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FUS stimulates microRNA biogenesis by facilitating co-transcriptional Drosha recruitment

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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.)

1st Editorial Decision 30 August 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 referees are positive about your manuscript, but they do raise a number of issues that you will have to address in an adequate manner before submitting a revised version. We agree with the requests made by the referees; however, it is not an absolute demand from our side that you address FUS-binding sequences in all affected miRNAs as requested by referee #1.

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 to the full satisfaction of the referees in this revised version. Please do not hesitate to contact me if you have questions related to the review process and the requests made by the referees.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, 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. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

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

In this interesting manuscript, the Bozzoni lab investigates the role of TLS/FUS in the posttranscriptional regulation of miRNA biogenesis.

During the purification of the Microprocessor complex, the Shiekhattar lab identified TLS/FUS as a component of the larger Microprocessor complex. This is suggestive of a role of this protein in miRNA biogenesis.

Here, Morlando and colleagues first tested the effect of siRNA-mediated depletion of TLS/FUS on miRNA expression in the neuroblastoma cell line SK-N-BE. Approximately half of the miRNAs analyzed were found to be deregulated more than 15%, with most of them being downregulated. TLS/FUS has been linked to familial forms of ALS; interestingly, among the miRNAs that are positively regulated by this protein, there are several with roles in neuronal function and synaptogenesis. Moreover, neuronal miRNAs, which were transiently expressed using U1 vectors, were also downregulated in HeLa cells depleted of TLS/FUS.

Next, the authors show that FUS binds in vitro to specific pri-miRNA transcripts. Despite the lack of a motif common to all miRNAs dysregulated upon FUS depletion the authors investigated a GU-rich sequence present in the miR-9-2 loop and found that it affected FUS binding. The authors went on to show that two mutations in FUS, commonly found in ALS patients, show no difference neither in miR-9-a binding in vitro nor in their interaction with Drosha. As reported previously, these mutant proteins relocalize to the cytoplasm.

Use of a ChIP assay revealed that TLS/FUS is recruited on the chromatin at the sites of transcription and facilitates recruitment of Drosha. Importantly, the authors show that extracts depleted of FUS still process pri-miRNAs efficiently. This is a negative result but an important one since this difficult experiment has been carried out very carefully.

Conceptually, the existence of posttranscriptional regulation of subsets of miRNAs exerted by RNA-binding proteins is not entirely novel. However, this work is very interesting from the point of view of altered subcellular localization of FUS found in ALS patients resulting in decreased biogenesis of subsets of miRNAs.

Specific comments

- On Fig. 1B, it would be more convincing to show that depletion of FUS results in accumulation of the respective pri-miRNAs

- On Fig. 1D, what are the effects of FUS depletion on HeLa endogenous miRNAs? Are these also downregulated upon decreased FUS levels?

- On Fig. 3B, the authors have to show whether the interaction of FUS with Drosha is RNA-dependent or not?

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- It remains unclear what is the sequence being recognized in all other pri-miRNAs bound by FUS. This has to be further explored.

Referee #2

The authors reasonably assume that FUS is involved in miRNA biogenesis. Microarray analysis of FUS-silenced SK-N-BE neuroblastoma cells identified altered detection of 166 out of 377 represented miRNAs at 15% fold-change cut-off. They select a number of miRNAs reported to be involved in neuronal functions, and confirm specific down-regulations of miR-9, miR-125b, miR-199a, miR-192, miR-212, miR-132, miR-143, but not miR-15 correlating with binding of recombinant GST-FUS to the corresponding pri-miRNAs. Mutagenesis of a potential FUS recognition sequence in the pri-miR-9-2 loop reduced FUS binding partially. Some mechanistic insight is provided for miR-9 and miR-125b as CHIP reveals binding of FUS. Interestingly, FUS knockdown reduces Drosha CHIP at these loci, in contrast to the FUS-independent control miR-15. A little effort is devoted to clinical FUS mutations, however not showing significant differences to wild-type FUS, precluding strong conclusions about pathogenic mechanisms. All in all, the manuscript clearly addresses an interesting and highly relevant question, and provides a solid starting point of a study that is too preliminary for publication in the EMBO Journal at present.

Specific Comments:
1. Results, first paragraph (page numbers missing):
a. Add: "PROTEIN LEVELS of microprocessor major components, Drosha and DGCR8, were unaffected" while Drosha activity seems to be affected by FUS knockdown.
b. "Other NON-NEURONAL species were more affected" is not worded ideally. They were detectable here in SK-N-BE neuroblastoma cells.
2. Provide supplementary information about quality control and purity checks of the recombinant GST-FUS proteins.
3. In Fig. 2A better use (e.g. GST) protein negative control instead of blank.
4. Show a total protein stain in Fig. 2B to appreciate the relative amount of FUS band compared to all binding proteins.
5. The study is completely devoid of essential rescue experiments. Do confirm that FUS re-transfection normalizes the effects on miRNAs. Compare such complementation activities of wild-type and mutant FUS. The strong NLS mutant P525L should be significantly compromised in recruitment and regulation of nuclear biogenesis of the identified miRNAs.
6. The defects of R521C are not clearly established and retention of pri-miRNA binding is not so surprising. Examine more closely predicted RNA recognition domains in the FUS protein.
7. Fig. 3A FUS wild-type and mutant input controls missing.
8. Fig. 3B shows that FUS wild-type and two C-terminal point mutants generally bind to Drosha in whole cell lysates where all cellular compartmentalization is broken down. This experiment would be more meaningful with nuclear extracts (where NLS mutant effects would be expected), and ideally subcellular interaction studies in situ.
9. Provide negative controls in Fig. 4. Particularly show absence of FUS recruitment to miR-15a.
10. Elaborate more on the mechanism how FUS recruits Drosha specifically to selected miRNAs. It does not become entirely clear in what order FUS targets promoter DNA and pri-miRNA. Does FUS binding to either alter its conformation to allow stronger Drosha binding, recruitment of adaptor proteins and/or particular complex assemblies, or is FUS constitutively bound within the microprocessor acting statically as a nucleic acid sequence scanner?
11. Another great deficiency is the complete absence of measurements of cellular consequences. Does FUS knockdown impair the featured neuronal functions and differentiation and viability in this system, and does selective miRNA complementation restore them?

Referee #3

The authors report that the FUS/TLS protein, associated with familial forms of Amyotrophic lateral sclerosis (ALS), is involved in processing of a subset of miRNA, including many miRNAs expressed specifically in neurons. They show that depletion of FUS/TLS in differentiating neuroblastoma cells grown in culture results in depletion of mature miRNA without affecting levels

of primary transcripts. Experiments are presented that demonstrate direct FUS/TLS binding to selected pri-miRNAs and also interaction of the protein with endogenous transcripts.
ChiP experiments demonstrate RNA-dependent association of FUS/TLS with miRNA gene chromatin and partial dependency of the Drosha interaction with chromatin on FUS/TLS.
These are all exciting findings. Although most of the experiments are well done and properly controlled, some additional data are needed to make the observations more complete and convincing. Detailed comments and recommendations:

1. The authors use only single siRNA to knock-down FUS. This does not exclude off-target effects. Another independent siRNA should also be tested in couple of key experiments. Alternatively, they should show, that miRNA phenotype is rescued by expression of the siRNA-resistant FUS.
2. The effects of FUS KD on miRNA biogenesis are generally mild (15-50% decrease). However, the knock-down has a very dramatic effect on chipping of FUS shown in Fig. 5B. Interactions of FUS are practically totally eliminated. Why this discrepancy?
3. How reliable are 15% changes in the levels of some miRNAs as observed by RT-PCR? For 2-3 selected miRNAs the authors should provide calibration curves, using appropriate synthetic miRNAs, showing that their procedure can indeed measure such minimal changes in miRNA levels.
4. The observation that effects of FUS observed in cell lines cannot be reproduced in vitro is disappointing. The authors should make some additional attempts to test the in vitro effects. For example, they could test Drosha immunoprecipitates, in the absence and presence of recombinant FUS. There are many pri-miRNA processing in vitro systems. Perhaps some of them would give positive results. Titration of the pri-miRNA substrate and the FUS protein imputs might also be of help in in vitro assays.
5. Fig. 2A. Addition of increasing amounts of FUS decreases rather than increases the binding to pri-miRNAs. Please comment. Perhaps lower concentrations of FUS should be tested to show concentration-dependence.
6. Is the FUS knock-down affecting SK-N-BE cells differentiation? Since many tested miRNAs are induced by differentiation, any effects of FUS on differentiation might have an effect on the miRNA levels.

Minor points:
1. Organization of manuscript text paragraphs should be improved. There are many single-sentence paragraphs in the paper. Spacing between paragraphs is not uniform.
2. Fig. S4: left lanes in the gel rather contain just a substrate alone than being mock reactions.

1st Revision - authors' response 16 October 2012

Referee #1

- On Fig. 1B, it would be more convincing to show that depletion of FUS results in accumulation of the respective pri-miRNAs
We have analysed such aspect without finding any significant correlation between FUS depletion and pri-miRNA increase. A figure is provided here below for the referee (REF. Fig.1) where it is shown that pri-miRNA tend to decrease in the absence of FUS. The lack of a direct correlation between microprocessor impairement and pri-miRNA accumulation has already been described by others (Davis et al. 2008 Nature, 454: 56; Kawai and Amano, 2012, J.Cell Biol., 197: 201; Kawahara and Mieda-Sato, 012 P.N.A.S., 109: 3347). Possible explanations are that in the absence of Drosha cleavage different pri-miRNA (also depending on their genomic organization) can be affected at different extent due to defects in 3' end-formation, stability or even decrease in transcription.

- On Fig. 1D, what are the effects of FUS depletion on HeLa endogenous miRNAs? Are these also downregulated upon decreased FUS levels?
We performed a high throughput miRNA analysis using TaqMan® Human MicroRNA Array. The data, presented in the new Supplementary Figure S1B and Table I, indicate that also in HeLa cells, FUS depletion impacts on miRNA biogenesis; however, the number of negatively affected species is lower than in neuronal cells.

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- On Fig. 3B, the authors have to show whether the interaction of FUS with Drosha is RNA-dependent or not?
We have performed such experiment, introduced in the new Figure 3C, showing that the FUS-Drosha interaction is resistant to RNase treatment.

-It remains unclear what is the sequence being recognized in all other pri-miRNAs bound by FUS. This has to be further explored.
We would suggest that this topic can be deeply analysed in subsequent studies since preliminary analyses have not identified a clear consensus among the class of FUS-affected pri-miRNA.

Referee #2

1. Results, first paragraph (page numbers missing):
a. Add: "PROTEIN LEVELS of microprocessor major components, Drosha and DGCR8, were unaffected" while Drosha activity seems to be affected by FUS knockdown.
Right, we have corrected the sentence

b. "Other NON-NEURONAL species were more affected" is not worded ideally. They were detectable here in SK-N-BE neuroblastoma cells.
Right, we have substituted non-neuronal with “Other species not restricted to neuronal cells”

2. Provide supplementary information about quality control and purity checks of the recombinant GST-FUS proteins.
The Coomassie staining showing the purity of the recombinant GST-FUS protein has been added in the new Supplementary Figure S3A.

3. In Fig. 2A better use (e.g. GST) protein negative control instead of blank.
In the experiment shown in Figure 2A, the sample “mock” indeed contained a control GST peptide. We have better specified this in the text and in the legend.

4. Show a total protein stain in Fig. 2B to appreciate the relative amount of FUS band compared to all binding proteins.
We have added what requested in Supplementary Figure S3B. However, in this experiment the bound FUS is not expected to be detectable by Ponceau staining.

5. The study is completely devoid of essential rescue experiments. Do confirm that FUS re-transfection normalizes the effects on miRNAs. Compare such complementation activities of wild-type and mutant FUS. The strong NLS mutant P525L should be significantly compromised in recruitment and regulation of nuclear biogenesis of the identified miRNAs.
We have performed such experiments in SK-N-BE cell lines carrying wild type or mutant FUS cDNAs, integrated in the genome through a Piggy Back vector. These constructs have an unrelated 3’UTR and are under the control of a Doxycycline-dependent promoter (described in Figure 3B). Using these lines, we obtained depletion of the endogenous FUS, with siRNA against the 3’UTR, and then induced exogenous FUS expression by Doxycycline (Dox) treatment.
The new Figure 4 shows that miR-132, miR-9 and miR-192 levels are decreased in cells treated with RNAi against FUS in the absence of Dox and are rescued upon activation of the exogenous wild type FUS. The results with the two FUS mutants are consistent with their delocalization phenotype: FUS^{R521C} which displays only a slight cytoplasmic delocalization is able to rescue miRNAs at levels similar to those of control, while FUS^{P525L}, which has a stronger delocalization phenotype, has a lower rescue activity. Note that these experiments have been necessarily carried out in overexpression conditions, therefore producing increased levels of FUS, including its nuclear fraction. This is true also for the FUS^{P525L} mutant which still has a conspicuous nuclear retention (see Supplementary Figure S4B) and ability to rescue miRNA biosynthesis.
These experiments now clearly demonstrate a direct involvement of FUS on miRNA biogenesis and again indicate that this ability directly correlates with the amount of nuclear FUS.

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6. The defects of R521C are not clearly established and retention of pri-miRNA binding is not so surprising. Examine more closely predicted RNA recognition domains in the FUS protein.
Indeed it is expected that FUS^{R521C} maintains its binding ability to RNA since the mutation affects specifically the NLS domain. The experiments of Figure 2A and B indeed prove that neither RNA nor Drosha binding are affected, again suggesting that the effects of this mutation on miRNA biogenesis could be due to decreased levels of FUS in the nucleus (see Supplementary Figure S4B).

7. Fig. 3A FUS wild-type and mutant input controls missing.

If the referee means the amount of WT and mutant proteins utilized, we have now provided the Coomassie staining of the purified proteins (Supplementary Figure S3A). Similar amount of proteins were utilized and this has been specified both in the legend and in material and methods.

8. Fig. 3B shows that FUS wild-type and two C-terminal point mutants generally bind to Drosha in whole cell lysates where all cellular compartmentalization is broken down. This experiment would be more meaningful with nuclear extracts (where NLS mutant effects would be expected), and ideally subcellular interaction studies in situ.
The experiment was indeed carried out with nuclear extracts as specified in the figure legend.

9. Provide negative controls in Fig. 4. Particularly show absence of FUS recruitment to miR-15a.
The experiment has been performed and the result added in the new Fig.5.

10. Elaborate more on the mechanism how FUS recruits Drosha specifically to selected miRNAs. It does not become entirely clear in what order FUS targets promoter DNA and pri-miRNA. Does FUS binding to either alter its conformation to allow stronger Drosha binding, recruitment of adaptor proteins and/or particular complex assemblies, or is FUS constitutively bound within the microprocessor acting statically as a nucleic acid sequence scanner?
The indication that FUS does not require RNA for Drosha interaction (new experiment of Figure 3B) and that, in the absence of FUS, Drosha binding to the chromatin is reduced, would suggest that FUS by recognizing the pri-miRNA during transcription cooperates for co-transcriptional Drosha recruitment.
We have added such comment both in result and discussion sections.

11. Another great deficiency is the complete absence of measurements of cellular consequences. Does FUS knockdown impair the featured neuronal functions and differentiation and viability in this system, and does selective miRNA complementation restore them?

In our analysis we have followed the differentiation of SK-N-BE up to 6 days. Under these conditions we have not observed any specific alteration in the abundance of neuronal differentiation markers N-Myc, VGF and ID2 (not shown). However, this is not surprising in consideration of the short time of analysis and to the slight reduction of miRNA. We suppose that visible/measurable effects on differentiation should require much longer times.

Referee #3

1. *The authors use only single siRNA to knock-down FUS. This does not exclude off-target effects. Another independent siRNA should also be tested in couple of key experiments.*

Alternatively, they should show, that miRNA phenotype is rescued by expression of the siRNA-resistant FUS.
This comment is satisfied by the new experiments shown in Figure 4 and Supplementary Figure S2B (see also reply to ref #2): a second siRNA (against the 3'UTR; siFUS-3') was utilized providing efficient FUS depletion. Under these conditions we observed the same miRNA downregulation as the one shown in Figure 1B. The results are included in Figure 4 and in Supplementary Figure S2B.

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2. The effects of FUS KD on miRNA biogenesis are generally mild (15-50% decrease). However, the knock-down has a very dramatic effect on chipping of FUS shown in Fig. 5B. Interactions of FUS are practically totally eliminated. Why this discrepancy?
It is difficult to answer this question since many factors can contribute to the discrepancy between the effect observed at the level of mature miRNA and at the level of chromatin. Among them: miRNA stability/turn-over, effect of FUS on transcription, elongation...More experiments in the future will be devoted to understand the effects of FUS/pri-miRNA interaction on transcription; this is certainly one of our major future challenges.

3. How reliable are 15% changes in the levels of some miRNAs as observed by RT-PCR? For 2-3 selected miRNAs the authors should provide calibration curves, using appropriate synthetic miRNAs, showing that their procedure can indeed measure such minimal changes in miRNA levels.
In consideration of these slight variations we have repeated the experiments 6 times. The 15% variations resulted highly reliable and reproducible.

4. The observation that effects of FUS observed in cell lines cannot be reproduced in vitro is disappointing. The authors should make some additional attempts to test the in vitro effects. For example, they could test Drosha immunoprecipitates, in the absence and presence of recombinant FUS. There are many pri-miRNA processing in vitro systems. Perhaps some of them would give positive results. Titration of the pri-miRNA substrate and the FUS protein imputs might also be of help in in vitro assays.
This has been indeed one of our major efforts: many different attempts have been made (besides those described, we also performed: in vitro processing with Flag-FUS Ip and nuclear extract titrations). One possible explanation could be the impossibility of reproducing in vitro the correct assembly of the “miRNA processing factory”. The quality of our extracts was however good as demonstrated by the fact that when depleted of Drosha they lacked their processing activity. These different conditions are reported in the figure for the referee (REF. Fig.2) provided here below.

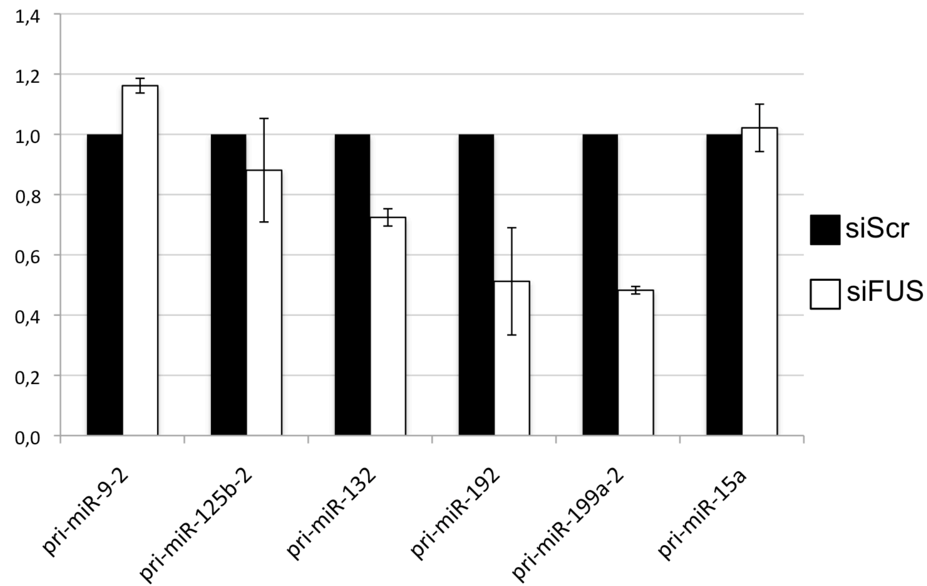
5. Fig. 2A. Addition of increasing amounts of FUS decreases rather than increases the binding to pri-miRNAs. Please comment. Perhaps lower concentrations of FUS should be tested to show concentration-dependence.
In the figure the increase is referred to the tRNA and not to the amount of FUS. This is better specified in the figure. An additional panel (new panel B) with a dose concentration assay has been added in Figure 2.

6. Is the FUS knock-down affecting SK-N-BE cells differentiation? Since many tested miRNAs are induced by differentiation, any effects of FUS on differentiation might have an effect on the miRNA levels.
Se reply to point 11 of ref.#2.

The suggestions for the minor points have been taken into consideration.

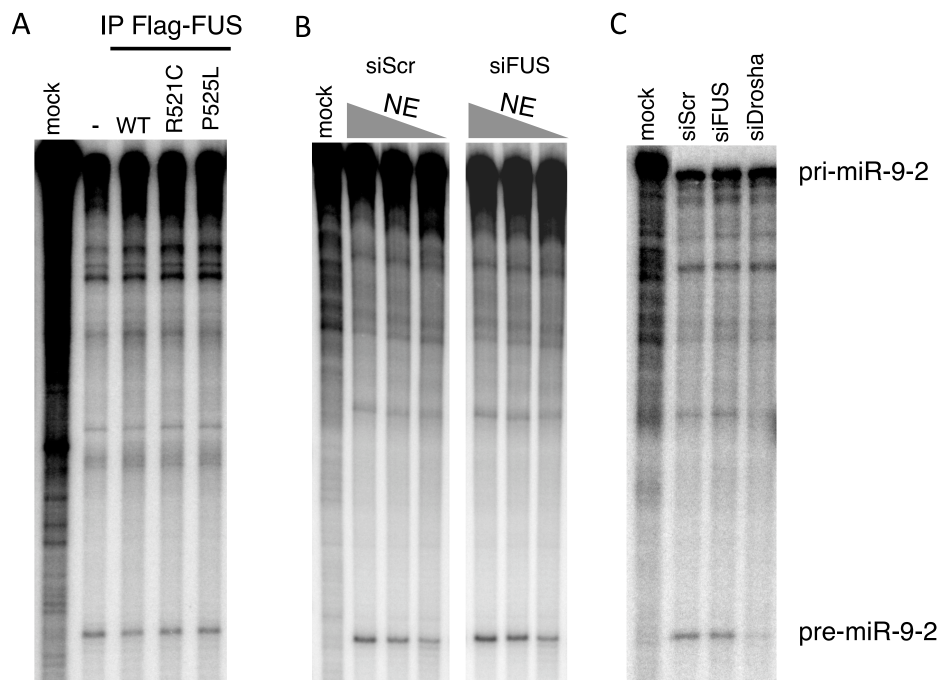
We hope that, with the new experiments performed and the modifications introduced, the paper is now suitable for publication.

REF Fig.1



REF Figure 1. Pri-miRNA levels in SK-N-BE cells treated with RA and with either scrambled siRNA (siScr) or siRNA against FUS (siFUS)

REF Fig.2



REF Figure 2. (**A**) *In vitro* processing with ^{32}P-UTP labelled pri-miR-9-2 using Flag - Immunoprecipitates from cells expressing FLAG-FUS^{wt}, FLAG-FUS^{R521C} and FLAG-FUS^{P525L}. In the control sample (-) the in vitro processing was carried out with SK-N-BE nuclear extracts. (**B**) *In vitro* processing with ^{32}P-UTP labelled pri-miR-9-2 using titration of nuclear extract (15,10 and 5

mg) from cells treated either with scrambled siRNA (siScr) or siRNA against FUS (siFUS). (**C**) *In vitro* processing with ^{32}P-UTP labelled pri-miR-9-2 using nuclear extract from cells treated with scrambled siRNA (siScr), siRNA against FUS (siFUS) or siRNA against Drosha.
The mock samples with no extract are used as controls.

2nd Editorial Decision 30 October 2012

Thank you for submitting a revised version of your manuscript. It has now been seen by one of the original referees whose comments are shown below.
As you will see the referee finds that all criticisms have been sufficiently addressed and he/she recommends the manuscript for publication pending a few minor changes. In addition to these minor issues, I would ask you to address a few editorial issues concerning text and figures.

The referee asks you to include additional statistics for the new data presented in figure 4; in addition I have to ask you to indicate the number of replicas used for calculating statistics and the values depicted by the error bars (S.D./S.E.) in all figure legends (currently missing or incomplete for fig. 1D, 3A, 4C).
Furthermore, 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. 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 REPORT

Referee #2

The authors have revised the manuscript satisfactorily. Although it is a pity and somewhat of a potential relevance limitation that pilot experiments did not reveal cellular (neuronal) consequences (Rev. #2.11 and Rev. #3.6), the suggested novel role of FUS as a regulator of co-transcriptional Drosha recruitment during miRNA biogenesis is of sufficient interest mechanistically.

Minor (discretionary) suggestions:

Original point 4: Fig. S3B is not referenced in the text. In fact, the Ponceau stainings hardly show any bound protein bands at all. What I was curious to see is the relative proportion of FUS among all binding proteins. This requires more sensitive protein stains. However, as this is a minor point I would agree to leave out Fig. S3B in the final version.

Original point 5: The rescue experiments are important and convincing. If the authors find the time, statistical analysis of Fig. 4C would be appreciable.

2nd Revision - authors' response 03 November 2012

Referee #2

-Original point 4: Fig. S3B is not referenced in the text. In fact, the Ponceau stainings hardly show any bound protein bands at all. What I was curious to see is the relative proportion of FUS among all binding proteins. This requires more sensitive protein stains. However, as this is a minor point Iwould agree to leave out Fig. S3B in the final version.

We agree with the referee therefore, we left out the Supplementary Figure S3B. We will refer to this Supplementary Figure as “Supplementary Figure S3” since now it contains only one panel.

- Original point 5: The rescue experiments are important and convincing. If the authors find the time, statistical analysis of Fig. 4C would be appreciable.

Statistical analysis based on 3 different measurements has been performed: significance was assessed by Unpaired Student’s t-test.

We also specified the number of replicas used for calculating statistics and the values depicted by the error bars (S.E.M) in all figure legends.

We hope that, with the new modifications introduced, the paper is now suitable for publication.

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