Arginine Methylation next to the PY-NLS modulates Transportin Binding and Nuclear Import of FUS

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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.)

Additional Correspondence 30 April 2012

Sorry for the delay in getting back to you with a decision on your MS 81514. I have now received the three reports on the paper and they are mixed - please see below. Before coming to a final decision if we can offer to consider a revised manuscript, I would like to give you an opportunity to consider the referees’ reports and to provide me with a point-by-point response including proposal for additional experiments that you can carry out.

The referees raise a number of different issues. One is that the mechanism of how FUS localization is regulated is not a novel concept as arginine methylation of PABPN1 has been reported to affect its interaction with Transportin (Fronz et al.). This does diminish the significance of the paper - referee #2. However, the more important issue is that there is too limited data provided to support the pathological implications of the findings - see referee #1 and 3. I recognize that the interest of the paper is from the disease angle, but at present this link is not strong enough. Bottom-line is that at the moment there is not strong enough support to consider publication here. If you have further data to strengthen the pathological implications of the findings then that would be a different matter. Therefore before taking the decision on this paper, I would like to ask you if you have data on hand or in progress to extend that aspect of the paper?

If you have any further questions please don’t hesitate to contact me.
Yours sincerely

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1

In this work, Dormann et al. report the impact on arginine methylation on FUS on its interaction with the import receptor Transportin through a novel TRN-binding motif (RGG3) N-terminal to the NLS signal. Although methylation seems to have a small effect on wild-type protein localization, the authors show that inhibition of methylation through a variety of means (AdOx treatment, KD of PRMT1) is capable of rescuing the effect of ALS-associated FUS mutants in the NLS signal of this protein and other FET family members. Overall, this study has been performed with technical competence and is very well written. Taken together, the results provide evidence that methylation of FUS in the RGG3 may be a strong modifier of nuclear/cytoplasmic localization of this protein in the presence of NLS-impairing mutations. However the following issues should be addressed before the manuscript is suitable for EMBO Journal.

1- The experiments in Fig.2 deal with the TAF15 and EWS proteins in which the authors inserted a mutation in their respective NLS signals to mimic the effect of the FUS-P525L mutation. Although the results are clear and consistent with the FUS data, this experiment would have been much more meaningful had the authors inserted in TAF15 and EWSR1 some mutations that have been shown to affect localization (ie. disease-associated mutants that result in cytoplasmic accumulation as those described by Couthouis et al., 2011, PNAS and Couthouis et al, 2012, HMG).

2- The potential pathological implications of these proteins has only been addressed in the Discussion (page 17 and 18). Although it is possible that an increased methylation of a mutated FUS might lead to an increased localization/aggregation in the cytoplasm, there is really no evidence that methylation levels may be altered in the brains of people affected by ALS or FTD. Furthermore, even if there was evidence of altered methylation levels in patient’s brains, the results in Fig.1 and 2 show very clearly that the almost complete nuclear localization of FUS, TAF, and EWS is hardly affected even by treatment with AdOx (an extremely strong inhibitor of all cellular methylation processes and thus unlikely to mimic any physiological condition, whether normal or pathological). If we then consider that many ALS/FTD patients with FUS inclusions do not carry any mutation in this protein it is really very hard to see how changes in methylation level could play a role in the pathological process.

Referee #2

The authors report that arginine methylation of the protein FUS reduces its affinity for the nuclear import receptor transportin. Inhibition of methylation in cells can rescue the nuclear import of FUS carrying mutations in its transportin binding site.

The paper is interesting and technically largely convincing. Its importance is slightly diminished by the fact that Fronz et al. (cited in the manuscript) already reported a weakening of the interaction between the protein PABPN1 and transportin by arginine methylation of the former. Thus, the claim that ‘a novel concept’ is revealed (abstract and first sentence of discussion) should be deleted. Nevertheless, the current manuscript goes significantly beyond the Fronz paper by showing effects on arginine methylation in cells, whereas the earlier paper was limited to in vitro binding and nuclear import assays. The possible role of arginine methylation in human genetic diseases, ALS and FTLD, is also interesting.

Specific comments:
1. The authors repeatedly make the point that arginine methylation is outside the nuclear localization signal (for example in the Discussion, p. 16). I find this misleading. First, they correctly state (p. 14, first paragraph of Discussion) that a PY-NLS has been previously described as a sequence of 30-40 amino acids. Thus, what they present as the NLS of FUS is clearly too short. Second, they show that the RGG domain of FUS, containing the methylated arginines, binds transportin. Thus, the RGG domain (or at least some part of it) should be considered part of the NLS. It would be more correct to suggest that an RGG domain can constitute a previously unknown part of the NLS.

In this context, arginine methylation of PABPN1 occurs both within the known consensus sequences of the PY-NLS and in more upstream regions. As the effect of arginine methylation on transportin binding is the same as in FUS, it appears artificial to make a distinction between PY-NLS and ‘outside’ sequences affecting transportin binding.

2. At least a selection of ITC data should be presented, e.g. in the Supplement. It would also be helpful if all dissociation constants were presented in a table. It is unexpected that the P525L mutation had no effect on the apparent transportin affinity of the 454-526 protein fragment (p. 12, first paragraph). In contrast, the same mutation had a very strong effect in the context of the shorter 489-526 peptide, independently of its methylation (bottom of p. 12). Do the authors have any thoughts about this? They might also wish to point out that binding of the longer peptide is significantly tighter.

The authors seem to imply that the effect of methylation is much stronger in the context of the P525L mutation compared to the wild-type, which would be a bit unexpected. However, the comparison seems unreliable, as the KD for the methylated mutant peptide could not be measured (unmethylated versus methylated: 172 versus higher than 200 microM; p. 12). Is this difference really bigger than that for the WT peptide (2.8 versus 7.8)?

3. Fig. 5: The data raise two obvious and related questions: First, if amino acids 514-526 are sufficient for nuclear import of FUS, independently of methylation, why is the RGG domain necessary? As the domain is present in three related proteins, it is probably there for a reason. Second, if arginine methylation in the RGG domain tends to inhibit nuclear import, what is the purpose of arginine methylation? In other words, having neither the arginine residues nor their methylation would seem more economical and more reliable with respect to nuclear import. These questions should be discussed.

4. Fig. 7: Although mutant and WT peptide were not compared side by side, there is no obvious difference in their ability to pull down transportin. Such a difference would be an important control that the pull-down assay reflects a biologically meaningful interaction. If the F525L mutation has no detectable effect, it would be important to show sensitivity of the interaction to Ran-GTP. The same control seems essential for transportin pull-down by the immobilized RGG peptide, as this is a novel transportin ligand.

Minor points:

p. 9, line 8: Cytosolic stress granules are visible in Fig. 4A, not B.

p. 9, line 10: The claim that GFP-M9M expression 'completely' blocked AdOx-induced nuclear import of FUS-P525L seems exaggerated - nuclear staining is still easily visible.

Fig. 6 and corresponding text: It was not clear to me which tag was present on the two peptides (454 - 526) used for NMR and ITC.

Referee #3

Arginine Methylation next to the PY-NLS modulates Transportin Binding and Nuclear Import of FUS

Overall, I think this is a well written paper that takes the previously published evidence that
inhibiting arginine methylation of ALS-causing FUS mutants can restore their nuclear import one step further, by indicating a mechanism by which this is likely to occur. Specifically the authors provide evidence indicating:

1) That this mechanism is transportin dependent

2) That it requires the presence of the PY-NLS sequence of FUS

3) That it also requires arginines in the RGG3 domain of FUS

4) That the ALS-causing P525L mutation in the PY-NLS-sequence of FUS causes weaker binding of the mutant PY-NLS sequence to transportin and that when de- methylated, the RGG3 domain of FUS interacts tightly with TRN, thereby rescuing the weaker binding of the mutant PY-NLS sequence.

5) That Arginine methylation in the RGG3 domain of FUS impairs transportin-binding

On the basis of these novel findings, I conclude that this paper should be accepted, providing the following amendments are made:

1) In the authors previous Embo publication (Dormann et al., 2010) it was stated in the materials and methods that in the nuclear and cytoplasmic localisation experiments the pictures and quantification presented were from one experiment, but were representative of several experiments. In the current manuscript it is stated that in experiments involving analysis of nuclear vs. cytoplasmic localisation of FUS in Hela cells, 30-50 cells were randomly selected and nuclear vs. cytoplasmic localisation of FUS was quantified and averaged across these cells. This statement suggests that only one experiment was carried out in each case. I assume that the authors carried out more than one experiment for each set of results in order to confirm their findings and think that the manuscript should be adjusted to specify that the results being presented were representative of several experiments.

2) There appears to be a mistake on page 16 in the following sentence: 'Indeed, a recent study reported that arginine methylation of the nuclear poly(A) binding protein (PABPN1) weakens its interaction with TRN and that several nuclear proteins, including FUS, show reduced TRN binding in PRMT1 knockout cells (Fronz et al., 2011)'. Having read the paper cited, I think that this sentence should say increased TRN binding in PRMT1 knockout cells.

3) In the following paragraph a lot of emphasis is placed on potential small changes seen in the amount of nuclear FUSWT upon AdOX treatment in Hela cells. Although I, like the authors think it is tempting to speculate that arginine methylation may be a common mechanism that modulates nuclear import of all FET proteins, I think that the results for WT FUS in this paper can not be used to make this argument as the changes seen are so small that they could very possibly have occurred due to chance. Furthermore, if data were only derived from one experiment then it is quite possible that these small changes would disappear when averaging across experiments was performed. Consequently I think this paragraph should be re-written to put less emphasis on these results and more emphasis on how this hypothesis could be investigated further. It would for example be interesting to investigate whether the methylation state of WT FUS is different to mutant FUS or whether it changes under conditions of stress etc.

It is still unclear why FUS and the other FET proteins (EWS and TAF15) are pathologically deposited in the cytoplasm in sporadic FTLD-FUS patients in the absence of NLS17mutations (Neumann et al., 2011). Our binding studies have shown that arginine methylation impairs TRN binding not only to mutant FUS, but also slightly decreases the affinity of TRN for wild-type FUS. Consistently, a small but reproducible increase in the amount of nuclear FUSWT can be observed upon AdOx treatment or PRMT1 knockdown (Fig. 1 and 3). Given our finding that nuclear localization of the FET protein mutants can be restored by AdOx treatment (Fig. 2) and the fact that all FET proteins are arginine methylated within the RGG domain next to the PY-NLS (Belyanskaya et al., 2001; Du et al., 2011; Hung et al., 2009; Jobert et al., 2009; Lee and
Bedford, 2002; Ong et al., 2004; Pahlich et al., 2005; Rappsilber et al., 2003), it is plausible that arginine methylation may be a common mechanism that modulates nuclear import of all FET proteins. Thus, it is tempting to speculate that altered arginine methylation of the FET proteins might contribute to their pathological mislocalization in sporadic FTLD-FUS. Hypermethylation of the FET proteins might lead to slightly reduced TRN binding, which slowly over time might result in cytoplasmic accumulation and abnormal deposition of the FET proteins in FTLD-FUS brains. Alternatively, hypomethylation of the FET proteins, which according to our data would lead to very tight binding of the FET proteins to TRN, might hamper dissociation of the transport complex and might lead to the co-deposition of the partner proteins in the cytoplasm. In line with this idea, TRN has recently been identified as an abundant component of the FUS-positive inclusions in FTLD-FUS patients (Brelstaff et al., 2011). In this context, it is also interesting to note that arginine methylation can affect protein aggregation, and in all reported cases hypomethylation favored protein oligomerization/aggregation (Ostareck-Lederer et al., 2006; Perreault et al., 2007; Yu et al., 2004). Thus, hypomethylation might contribute to the pathological deposition of the FET proteins by affecting both their nuclear import and their aggregation behavior.

Thank you for your interest in our manuscript “Arginine methylation next to the PY-NLS modulates Transportin-mediated nuclear import of FUS”. We appreciate your helpful comments and believe that we can fully address all of the concerns that you and the reviewers have raised.

As mentioned before, we have generated novel monoclonal antibodies specific for methylated FUS. This unique tool will allow us to examine the methylation status of FUS in cells and human post mortem brains. By now, we have thoroughly characterized the antibodies and can show that they are specific for methylated FUS and work in immunoblot and immunocytochemistry (Fig. R1A and B).

Moreover, we can show that ALS-associated FUS mutants are indeed methylated (Fig. R1C), in line with their poor nuclear import (Dormann et al., EMBO Journal 2010). Strikingly, we find that stress granules, the potential precursors of pathological FUS inclusions in ALS/FTLD (Dormann & Haass, TINS 2011), contain methylated FUS (Fig. R1C), supporting our hypothesis that arginine methylation might be involved in the pathogenesis of ALS with FUS mutations (last section of our discussion). These data are ready to be included in a revised manuscript. We are currently also testing if our antibodies work on paraffin sections and are planning to use them for IHC on post mortem brains of FTLD/ALS-FUS patients. If they do, we will investigate if cytosolic deposits in FTLD/ALS-FUS brains contain methylated FUS.

In addition to the potential pathological impact, our data also provide novel insights into the function of arginine methylation in nuclear transport of FET proteins, as pointed out by the reviewers. First of all, our data identify the RGG domain of FUS as a previously unknown TRN binding epitope (epitope 4 in Fig. 8) and suggest a novel model of TRN-substrate recognition, where the previously described epitopes 1-3 anchor the substrate in the TRN binding pocket and the adjacent RGG repeats (epitope 4) stabilize the interaction. Second, we find a small, but statistically significant effect of methylation inhibition on nuclear import of wild-type FUS (p<0.05; unpaired two-tailed t-test). We thus suggest that arginine methylation may fine-tune nucleocytoplasmic localization of FUS, which is thought to have essential functions both in the nucleus and the cytoplasm and this most likely requires an accurately controlled nuclear import mechanism.

To address the additional issues raised by the reviewers, we have completed or started the following experiments:

Referee #1:
1- We examined some of the TAF15 mutations described by Couthouis et al., PNAS 2011. However, we could not reproduce the cytoplasmic mislocalization reported for these mutants and therefore had to introduce a point mutation in the NLS of TAF15 in order to demonstrate an effect of AdOx on nuclear import of TAF15.
2- As described above, we will use our novel meFUS-specific antibodies to show that ALS-associated FUS mutants are indeed methylated.
Referee #2:
1- As suggested by the reviewer, we will revise our definition of the PY-NLS and will discuss the interesting questions brought up by the reviewer.
2- We will present a selection of the ITC binding curves in the supplement and will put all dissociation constants in a table (Fig. R2). Moreover, we measured binding of two new peptides (504-526-WT and P525L) to TRN. This demonstrates that the WT peptide binds to TRN with much higher affinity than the mutant and shows that addition of the RGG3 region strongly enhances binding to TRN (Fig. R2).
3- We will analyze the RGG3-TRN interaction for sensitivity to Ran-GTP.

Referee #3:
1- We will include a statement that all results shown are representative of several experiments.
2- We will show statistics demonstrating that the small differences in nuclear localization of FUS-WT with and without AdOx did not occur by chance, but are statistically significant.
3- We will use our novel meFUS-specific antibodies to provide evidence that ALS-associated FUS mutants are methylated. Thus we believe that we can address all questions raised by the reviewers and demonstrate important mechanistic insights into the arginine methylation-dependent regulation of nuclear import of FUS and its potential role in disease.

1st Editorial Decision 23 May 2012

Thank you for sending me your detailed point-by-point response I have now had a chance to take a look at it.

I find that the inclusion of the data using the developed monoclonal antibodies against methylated FUS will clearly strengthen the paper and add more support for that arginine methylation is involved in pathogenesis. Adding data using the Abs on FTLD/ALS-FUS patient samples would also significantly add to the paper. Given this I would like to ask you to submit a suitably revised manuscript that includes the data as indicated in your point-point response.

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

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

Yours sincerely,

Editor
The EMBO Journal

1st Revision - authors' response 03 August 2012

Point-by-point response to the reviewers:

Referee #1

We are happy that this reviewer finds that our “study has been performed with technical competence and is very well written. Taken together, the results provide evidence that methylation of FUS in the RGG3 may be a strong modifier of nuclear/cytoplasmic localization of this protein in the presence of NLS-impairing mutations.”
Specific points:

1- The experiments in Fig. 2 deal with the TAF15 and EWS proteins in which the authors inserted a mutation in their respective NLS signals to mimic the effect of the FUS-P525L mutation. Although the results are clear and consistent with the FUS data, this experiment would have been much more meaningful had the authors inserted in TAF15 and EWSR1 some mutations that have been shown to affect localization (i.e. disease-associated mutants that result in cytoplasmic accumulation as those described by Couthouis et al., 2011, PNAS and Couthouis et al, 2012, HMGB).

   We followed the reviewer’s advice and examined some of the TAF15 mutations described by Couthouis et al., PNAS 2011 (M368T, D386N, G391E and G473E). However, we did not observe the cytoplasmic accumulation referred to by the reviewer and therefore had to use a TAF15 mutant with a PY-NLS mutation (P591L) in order to demonstrate the effect of AdOx on nuclear import of TAF15.

2- The potential pathological implications of these proteins has only been addressed in the Discussion (page 17 and 18). Although it is possible that an increased methylation of a mutated FUS might lead to an increased localization/aggregation in the cytoplasm, there is really no evidence that methylation levels may be altered in the brains of people affected by ALS or FTD. Furthermore, even if there was evidence of altered methylation levels in patient’s brains, the results in Fig. 1 and 2 show very clearly that the almost complete nuclear localization of FUS, TAF, and EWS is hardly affected even by treatment with AdOx (an extremely strong inhibitor of all cellular methylation processes and thus unlikely to mimic any physiological condition, whether normal or pathological). If we then consider that many ALS/FTD patients with FUS inclusions do not carry any mutation in this protein it is really very hard to see how changes in methylation level could play a role in the pathological process.

   The reviewer criticized that the pathological implications of our findings have only been addressed in the discussion. We therefore generated monoclonal antibodies specific to methylated FUS (new Fig. 8) and examined the methylation status of FUS in our cellular models and post mortem tissue of ALS-FUS and FTLD-FUS patients. Using these unique tools, we can now show that ALS-associated FUS mutants are indeed methylated and that stress granules, the potential precursors of pathological FUS inclusions (Dormann et al, EMBO J 2010; Bosco et al, Hum Mol Gen 2010, Gal et al, Neurobiol Aging 2010), contain methylated FUS as well (new Fig. 8). More importantly, we find that FUS inclusions in ALS-FUS patients are strongly co-labeled with our meFUS-specific antibodies (new Fig. 9A), supporting our hypothesis that arginine methylation is required for the pathological mislocalization of ALS-associated FUS mutants. Interestingly, we found that in contrast to ALS-FUS, FUS inclusions in FTLD-FUS patients are unmethylated (new Fig. 9B), suggesting that hypomethylation of FUS and the related EWS and TAF15 proteins (FET proteins) may be responsible for the co-deposition of FET proteins and TRN in pathological inclusions. Even though we agree with the reviewer that localization of FUS, TAF, and EWS is only very slightly affected by AdOx treatment (Fig. 1 and 2), our in vitro pulldown experiments and ITC data (Fig. 7C) clearly demonstrate that binding of FUS-WT to TRN is affected by arginine methylation. Even though differences in TRN binding affinity of methylated versus unmethylated FUS-WT may be small, it seems possible that over long periods of time, a slight increase in FET-TRN binding may lead to a co-deposition of FET proteins and TRN in this late-onset neurodegenerative disease. This may be analogous to the very subtle changes in amyloid β-peptide metabolism in sporadic Alzheimer cases as compared to the dramatic differences observed in familial cases.

Referee #2

This reviewer finds our paper “interesting and technically largely convincing. Its importance is slightly diminished by the fact that Fronz et al. (cited in the manuscript) already reported a weakening of the interaction between the protein PABPN1 and transportin by arginine methylation of the former. Thus, the claim that 'a novel concept' is revealed (abstract and first sentence of discussion) should be deleted. Nevertheless, the current manuscript goes significantly beyond the Fronz paper by showing effects on arginine methylation in cells, whereas the earlier paper was limited to in vitro binding and nuclear import assays. The possible role of arginine methylation in human genetic diseases, ALS and FTLD, is also interesting.
As requested by the reviewer, we deleted the claim of ‘a novel concept’ and now only state that a “novel TRN binding epitope” is revealed.

Specific points:

1- The authors repeatedly make the point that arginine methylation is outside the nuclear localization signal (for example in the Discussion, p. 16). I find this misleading. First, they correctly state (p. 14, first paragraph of Discussion) that a PY-NLS has been previously described as a sequence of 30-40 amino acids. Thus, what they present as the NLS of FUS is clearly too short. Second, they show that the RGG domain of FUS, containing the methylated arginines, binds transportin. Thus, the RGG domain (or at least some part of it) should be considered part of the NLS. It would be more correct to suggest that an RGG domain can constitute a previously unknown part of the NLS. In this context, arginine methylation of PABPN1 occurs both within the known consensus sequences of the PY-NLS and in more upstream regions. As the effect of arginine methylation on transportin binding is the same as in FUS, it appears artificial to make a distinction between PY-NLS and ‘outside’ sequences affecting transportin binding.

We have previously shown that an even shorter sequence of FUS (residues 514-526) is necessary and sufficient for nuclear import of FUS (Dormann et al., EMBO J 2010) and a recent X-ray crystallography study by Chook and colleagues showed that epitopes 1-3 of TRN are located in residues 508-526 (Zhang et al, PNAS 2012). Therefore, we think that it is correct to designate the residues C-terminal of the RGG3 repeat region (506-526) as PY-NLS harboring epitopes 1-3 (see schematic diagrams in Fig. 1A and 10A).

We show for the first time that the RGG repeat domain of FUS can be considered a new TRN binding epitope (epitope 4 in Fig. 10A). We agree with the reviewer that in the future this domain should be considered part of the PY-NLS of FUS. However, in our manuscript we still make the distinction between PY-NLS (epitopes 1-3, light red) and RGG repeat region (epitope 4, light green) for historic reasons and better comprehension.

2- At least a selection of ITC data should be presented, e.g. in the Supplement. It would also be helpful if all dissociation constants were presented in a table. It is unexpected that the P525L mutation had no effect on the apparent transportin affinity of the 454-526 protein fragment (p. 12, first paragraph). In contrast, the same mutation had a very strong effect in the context of the shorter 489-526 peptide, independently of its methylation (bottom of p. 12). Do the authors have any thoughts about this? They might also wish to point out that binding of the longer peptide is significantly tighter.

The authors seem to imply that the effect of methylation is much stronger in the context of the P525L mutation compared to the wild-type, which would be a bit unexpected. However, the comparison seems unreliable, as the KD for the methylated mutant peptide could not be measured (unmethylated versus methylated: 172 versus higher than 200 microM; p. 12). Is this difference really bigger than that for the WT peptide (2.8 versus 7.8)?

As suggested by the reviewer, we now show the experimental ITC curves for FUS454-526WT and FUS454-526P525L in a new supplementary figure (new Fig. S1) and present all the Kds measured for the unmethylated FUS peptides/proteins in a schematic diagram (new Fig. 6D). We also studied two new peptides that comprise the PY-NLS only and no RGG repeats (FUS504-526WT and FUS504-526P525L). The comparison of all Kds obtained for the different peptides shows that especially the N-terminal residues of the RGG repeat region (454-488) have a strong contribution to TRN binding, since the presence of this sequence strongly enhances TRN binding, especially in the context of the P525L mutation, where it restores binding of the P525L mutant to wild-type levels (new Fig. 6D).

The fact that methylation has a stronger effect in the context of the P525L mutation compared to the WT is supported by our pulldown assays (Fig. 7B and C), where methylation has a stronger effect on the P525L mutant peptides compared to the WT peptides.

3- Fig. 5: The data raise two obvious and related questions: First, if amino acids 514-526 are sufficient for nuclear import of FUS, independently of methylation, why is the RGG domain necessary? As the domain is present in three related proteins, it is probably there for a reason. Second, if arginine methylation in the RGG domain tends to inhibit nuclear import, what is the purpose of arginine methylation? In other words, having neither the arginine residues nor their
methylation would seem more economical and more reliable with respect to nuclear import. These questions should be discussed.

The reviewer brings up some very interesting questions. As requested, we now discuss these points in our revised discussion (last paragraph).

4- Fig. 7: Although mutant and WT peptide were not compared side by side, there is no obvious difference in their ability to pull down transportin. Such a difference would be an important control that the pull-down assay reflects a biologically meaningful interaction. If the P525L mutation has no detectable effect, it would be important to show sensitivity of the interaction to Ran-GTP. The same control seems essential for transportin pull-down by the immobilized RGG peptide, as this is a novel transportin ligand.

Yes, WT and mutant peptides were compared side by side: First of all, their Kds were determined by ITC, allowing a direct quantitative comparison. This showed that both FUS489-526WT and FUS504-526WT bind to TRN much better than their mutant counterparts (new Fig. 6D). Second, FUS489-526WT and FUS489-526P525L were also compared side by side in a pulldown assay (see Fig. 7C right lane labeled P525L, this refers to the FUS489-526P525L peptide). This also demonstrates a much better binding of the WT peptide compared to the P525L peptide. Only in the much longer FUS454-526 construct, which includes the entire RGG3 region, the WT and P525L mutant show equal TRN binding (Fig. 6D), presumably due to the tight interaction of the RGG repeat region with TRN (Fig. 6C).

Minor points:

- p. 9, line 8: Cytosolic stress granules are visible in Fig. 4A, not B.
  We corrected this mistake.

- p. 9, line 10: The claim that GFP-M9M expression 'completely' blocked AdOx-induced nuclear import of FUS-P525L seems exaggerated - nuclear staining is still easily visible.
  We rewrote this sentence.

- Fig. 6 and corresponding text: It was not clear to me which tag was present on the two peptides (454-526) used for NMR and ITC.
  As stated in the supplementary material and methods (under “Recombinant protein expression and purification”), FUS454-526WT and FUS454-526P525L were purified as ZZ-His6-tagged fusion proteins and the tag was subsequently cleaved off and eliminated in a second affinity purification step, so untagged FUS454-526 proteins were used for NMR and ITC measurements.

Referee #3

Overall, I think this is a well written paper that takes the previously published evidence that inhibiting arginine methylation of ALS-causing FUS mutants can restore their nuclear import one step further, by indicating a mechanism by which this is likely to occur. Specifically the authors provide evidence indicating: 1) That this mechanism is transportin dependent; 2) That it requires the presence of the PY-NLS sequence of FUS; 3) That it also requires arginines in the RGG3 domain of FUS; 4) That the ALS-causing P525L mutation in the PY-NLS sequence of FUS causes weaker binding of the mutant PY-NLS sequence to transportin and that when de-methylated, the RGG3 domain of FUS interacts tightly with TRN, thereby rescuing the weaker binding of the mutant PY-NLS sequence; 5) That Arginine methylation in the RGG3 domain of FUS impairs transportin-binding.

On the basis of these novel findings, I conclude that this paper should be accepted, providing the following amendments are made:

Specific points:

1 - In the authors previous Embo publication (Dormann et al., 2010) it was stated in the materials and methods that in the nuclear and cytoplasmic localisation experiments the pictures and quantification presented were from one experiment, but were representative of several experiments. In the current manuscript it is stated that in experiments involving analysis of nuclear vs. cytoplasmic localisation of FUS in Hela cells, 30-50 cells were randomly selected and nuclear vs. cytoplasmic localisation of FUS was quantified and averaged across these cells. This statement
suggests that only one experiment was carried out in each case. I assume that the authors carried out more than one experiment for each set of results in order to confirm their findings and think that the manuscript should be adjusted to specify that the results being presented were representative of several experiments.

We apologize for omitting the information that pictures and quantification shown are from one experiment, but are representative of at least three independent experiments. We now state this in the materials and methods (under “Fluorescence image acquisition” and “Image quantification and statistics”).

2- There appears to be a mistake on page 16 in the following sentence: “Indeed, a recent study reported that arginine methylation of the nuclear poly(A) binding protein (PABPN1) weakens its interaction with TRN and that several nuclear proteins, including FUS, show reduced TRN binding in PRMT1 knockout cells (Fronz et al., 2011)”. Having read the paper cited, I think that this sentence should say “increased TRN binding in PRMT1 knockout cells”.

We corrected this mistake.

3- In the following paragraph a lot of emphasis is placed on potential small changes seen in the amount of nuclear FUS-WT upon AdOX treatment in Hela cells. Although I, like the authors, think it is tempting to speculate that arginine methylation may be a common mechanism that modulates nuclear import of all FET proteins, I think that the results for WT FUS in this paper can not be used to make this argument, as the changes seen are so small that they could very possibly have occurred due to chance. Furthermore, if data were only derived from one experiment then it is quite possible that these small changes would disappear when averaging across experiments was performed. Consequently I think this paragraph should be re-written to put less emphasis on these results and more emphasis on how this hypothesis could be investigated further. It would for example be interesting to investigate whether the methylation state of WT FUS is different to mutant FUS or whether it changes under conditions of stress etc.

As explained under specific point 2 of reviewer 1, we have used novel meFUS-specific monoclonal antibodies to show that both FUS-WT and ALS-associated FUS mutants are methylated (new Fig. 8) and that inclusions in ALS-FUS patients contain methylated FUS, while inclusions in FTLD-FUS are unmethylated (new Fig. 9). Moreover, our in vitro pulldown experiments and ITC data clearly demonstrate that binding of FUS-WT to TRN is affected by arginine methylation (Fig. 7C). Even though differences in TRN binding affinity of methylated versus unmethylated FUS-WT may be small, it seems possible that over long periods of time, a slight increase in FET-TRN binding may lead to a co-deposition of FET proteins and TRN in FTLD-FUS. Taken together, we now provide ample experimental evidence that arginine methylation may play a role in FTLD-FUS. Based on our new findings, we have substantially rewritten the paragraph referred to by the reviewer and now discuss the interesting differences in the neuropathology of ALS-FUS and FTLD-FUS and the idea that the two disorders may be caused by different pathomechanisms.

Additional Correspondence 17 August 2012

Thank you for submitting your revised manuscript to the EMBO Journal. Your revision has now been seen by referees #1 and 2. As you can see below, both referees appreciate the introduced changes and support publication here. I am therefore very pleased to proceed with the acceptance of the paper. I also would like to add that I appreciate very much the extra efforts that you undertook to address the concerns raised.

There are just a few formalities we need to take care of before the paper can be transferred to the publisher.

1) Referee #2 has a few minor text suggestions. You can send me a modified text by email address.

2) We also now encourage the publication of source data, particularly for electrophoretic gels and blots. Would you be willing to provide a PDF file per figure that contains the original, uncropped and unprocessed scans of all or key gels used in the figure? The PDF files should be labeled with the appropriate figure/panel number, and should have molecular weight markers; further annotation could be useful but is not essential. The PDF files will be published online with the article as supplementary “Source Data” files. If you have any questions regarding this just contact me.
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4) Please also note: A new policy at The EMBO Journal is for the transcript of the editorial process (containing referee reports and your response letter) to be published as an online supplement to each paper. If you do NOT want this in this case, you will need to inform the Editorial Office via email immediately (i.e. at the stage of providing licenses and/or confirming whether you take the EMBO OPEN option, see below).

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If you have any questions, please do not hesitate to call or email us.

Yours sincerely

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1:
The authors have answered this reviewer's questions to complete satisfaction and should be commended for the additional work undertaken to perform this task at the experimental level.

Referee #2:
In the revision, the authors have taken care of my criticism. This is a good paper that should be published.

Before it goes to print, a few minor points can be taken care of:

p. 11, first line: 'To prove', not 'proof'

A few lines down: 'Lack of stable tertiary and secondary structure is a common feature of known PY-NLSs and allows for highly specific binding of a great variety of different cargo proteins.' If I understand correctly what the authors wish to say, it would be better to say '....of different transportin cargo proteins'.
p. 17, second paragraph: 'This suggests that the C-terminal TRN binding epitopes might anchor the protein and for further...' - this sentence seems to be screwed up. Delete 'and'?

p. 19-20: The authors state that PABPN1 is not deposited in inclusions in FTLD-FUS. They use this as an argument 'against a general defect in PRMT1-dependent arginine methylation'. However, Fronz et al. (reference cited in the manuscript) cite an earlier paper according to which PABPN1 is methylated even in PRMT1 knock-out cells. Thus, the behavior of PABPN1 cannot be used to judge PRMT1-dependent arginine methylation.