) A miRNA-target mutant reporter should be added to the LIMK1 3'UTR experiment in Figure 3E.

Referee #2:

In this manuscript, Störchel, Thümmler and colleagues identify two novel proteins involved in the regulation of miRNA function in neurons. They first perform a screen to identify proteins important in miR134 function and come up with some candidates that they then proceed to test exhaustively for expression and function in primary neurons. They provide solid evidence for a general role of Nova1 in regulating miRNA function through interaction with the RISC complex and for a more

specific role of Ncoa3 in regulating expression of important components of the same complex. The manuscript is well-structured and presents relevant, highly important data with appropriate controls. The data will certainly be of great interest to the readership of EMBO J. There are, nonetheless, some issues that should be clarified.

1. There seems to be a lack of consistency in the specific timings (DIV) used for each experiment. I wonder why the authors decided to do for example the stainings for Nova1 and Ncoa3 at 14DIV but the WB at 7DIV. As neurons in vitro develop very fast and show significant differences between early and late time points, the authors should clarify why they decided to use different time points for different experiments.

2. Similarly, while the majority of experiments was performed in rat cortical neurons, some are performed in hippocampal cultures or mouse cells. Moreover the co-IP experiment presented in figure 5A was performed in adult hippocampal tissue. While I agree that it might be of ultimate important to eventually prove a biological function in vivo rather then in cell culture, the rational for using a particular cell type or tissue is at present not entirely clear and should be explained.

3. For some siRNA experiments neurons (e.g. Fig1) neurons were transfected with lipofectamine or alike. Transfection efficiency in neurons is generally quite low, so despite the fact that luciferase is a very sensitive assay, I wonder how efficient the transfection was to see a biochemical effect on the whole population. Could the authors show a representative picture of a transfected culture or give an estimate of the efficiency?

4. The authors state that they observe a consistent 40% reduction in the luciferase levels of the UBE3A reporter in the initial screen. The results are then based on the derepression of this reduction. I would expect the same derepression effect in all of the subsequent luciferase experiments. The authors state that all experiments are compared to a basal level (that =1) measured in the absence of any shRNA plasmid. I would expect that the in the absence of a Nova1 or Ncoa3 shRNA, introduction of the luciferase reporters should be repressed. I wonder whether for example in Fig3A, the ~0.8 level for the control shRNA is indeed this repression and whether the transfection of the luciferase reporters alone (what the authors call the 'basal' condition) would be even below this 0.8 level.

5. The expression pattern of Ncoa3 seems to be clearly age-dependent in neurons (Fig. 2B), with very low levels detected at 18DIV. Coming back to the first question about performing experiments at different time points, I wonder for example about the expression levels of Ncoa3 at 19DIV, when the initial experiments for spine volume (Fig. 4C) were performed. The WB shown in the same figure (Fig 4B) is done at 14DIV. Is that still true at 19DIV?

6. Figure 5F with the specific need of nuclear Ncoa3 for its activity is not entirely convincing at present. It seems that the rescue by the shRNA-resistant construct expressed throughout the neuron is not really enough to reinstate normal levels but rather brings it down minimally and the difference between the NLS and NES constructs is also quite minimal.

7. The initial screening was performed on the basis of the activity of miR-134. The authors then examine miR-138 and let-7 as additional miRNAs whose activity is regulated by Nova1 and Ncoa3. To this end, they use the prototypical targets of these miRNAs (LIN41, APT1, and LIMK1). How general is this effect? Is it thought to also affect other target genes of the same miRNAs? The authors may consider performing experiments with additional targets of the same miRNAs

8. It is unclear whether the microarray analysis was done using multiple testing correction or not. The authors state that a moderated t-test was performed, but should clearly state whether the genes reported as significant have been selected on the basis of adjusted or unadjusted p-values. If unadjusted p-vales have been used, authors have to reassess the data to provide a more restrictive list based on multiple testing correction.

9. Although miRNA-134 leads to spine shrinkage as documented in Fig 4C-D, inhibition of Ncoa3 also has the same effect on spine volume (Fig 4C-D and Fig 7A-B). This seems counterintuitive and contradictory at first given that according to the findings in this manuscript Ncoa3 is supposed to facilitate miRNA-134 function. The authors should discuss this.

10. The fact that there is no effect of Drosha KD on dendritic branching is rather surprising, considering that it affects all miRNAs in the cell. The authors state in the discussion that this may be due to compensatory effects by other dendrite growth-promoting miRNAs, but these would be expected to be equally affected by Drosha KD? 11. The UBE3A-luc construct is cited to be unpublished. Should this be made available for others to use? Or is it expected to be released soon?

Minor points:
1. Reading the authors' conclusions one gets an impression that Ncoa3 affects miRNA function is solely through transcriptional regulation of Ago2. I would suggest to also point out that there might be other explanations. May Ncoa3 also bind to mRNA directly and be a part of miRISC complex? It would be interesting to test for a possible interaction with Ago2 at the protein level as it has been performed in Fig 5A for that of Nova1.

One observation which probably biases the authors towards the transcriptional regulation of Ago2 by Ncoa3 is the observation in in Fig 5F that Ncoa3R-GFP-NLS does significantly rescue the luciferase inhibition, whereas Ncoa3R-GFP-NES does not. However, in both cases the trend is similar although in the latter case the difference does not reach significance.

Referee #3:

Storchel et al.

A large-scale functional screen identifies Nova1 and Ncoa3 as regulators of neuronal miRNA function

In this manuscript, the authors describe a novel role for the RNA binding protein Nova1 and Ncoa3 in miRNA-guided gene regulation in neurons. Using an RNAi screening approach, they identify Nova1 and Ncoa3 as factors that affect miRNA function in neurons. In further experiments, they show that knock down of Ncoa3 and Nova1 affects spine volume. They studied Nova1 in more detail and find that it interacts with Ago2 and tethering of Nova1 to mRNAs inhibits expression. For Ncoa3, the authors present data pointing towards a nuclear function in gene silencing. Knock down of Ncoa3 leads to an increase of neuronal complexity, reduced spine volume and a reduced excitatory postsynaptic current. Finally, the authors knocked down Ncoa3 and analyzed expression changes. They find that Ncoa3 affects Ago2 expression and over expression Ago2 can rescue the increase of neuronal complexity upon Ncoa3 knock down.

This manuscript addresses an interesting topic and the results are presented clearly. However, most of the observed effects are rather mild and some might be questionable. In addition, several results appear to be inconsistent. Some of the major points are listed below.

1. The screen for RNA binding proteins that affect miRNA regulation lacks a clear positive control. Ago or TNRC6 proteins form the core of the miRNA system and knock down of these proteins should have the strongest effect. It is probably worth combining siRNAs against the individual members of the TNRC6 family in this assay. In case these proteins are redundant, double or triple knock downs might give stronger effects.

2. Figure 1: siRNA1 against Nova1 shows the strongest effect of all on the reporter gene. However, the knock down efficiency is rather poor. Moreover, siRNA3 produces a very strong knock down but has a much weaker effect on the reporter. This is not consistent and obviously the dynamic range of this system appears to be very broad and poorly controlled.

3. Figure 2: why is a pan-Nova antibody used in 2E and a Nova1-specific one in A? Which one is used in C? Unlike the authors claim in the text, Nova1 seems to be mainly nuclear in Figure 2E (if at all, there is a very small portion in the cytoplasmic fraction, which could also represent contaminations). Again, unlike the authors claim in the text, Ncoa3 is not expressed throughout the in vitro differentiation process as shown in Figure 2B. Furthermore, in Figure 2F/G, cells were in

vitro differentiated for 14 days and the signal in Figure 2B is almost gone at this time point? These results question the specificity of the antibodies used.

4. Figure 3: all Ncoa3 knock down experiments were performed at day 14 of in vitro differentiation. As mentioned above, according to Figure 2B, there is almost no expression of Ncoa3 at this time point. Since most effects are mild and Ncoa3 is low abundant, it cannot be excluded that the observed effects are within the noise range of this system. It should be noted that only one shRNA has been used for all these assays.

5. Figure 3 and 4: the Nova1 shRNA has a rather low efficiency (Figure E3A; why does the western blot presented here look so different compared to the one in Figure 2A?). In Figure 4A, the Nova1 knock down is rather weak but the effect on LimK1 is strong. In B, there seems to be a very efficient knock down of Ncoa3 but effects are hardly visible (maybe quantification of the signal could make it clearer).

6. Figure 5: the observed interaction with Ago2 is weak and only a very small portion of Nova1 is associated with Ago2 (compared to the input lane, how much input is loaded compared to the material used for IP?). To convincingly demonstrate that the two proteins interact, both proteins should be IPed and the respective binding partner assessed by western blotting. How is the repressive effect of Nova1 tethering conceptual related to miRNA-guided gene silencing? It is probably more realistic that Nova1 is a repressor itself and the observed effects might be independent of the miRNA pathway.

7. Figure 5D-F is not conclusive at all. The effect of the NES fusion construct is very mild. Since the GFP version is much stronger expressed (Figure 5E), the two constructs cannot be compared.

8. Figure 6D: it is interesting that Drosha knock down is very similar to the control knock down and has no effect on neuronal complexity at all. That means that miRNA regulation is generally irrelevant for this phenotype? MiRNA loss should be validated by Northern blotting or at least qPCR if not enough material can be generated.

9. Figure 9: the effect of Ncoa3 knock down on Ago2 mRNA levels is very weak and it is difficult to imagine that the strong effect observed in the screen and in the functional assays can be assigned to such a mild change of Ago2. How strong is Ago2 over expressed in Figures 9C-F? Obviously, the other Ago proteins are important as well otherwise a strong effect of Ago2 knock down would be observed in the RNAi screen presented in Figure 1.

1st Revision - authors' response 08 May 2015

Response to referees:

Referee #1:

We are glad that this referee states that „the strategy and assays used in the study are generally considered appropriate and the identification of factors required for the miRNA pathway in neurons is an interesting topic that could in principle be appropriate for publication in EMBO J.“

Major concerns:
1) Although the authors show that Nova1 can associate with Ago2 in co-IP experiments, the role of Nova1 in the miRNA pathway is obscure throughout the manuscript even though they concluded and discussed that Nova1 regulates miRISC activity. What possible role might Nova1 have? Considering the tethering data (Figure 5B) it is possible that Ago2 recruits Nova1 to target 3' UTRs. Does Nova1 associate with target mRNA? Is this possible recruitment miRNA/Ago2 dependent? This could be addressed by Nova1 pull-down and q.RT-PCR. Does ectopic Nova1 expression enhance miRISC activity in non-neuronal cells? Additional insight into the role of Nova1 in miRISC regulation is needed.

We fully agree that additional insight into the mechanism of Nova1 function is desirable. Following the suggestion of this referee, we have now performed a series of experiments to further elucidate Nova1 function. First, we performed RNA-IP experiments from brain lysate obtained from adult rats using a Nova1 specific antibody. Thereby, we could detect a robust interaction with the endogenous Limk1 mRNA and a previously reported Nova1 target (Rgs4), whereas Gapdh mRNA was not enriched above control levels (new Fig. 5B). We conclude that Nova1 interacts with the endogenous Limk1 mRNA in neurons (either directly or indirectly via Ago), consistent with the gene regulatory effects of Nova1 knockdown on Limk1 expression (Fig. 3E, 4C). Second, we have performed additional reporter assays in the context of BDNF stimulation and found that the stimulatory effect of BDNF was occluded in the Nova1 knockdown condition (new Fig. 5C). This result indicates that Nova1 could be involved in the dynamic regulation of Limk1 synthesis in response to BDNF. Third, we have performed a Nova1 deletion analysis in the context of luciferase reporter assays (new Fig. 5E). We found that deletion of both the N- and C-terminal parts of Nova1 impairs repression of the reporters, suggesting that domains from both parts (e.g. KH domains 1-3) contribute to the gene regulatory function. Fourth, we found that ectopically expressed Nova1 repressed reporter gene activity in tethering assays in HEK293 cells, similar to the known miRISC component Tnrc6c (new Fig. E5H). This suggests that a Nova1-containing translational repressor complex can be assembled in non-neuronal cells.
Concerning miRNA dependence, we already showed reporter assays (lin-41, 138 sponge) where mutations of the miRNA binding sites largely abolished the effect of Nova1 shRNA (Fig. 3 C, F). We have now further included data from a Limk1-luc reporter with a mutated miR-134 binding site that also supports miRNA dependence of the Nova1 gene regulatory function (Fig. 3E). We note however that miRNA mutant reporters display a residual increase in activity upon Nova1 knockdown, suggesting that Nova1 has an additional, miRNA-independent role in translational repression. Similar observations have been made for other miRISC accessory proteins (e.g. DDX6/RCK, FMRP). We have now discussed this point on p. 19 of the revised manuscript.

2) Similarly, since the authors conclude that Ncoa3 might be required for Ago2 expression it seems preliminary (based on the presented data) to suggest that this is by the direct transcriptional regulation of Ago2 expression. The inclusion of Ncoa3 chromatin IPs and functional assays using luciferase reporters containing Ago2 promoter/regulatory sequences combined with Ncoa3 knockdown are considered necessary to make this conclusion that Ncoa3 directly regulates Ago2 expression. Also, since not all 3' UTRs tested were responsive to Ncoa3 depletion, how can the apparent target site specificity be explained is Ago2 is required for miRISC function? Why are only some sites responsive to Ncoa3 depletion? What determines the observed specificity?

We agree that our previous dataset did not allow a definite conclusion regarding a direct transcriptional regulation of Ago2 by Ncoa3. Following the suggestion of this reviewer, we have now performed Ncoa3 ChIP (Fig. 8E, E8E, F), and found in three independent experiments an enrichment of DNA fragments corresponding to the Ago2 promoter region in Ncoa3 IP compared to control IgG IP. The enrichment was consistently reduced by Ncoa3 knockdown, demonstrating that this result is not due to non-specific binding of antibodies. We also performed luciferase reporter assays with a potential retinoic acid receptor binding site within the Ago2 promoter that we identified bioinformatically (JASPAR database; jaspar.genereg.net). Thereby, we found that induction of the reporter by retinoic acid was completely abolished in Ncoa3 knockdown neurons (Fig. 8F). Together, we are confident that these results provide more conclusive evidence for a direct transcriptional control of Ago2 by Ncoa3.
We agree that the apparent target site specificity of Ncoa3/Ago2 is highly interesting and needs further mechanistic support. However, we feel that the experiments required to answer this complex issue are clearly beyond the scope of the present manuscript. For example, to work out potential commonalities between the miRNA-target interactions that are sensitive to Ncoa3 knockdown will require genome-wide approaches, e.g. CLIP-seq with Ago1-4 specific antibodies in combination with Ncoa3 or control shRNA. Thereby, one could get an impression what sites are bound by which Ago protein, and which of the associations are Ncoa3-dependent. Bioinformatics on differentially occupied sites could then lead to the identification of common motifs within or in the vicinity of these sites.

3) If miRNAs are important in this context (as would be concluded from the experiments with Nova1 or Ncoa3 knockdown in this manuscript) then how do the authors explain the lack of any phenotype with Drosha knockdown (Figure 6D-F)? This seems at odds with the conclusion of this study and

especially relevant given the presented data that Ago2 expression can rescue Ncoa3 knockdown phenotypes.

The absence of a dendritic phenotype in Drosha knockdown cells is indeed surprising at first glance, and we have extensively discussed this issue in the revised version of the manuscript (p. 21-22). Briefly, our data is consistent with the presence of two groups of miRNAs, one promoting dendritogenesis (as shown e.g. for miR-132) and one inhibiting dendritogenesis (as shown e.g. for miR-137). If these activities are in equilibrium under basal conditions, manipulations that affect all miRNAs equally (e.g. Drosha or Nova1 knockdown) are not expected to alter dendritogenesis, which is what we observe experimentally. On the other hand, if a manipulation preferentially targets one pool (e.g. the growth-inhibitory miRNAs in the case of Ncoa3 knockdown), the balance would tip to the other side (e.g. increased dendritogenesis). Our findings that Ago2 can rescue the Ncoa3 phenotype therefore suggest that Ago2 is preferentially involved in the regulation of the Ncoa3-sensitive, growth inhibitory miRNAs (as discussed on p. 20-21, see also our comments to major concern 2).

Other concerns:
1) Why did the authors use a pan-Nova antibody for the Western blot in Figure 2E? There are so many bands detected with this antibody that the evidence for any specific interaction between Ago2 and Nova1 is unconvincing. It would be reassuring to include a cleaner blot using the antibody that specifically recognizes Nova1 (i.e. the same antibody used for the IHC in Figure 2D).

Following the suggestion of this referee, we have now replaced the original blot with a new blot using a Nova1 specific antibody (Fig. 2G). Thereby, we could replicate our previous findings that altough the majority of Nova1 localizes to the nucleus, a significant pool is also present in the cytoplasm. The specificity of the Nova antibodies used was validated by Western blotting with lysates from Nova knockout mouse brain (Fig. E2B, E5E).

2) Figure 4A, 4B: The authors mention that Limk1 protein level is significantly elevated with knockdown of Nova1 and Ncoa3, but the data supporting this is also unconvincing. Cleaner Western blots are required. Why are two Limk1 bands detected in Figure 4A whereas a single band is shown in Figure 4B? Also, isn't the authors' conclusion that both Nova1 and Ncoa3 depletion can lead to elevated Link1 inconsistent with the limk1-luc assay (in Figure 3E)?

In the case of Ncoa3 knockdown, we see a small, but highly reproducible increase in Limk1 protein levels, which is also supported by our quantification of three independent experiments (Fig. 4B; Fig. E4C-E). This is consistent with our new results from luciferase assays, where we now see also a small, but statistically significant increase upon Ncoa3 knockdown specifically for the wild-type, but not for the miR-134 mutant reporter (new Fig. 3E). Altogether, we conclude that Limk1 expression is regulated by Ncoa3, although the observed phenotypes caused by Ncoa3 knockdown are likely due to other deregulated miRNA-target interactions, since miR-134 negatively regulates spine size and is required for activity-dependent dendritogenesis.
Concerning Nova1 knockdown, we have performed additional experiments using infection of neurons with rAAV expressing shRNAs (new Fig. 4A). This led to a more efficient Nova1 knockdown and more reliable upregulation of Limk1 protein expression in independent experiments (Fig. 4A, Fig. E4A, B).

3) A miRNA-target mutant reporter should be added to the LIMK1 3'UTR experiment in Figure 3E.

Following the suggestion of this referee, we have now included data using a Limk1-luc reporter containing a mutated miR-134 binding site in the context of Nova1 and Ncoa3 knockdown (new Fig. 3E). Thereby, we found that the shRNA-mediated upregulation was either completely (in the case of Ncoa3) or largely (in the case of Nova1) diminished, consistent with a miRNA dependence of the observed effects.

Referee #2:

We are pleased that this referee states: "The manuscript is well-structured and presents relevant, highly important data with appropriate controls. The data will certainly be of great interest to the readership of EMBO J. There are, nonetheless, some issues that should be clarified."

1. There seems to be a lack of consistency in the specific timings (DIV) used for each experiment. I wonder why the authors decided to do for example the stainings for Nova1 and Ncoa3 at 14DIV but the WB at 7DIV. As neurons in vitro develop very fast and show significant differences between early and late time points, the authors should clarify why they decided to use different time points for different experiments.

Stainings were performed at 14DIV, since dendrite and spine development is already rather advanced at this time and the subcellular localization of proteins in the cell body, dendrites and spines can be nicely resolved. We have performed an additional time-course of Ncoa3 protein expression using Western blotting to demonstrate that Ncoa3 protein is still expressed at 14 DIV (new Fig. E2C). The Western blot in Fig. 2G was performed at 7DIV, since this is the peak expression of Ncoa3 (Fig. 2B) and Nova1 is also expressed to high levels at this time point. We also repeated Ncoa3 immunocytochemsitry at 7DIV and found an identical subcellular distribution of Ncoa3 compared to 14 DIV (new Fig. E2D).

2. Similarly, while the majority of experiments was performed in rat cortical neurons, some are performed in hippocampal cultures or mouse cells. Moreover the co-IP experiment presented in figure 5A was performed in adult hippocampal tissue. While I agree that it might be of ultimate important to eventually prove a biological function in vivo rather then in cell culture, the rational for using a particular cell type or tissue is at present not entirely clear and should be explained.

We completely agree that performing experiments in the same species, cell type and even developmental stage would be highly advantageous. However, specific experimental demands, such as cell availability or the degree of characterization of a system, sometimes necessitate the use of a specific species /cell type. For example, the initial screen was performed in mouse cells, since the genome is still better annotated in this species, making the design of specific siRNAs more reliable. Also, the large quantities of cells needed for the screen made it impossible to use primary hippocampal neurons, so that mixed cultures (hippocampus+cortex) had to be used. For follow-up experiments, we used rat cells, since these cells are more robust in long-term cultures based on our experience and can be obtained at higher quantities. In general, we prefer the more homogenous hippocampal cultures, in particular for neuromorphological analysis. However, Co-IP experiments, in particular those investigating relatively weak interactions, require high protein amounts as starting material, which could only be achieved by using hippocampal tissue from adult animals.

3. For some siRNA experiments neurons (e.g. Fig1) neurons were transfected with lipofectamine or alike. Transfection efficiency in neurons is generally quite low, so despite the fact that luciferase is a very sensitive assay, I wonder how efficient the transfection was to see a biochemical effect on the whole population. Could the authors show a representative picture of a transfected culture or give an estimate of the efficiency?

As indicated by this referee, transfection efficiency of primary neurons with lipofectamine is quite low (max. 5%), making it impossible to perform biochemical experiments with this method. However, this is not an issue in luciferase assays (e.g. in Fig. 1), since in these experiments, co-transfection of luciferase reporter plasmids and siRNAs ensures that luciferase activity is only measured from manipulated cells. Therefore, the population of cells whose activity can be measured by luminometry is expected to be homogenous. In contrast, if we measured an endogenous parameter (such as RNA or protein), high-efficiency transfections were required to manipulate the majority of cells. This was achieved by rAAV infection (max. 90% efficiency; Fig. E8A).

4. The authors state that they observe a consistent 40% reduction in the luciferase levels of the UBE3A reporter in the initial screen. The results are then based on the derepression of this reduction. I would expect the same derepression effect in all of the subsequent luciferase experiments. The authors state that all experiments are compared to a basal level (that =1) measured in the absence of any shRNA plasmid. I would expect that the in the absence of a Nova1 or Ncoa3 shRNA, introduction of the luciferase reporters should be repressed. I wonder whether for example in Fig3A, the ~0.8 level for the control shRNA is indeed this repression and whether the transfection of the luciferase reporters alone (what the authors call the 'basal' condition) would be even below this 0.8 level.

As indicated by the referee, we indeed observe a consistent 40% inhibition of the Ube3a-luc reporter in the initial screen. A similar or even higher repression is also observed in the following validations (Fig. 1C, D), where we compared conditions transfected with miRNA duplex (+miR) to conditions without (-miR). However, in Figure 3, we did not transfect miRNA duplex, but only measure regulation by the endogenous miRNA. The reporters are expected to be repressed even under basal conditions without shRNA (which was set to 1). The only way to visualize the repression brought about by the endogenous miRNA would be to compare unnormalized values of wt vs. mut reporters, which was not performed. Therefore, the small (max. 20%) reduction in the control shRNA compared to the basal levels likely reflects a non-specific effect on luciferase expression by simply expressing any shRNA. This observation illustrates the importance of using an shRNA of unrelated sequence as a control for these experiments.

5. The expression pattern of Ncoa3 seems to be clearly age-dependent in neurons (Fig. 2B), with very low levels detected at 18DIV. Coming back to the first question about performing experiments at different time points, I wonder for example about the expression levels of Ncoa3 at 19DIV, when the initial experiments for spine volume (Fig. 4C) were performed. The WB shown in the same figure (Fig 4B) is done at 14DIV. Is that still true at 19DIV?

Based on our time-course experiments (Fig. 2B, E2C), Ncoa3 peaks at 7DIV, afterwards declines until 14 DIV and is basically undetectable at 18 DIV. As stated correctly by this referee, spine volume was assessed at 19 DIV (Fig. 4C, D), but we want to stress that cells were transfected with the Ncoa3 shRNA for spine analysis (Fig. 4, 7) already at 13 DIV, at a developmental time when Ncoa3 protein is still detectably expressed. Therefore, the resulting phenotypes detected at 19DIV are likely a result of processes that were already initiated shortly after the transfection at 13 DIV.

6. Figure 5F with the specific need of nuclear Ncoa3 for its activity is not entirely convincing at present. It seems that the rescue by the shRNA-resistant construct expressed throughout the neuron is not really enough to reinstate normal levels but rather brings it down minimally and the difference between the NLS and NES constructs is also quite minimal.

We agree that the rescue obtained with the shRNA-resistant construct is incomplete, which also leads to a small difference between the NES and NLS constructs. This suggests that expression of a GFP-Ncoa3 fusion protein cannot fully replace the lack of endogenous Ncoa3 in this assay, e.g. due to partial loss-of-function or mislocalization of the fusion protein. Although we are still convinced that this figure argues for a preferential nuclear function of Ncoa3 in miRNA control, also in conjunction with a number of other assays (Fig. 8), we decided to remove this figure from the manuscript altogether.

7. The initial screening was performed on the basis of the activity of miR-134. The authors then examine miR-138 and let-7 as additional miRNAs whose activity is regulated by Nova1 and Ncoa3. To this end, they use the prototypical targets of these miRNAs (LIN41, APT1, and LIMK1). How general is this effect? Is it thought to also affect other target genes of the same miRNAs? The authors may consider performing experiments with additional targets of the same miRNAs.

Following the suggestion of this reviewer, we have now investigated another let7 target, HMGA2. In contrast to Lin-41, this reporter was only responsive to Nova1, but not Ncoa3 knockdown (Fig. E3D). Concerning miR-134, we also investigated Ube3a, and found that it was similarly regulated by Nova1, but not Ncoa3 (Fig. E3C). In addition, we found that Iqgap1, a target of the neuronal miR-124, was only responsive to Nova1, but not Ncoa3 knockdown (Fig. E3E). For miR-138, we currently do not have additional targets that respond to miR-138 overexpression in neurons. Taken together, our data suggest that all studied miRNA/target combinations are subject to regulation by Nova1, whereas only specific ones are regulated by Ncoa3. Follow-up experiments will be required to characterize the molecular underpinnings of this specificity.

8. It is unclear whether the microarray analysis was done using multiple testing correction or not. The authors state that a moderated t-test was performed, but should clearly state whether the genes reported as significant have been selected on the basis of adjusted or unadjusted p-values. If unadjusted p-vales have been used, authors have to reassess the data to provide a more restrictive list based on multiple testing correction.

We have initially performed a very stringent analysis of the microarray data using correction for multiple testing (FDR), but this analysis did not yield any statistically significant hits. This is not uncommon for microarray data, given that the latest versions of arrays contain thousands of probe sets, so that even relatively robust changes in gene expression become statistically insignificant after correcting for multiple testing. We think that the list obtained with the less stringent moderate t-test available from the GeneSpring software is still meaningful, since we were able to validate the top 4 downregulated genes, as well as Ago2 (#14), by qPCR (Fig. 8C). We are therefore confident that the vast majority of the top15 downregulated genes are true positives, although each of them will have to be validated by independent approaches.

9. Although miRNA-134 leads to spine shrinkage as documented in Fig 4C-D, inhibition of Ncoa3 also has the same effect on spine volume (Fig 4C-D and Fig 7A-B). This seems counterintuitive and contradictory at first given that according to the findings in this manuscript Ncoa3 is supposed to facilitate miRNA-134 function. The authors should discuss this.

We agree that the spine phenotype observed upon Ncoa3 knockdown cannot be solely explained by the positive regulation of miR-134 by Ncoa3. However, we obtained evidence that Ncoa3 regulates Ago2, which in turn affects the activity of many other miRNAs besides miR-134. For instance, it is conceivable that Ncoa3-dependent regulation of a spine growth promoting miRNA (e.g. miR-132) is dominant in this context. The regulation of a specific pool of miRNA activities by the Ncoa3-Ago2 pathway has now been extensively discussed in the revised manuscript (p. 21-22).

10. The fact that there is no effect of Drosha KD on dendritic branching is rather surprising, considering that it affects all miRNAs in the cell. The authors state in the discussion that this may be due to compensatory effects by other dendrite growth-promoting miRNAs, but these would be expected to be equally affected by Drosha KD?

Please see our comment to Referee #1, major concern 3), regarding this issue.

11. The UBE3A-luc construct is cited to be unpublished. Should this be made available for others to use? Or is it expected to be released soon?

The UBE3A-luc construct has now been published (Valluy et al., Nature Neuroscience 2015), and we have included the citation in the revised manuscript.

Minor points:
1. Reading the authors' conclusions one gets an impression that Ncoa3 affects miRNA function is solely through transcriptional regulation of Ago2. I would suggest to also point out tha there might be other explanations. May Ncoa3 also bind to mRNA directly and be a part of miRISC complex? It would be interesting to test for a possible interaction with Ago2 at the protein level as it has been performed in Fig 5A for that of Nova1.
One observation which probably biases the authors towards the transcriptional regulation of Ago2 by Ncoa3 is the observation in in Fig 5F that Ncoa3R-GFP-NLS does significantly rescue the luciferase inhibition, whereas Ncoa3R-GFP-NES does not. However, in both cases the trend is similar although in the latter case the difference does not reach significance.

We agree with this referee that post-transcriptional mechanisms could be involved in the regulation of miRNA function by Ncoa3, but we have no evidence supporting this at present. First, we could not detect Ncoa3 in Ago IPs (new Fig. 5A, lower panel). Second, we did not observe any effect on reporter gene expression upon tethering of Ncoa3 (new Fig. 5F). Finally, as indicated by the referee, we observed a more efficient rescue of miRNA activity by a nuclear-restricted Ncoa3 compared to a cytoplasmic construct (Fig. 5 of the original manuscript). It should also be noted that Ncoa3 does not possess a canonical RNA binding domain, and that we were not able to demonstrate specific interaction of Ncoa3 with endogenous miRNA target mRNAs in RNA-IP (data not shown). This has now been extensively discussed on p. 20 of the revised manuscript.

Referee#3:

We are pleased that this referee states: "This manuscript addresses an interesting topic and the results are presented clearly."

1. The screen for RNA binding proteins that affect miRNA regulation lacks a clear positive control. Ago or TNRC6 proteins form the core of the miRNA system and knock down of these proteins should have the strongest effect. It is probably worth combining siRNAs against the individual members of the TNRC6 family in this assay. In case these proteins are redundant, double or triple knock downs might give stronger effects.

We agree that knockdown of core components of miRISC should have a strong effect in the screen, but we think the reason why we did not identify most of these proteins is due to the high redundancy in the mammalian system. For example, knockdown of all of the four Ago members (Ago1-4) had some effect on reporter gene expression, which however did not reach the magnitude required to be classified as hits (Fig. 1B). However, we want to stress that we identified two known RISC components, one Trnc6/GW182 member (Tnrc6c) and Ddx6 (RCK/p54) (Chu&Rana, Plos Biol. 2006), in the screen. These proteins in our opinion represent the requested positive controls and validate the screen.

2. Figure 1: siRNA1 against Nova1 shows the strongest effect of all on the reporter gene. However, the knock down efficiency is rather poor. Moreover, siRNA3 produces a very strong knock down but has a much weaker effect on the reporter. This is not consistent and obviously the dynamic range of this system appears to be very broad and poorly controlled.

We agree that the knockdown efficiency of the Nova1 siRNA2 does not correlate with the degree of derepression observed in the luciferase screen. Given the poor knockdown efficiency achieved with siRNA2, it is likely that the stimulatory effect on luciferase expression is mainly due to an off-target effect of this particular siRNA. We therefore did not consider siRNA2 for any further experiments and focused instead on siRNA3, which leads to an almost complete knockdown of Nova1 (Fig. E1B) and has a strong effect (84.1% derepression) on luciferase expression (Fig. 1B). Importantly, the specificity of siRNA sequences (Nova siRNA3, Ncoa3 siRNA2) could be validated by rescue experiment using respective shRNAs together with the expression of shRNA-resistant GFP-fusion proteins (Fig. 3A, B). In general, it should be considered that it is impossible to validate the efficiency and specificity of all individual siRNAs used in such a large-scale screen (almost 1000 siRNAs were used in total). For this reason, we cannot entirely rule out that some of the proteins that scored positive in the initial screen were in fact false positives.

3. Figure 2: why is a pan-Nova antibody used in 2E and a Nova1-specific one in A? Which one is used in C? Unlike the authors claim in the text, Nova1 seems to be mainly nuclear in Figure 2E (if at all, there is a very small portion in the cytoplasmic fraction, which could also represent contaminations). Again, unlike the authors claim in the text, Ncoa3 is not expressed throughout the in vitro differentiation process as shown in Figure 2B. Furthermore, in Figure 2F/G, cells were in vitro differentiated for 14 days and the signal in Figure 2B is almost gone at this time point? These results question the specificity of the antibodies used.

Following the suggestion of this referee, we have now replaced pan-Nova blots (original Fig. 2E) with blots using a Nova1-specific antibody (new Fig. 2G). This blot shows a clear signal for Nova1 in the cytoplasm which unlikely represents contamination, given the complete absence of a nuclear protein (HDAC2) from the cytosolic fraction. The specificity of the Nova1 antibodies was confirmed using protein extracts from Nova knockout mice (Fig. E2B). With regard to Ncoa3 expression, please refer to our comments for referee #2, 5. The specificity of the Ncoa3 antibody in immunocytochemistry and Western blotting was further validated by Ncoa3 shRNA transfection (Fig. E2E, F; E3B).

4. Figure 3: all Ncoa3 knock down experiments were performed at day 14 of in vitro differentiation. As mentioned above, according to Figure 2B, there is almost no expression of Ncoa3 at this time point. Since most effects are mild and Ncoa3 is low abundant, it cannot be excluded that the observed effects are within the noise range of this system. It should be noted that only one shRNA has been used for all these assays.

Please refer again to our comments for referee #2, 5., regarding expression of Ncoa3 at 14 DIV. Since the effects of Ncoa3 knockdown on neuronal morphology are highly reproducible and statistically significant based on rigorous statistical analysis, we consider it highly unlikely that “the

observed effects are within the noise range of this system." We further want to stress that the specificity of the shRNAs was validated by rescue experiments using an shRNA-resistant GFP-Ncoa3 expression construct (Fig. 3B; Fig. 6C; Fig. 7B), which is generally considered the "gold standard" for evaluating shRNA specificity. In conclusion, we are confident that our experiments provide sufficient evidence to support a specific function of Ncoa3 in the miRNA-dependent regulation of neuronal morphology.

5. Figure 3 and 4: the Nova1 shRNA has a rather low efficiency (Figure E3A; why does the western blot presented here look so different compared to the one in Figure 2A?). In Figure 4A, the Nova1 knock down is rather weak but the effect on LimK1 is strong. In B, there seems to be a very efficient knock down of Ncoa3 but effects are hardly visible (maybe quantification of the signal could make it clearer).

In Fig. E3A, we used nucleofection of shRNAs, which has to be performed in young cortical neurons to achieve high efficiency knockdown. However, in young cortical neurons, in contrast to more mature cortical neurons or hippocampal neurons (cf. fig. E3A with E2A, 2A), we routinely observe three bands when using the anti-Nova1 antibody in Western blotting. Only the middle of the three bands represents Nova1, since this band is specifically reduced by the Nova1 shRNA. The other two bands represent unidentified proteins that cross-react with the Nova1 antibody. In addition, we now show that rAAV-mediated delivery of the Nova1 shRNA leads to a near complete loss of Nova1 protein in more mature hippocampal neurons (new Fig. 4A). In these cells, cross-reactive bands are barely visible. The fact that Nova1 knockdown results in a stronger effect on Limk1 protein expression compared to Ncoa3 knockdown (Fig. 4A, B) is consistent with our results from luciferase assays (Fig. 3E). Although the effects of Ncoa3 knockdown on Limk1 are mild, they are statistically significant, as shown by our quantification of three independent rAAV infections (Fig. 4B, E4C-E).

6. Figure 5: the observed interaction with Ago2 is weak and only a very small portion of Nova1 is associated with Ago2 (compared to the input lane, how much input is loaded compared to the material used for IP?). To convincingly demonstrate that the two proteins interact, both proteins should be IPed and the respective binding partner assessed by western blotting. How is the repressive effect of Nova1 tethering conceptual related to miRNA-guided gene silencing? It is probably more realistic that Nova1 is a repressor itself and the observed effects might be independent of the miRNA pathway.

We agree with this referee that the interaction of Nova1 with Ago2 is weak. In the blots shown in Fig. 5A and E5A, 10% of the input is loaded compared to the IP. Quantification of the signals revealed that about 2% of Nova1 protein that was originally present in the input was recovered in the Ago-IP (Fig. E5B). One possible reason for the low recovery is that it was extremely challenging to solubilize Ago, which could be due to the reported association of Ago with cellular membranes (Gibbings et al., NCB 2009). Therefore, the relatively harsh lysis conditions used for the Co-IP might be unfavorable for the preservation of the weak Ago-Nova1 interaction. Unfortunately, we were unable to detect Ago proteins in Nova1-IPs using either anti-Ago2 or anti pan-Ago antibodies for detection (data not shown). Since we do not know the binding interface between Nova1 and Ago, it is possible that binding of the Nova1 antibody to Nova1 interferes with the Nova1-Ago interaction. Alternatively, the efficiency of the Nova1 antibody in IP might be too low to be able to detect weak interactions. Nevertheless, we want to stress that the Co-IP of Nova1 with Ago in anti-Ago IPs was highly reproducible (Fig. 5A, E5A-D).
Concerning the tethering data, we would argue that it supports a mechanism whereby Ago recruits Nova1 to target RNAs, since we observe similar results for Nova1 and the known Ago interacting protein Tnrc6/GW182 in this assay. Although we observed miRNA dependence of Nova1 by using multiple reporters (Lin41, Limk1, 138-sponge, Iqgap1; Fig. 3 and E3), we do not rule out that Nova1, similar to other known RISC-associated factors, has also more general roles in translational regulation (see also our comment to Referee #1, 1).

7. Figure 5D-F is not conclusive at all. The effect of the NES fusion construct is very mild. Since the GFP version is much stronger expressed (Figure 5E), the two constructs cannot be compared.

Please see our comment to Referee #2, 6), regarding this concern.

8. Figure 6D: it is interesting that Drosha knock down is very similar to the control knock down and has no effect on neuronal complexity at all. That means that miRNA regulation is generally irrelevant for this phenotype? MiRNA loss should be validated by Northern blotting or at least qPCR if not enough material can be generated.

Please see our comment to Referee #1, major concern 3), regarding this concern. Following the suggestion of this reviewer, we have now validated miRNA loss in Drosha knockdown neurons using rAAV-mediated delivery of Drosha shRNA followed by TaqMan qPCR (new Fig. E6E-F). Interestingly, the reduction in mature miRNA levels upon Drosha knockdown appears to anti-correlate with miRNA abundance, suggesting that lower expressed miRNAs, such as miR-134, are more severely affected by Drosha knockdown compared to highly abundant miRNAs (e.g. let-7).

9. Figure 9: the effect of Ncoa3 knock down on Ago2 mRNA levels is very weak and it is difficult to imagine that the strong effect observed in the screen and in the functional assays can be assigned to such a mild change of Ago2. How strong is Ago2 over expressed in Figures 9C-F? Obviously, the other Ago proteins are important as well otherwise a strong effect of Ago2 knock down would be observed in the RNAi screen presented in Figure 1.

We agree that the reduction in Ago2 mRNA levels upon Ncoa3 knockdown is rather mild, but highly reproducible (Fig. 8B, C). Interestingly, effects on Ago2 protein levels appear to be larger (Fig. 8D), as shown by quantification of three independent Western blots (Fig. E8D). Nevertheless, re-expression of Ago2 in the context of Ncoa3 shRNA nearly completely rescues defective miRNA function (Fig. 9B) and dendritogenesis (Fig. 9C-E), strongly suggesting that the regulation of Ago2 contributes in an important manner to Ncoa3-dependent phenotypes. Following the suggestion of this referee, we have now quantified the degree of Ago overexpression achieved by Flag/HA-Ago2 transfections using anti-Ago2 immunocytochemistry (new Fig. 9A, E9C). We found that Flag/HA-Ago2 expression in the context of the Ncoa3 shRNA resulted in total Ago2 levels that were indistinguishable from non-transfected control cells. Therefore, we can rule out that the observed rescue effects are due to a massive overexpression of Ago2 compared to control cells.
Concerning the importance of other Ago members, we agree that they display a high degree of redundancy based on our results from the RNAi screen (Fig. 1B). However, the screen was performed with transfection of miRNA in the form of synthetic duplex RNA, and the high amounts of transfected miRNA might lead to a non-specific loading of miRNAs into Ago proteins if the latter are present at limiting concentrations. In contrast, the regulation of Ube3a by endogenous miR-134 was not sensitive to Ncoa3 knockdown (Fig. E3C), which argues that the Ncoa3-Ago2 pathway does not play a major role in this context. Consistently, the Ncoa3 phenotype is not in agreement with deregulated miR-134 activity. Overall, we do not think that the results from the screen contradict an important contribution of Ncoa3-dependent regulation of Ago2 expression to the regulation of specific miRNA-target interactions and neuronal morphology.

2nd Editorial Decision 20 May 2015

Thank you for submitting the revised version of your manuscript. It has now been seen by two of the original referees (comments included below) and as you will see they are both satisfied with the amended manuscript. I am therefore happy to tell you that your study has been officially accepted for publication in The EMBO Journal.

Before we can transfer your manuscript files for production I do have to ask you to address the following editorial points:

-> Could you provide us with the accession number for the microarray data? If the final number is not yet available you can provide a temporary replacement and the final number can be included at proof stage.

-> Papers published in The EMBO Journal include a 'Synopsis' to further enhance its discoverability. Synopses are displayed on the html version of the paper and are freely accessible to all readers. The synopsis will include a short standfirst - written by the handling editor - as well as 2-

5 one sentence bullet points that summarise the paper and are provided by the authors. I would therefore ask you to provide your suggestions for bullet points.

-> We 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, labelled 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".

If you have any questions, please feel free to contact me. Thank you for your contribution to The EMBO Journal and congratulations on this nicely executed work!

REFEREE COMMENTS

Referee #1:

The authors have done a very nice job of addressing the previous concerns and this improved manuscript is considered a strong candidate for publication in EMBO J.

Referee #2:

The authors have addressed all points raised previously and provide adequate responses. they show now data or have removed some data that was rather misleading.

I my view the manuscript should be published as it is.

