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G-quadruplex RNA structure as a signal for neurite mRNA targeting

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1st Referee Reports

REFEREE REPORTS

Referee #1 (Remarks to the Author):

In this manuscript Subramanian and colleagues describe the identification of G(uanine)-quartet structures as cis-acting dendritic-localization elements (DTEs) present within neuronal mRNAs. The Moine group and others previously identified G-quartets as binding sites for Fragile-X Mental Retardation protein (FMRP), a factor involved in activity-dependent mRNA transport and translation in neurons, and here test the hypothesis that these structures could act as mRNA localization signals. Indeed, the authors demonstrate that G-quartet consensus sequences are over-represented in transcripts transported into dendrites when compared to a random pool of mRNAs. The authors select two mRNAs, PSD-95 and CamKIIa, for detailed molecular analysis. Using various biochemical assays the authors demonstrate that these two transcripts indeed harbor G-quartet structures in their 3'UTRs. They go on to test for a functional requirement of these sequences using a previously described GFP-based mRNA tracking assay, coupled to welcome validation of the key results by in situ hybridization. Deleting the G-quartet forming sequences within the 3'UTR resulted in both cases in loss of dendritic targeting. Moreover, isolated G-quartet forming sequences could confer dendritic localization to heterologous sequences, providing strong evidence that G-quartet structures can act as dendritic-localization elements. The authors continue by showing that FMRP binds to PSD-95 and CamKII mRNAs and colocalizes with these transcripts in dendrites. However, absence of FMRP does not interfere with dendritic localization of mRNAs at steady-state and has only a minor role in activity induced transport.

The identification of G-quartet structures as dendritic localization elements is an important

contribution towards a better understanding of the molecular mechanisms that control mRNA transport in neurons. The experiments demonstrating the presence and importance of G-quartets are sound and well controlled and the results presented clearly support the drawn conclusions. However, I have major concerns regarding the section of the manuscript describing the role of FMRP as well as with the overall structure and novelty of this manuscript. I shall outline these in a point-by-point manner.

1. The role of FMRP is over-represented in the current version of the manuscript. Two figures and much of the text (particularly the discussion) are devoted to the role of FMRP, despite the fact that this protein appears to be of only minor importance for G-quartet mediated transport. What is more is that some of the key observations and conclusions drawn from the FMRP experiments are not novel. Two previous papers (Dictenberg et al., 2008 and Kao et al., 2010) have studied mRNA transport in FMRP deficient neurons. These studies present data showing that FMRP colocalizes with relevant mRNAs (here confirmed in Fig. 5a) and that loss of FMRP leads to decreased mRNA transport in stimulated but not unstimulated neurons (here reproduced in Fig. 5b). Although replication of published results is important for the field, the reduced novelty of these sections counts substantially against the overall suitability for publication of this manuscript in EMBO Journal. Moreover, I believe that placing the FMRP data in such a prominent position unnecessarily diverts attention from the strong point of the paper - the identification of a novel dendritic targeting element.

2. Discrepancies between the current study and previous findings are not appropriately discussed. The effect of the G-quartets for mRNA localization reported here is very striking. Deleting exclusively the G-quartet forming sequence virtually eliminates dendritic localization, whereas the same sequence can mediate strong localization on its own. In particular in the case of CamKIIa these results are at odds with previous results of other groups that have found DTEs in other parts of the 3'UTR. For example, Huang et al. showed in 2003 that the cytoplasmic polyadenylation element (CPE) present in the CamKIIa 3'UTR is sufficient for dendritic targeting. This CPE is still present in the CamK2a3112 G construct that does not show any dendritic localization in the present study, in apparent contradiction to the aforementioned paper. The authors of the current manuscript appear to control their mapping experiments very well, and their conclusions therefore seem sound. However, the reader should be made more aware of previously identified DTEs present within the used transcripts and interpretation should be offered to why these might have only little to no effect in the current study.

3. On page 9 the authors note that "...a very stable 4-layer G-quartet was a better DTE than a comparable less stable 3-layer G-quartet...". However, in Figure 2b it is shown that constructs with G4-quartets localize in 60+-5% (G4-A) and 35+-4% (G4-T) of cells, whereas G3-A containing constructs localize in 80+-5% of cells. Also in Fig. 3c G3-A containing constructs appear to localize at least as efficient as G4-A containing ones. Such discrepancies between text and experimental data are not acceptable.

4. The manuscript would greatly benefit from careful editing. There are multiple mistakes in spelling and grammar and it would be advisable to have the manuscript corrected by a native English speaker. Other parts are mixed up (for example legend to figure 2) and require proofreading. In addition, the composition of the figures does not always follow a clear logic (Fig. 3a is mentioned in the text before Fig. 2, Fig. 3c reports visually on the same data summarized in Fig. 2b), and it is not clear where the results end and the discussion begins.

Overall, this manuscript contains important findings about the role of the G-quartet in neuronal mRNA targeting. However, in the absence of information about the factor responsible for binding this feature and mediating localization, this manuscript is, in my opinion, more suitable for publication in another journal (possibly in a shorter format). If they had this factor the authors would probably wish to send the manuscript even higher, but that does not mean that in its current form it is acceptable for EMBO Journal.

Referee #2 (Remarks to the Author):

In their study Subramanian et al. identify g-quartets in the 3'-UTRs of dendritically localized mRNAs and validate their role as dendritic targeting elements (DTEs) promoting the transport of reporter transcripts. The study embarks on the established association of FMRP with g-quartets and hypothesizes that these act as cis-elements promoting dendritic localization of mRNAs. The biochemical studies validating g-quartets in CamkII and PSD-95 3'-UTRs convincingly demonstrate potassium sensitivity. Using a lambda-tagging approach, the authors demonstrate that reporter transcripts comprising the g-quartet are localized to dendrites whereas they observe a striking restriction to the cell body upon q-quartet deletion. These findings are supported by FISH studies and the observation that g-quartet dependent localization of transcripts is DHPG-responsive. Finally, the authors demonstrate that FMRP associates with the CamkII as well as PSD-95 3'-UTRs in a g-quartet and potassium-dependent manner. Although exogenous FMRP co-localizes with reporter transcripts, the protein appears to be dispensable for g-quartet dependent dendritic localization of reporter transcripts.
The presented study provides evidence for g-quartet dependent dendritic localization of mRNAs but fails to identify a key trans-acting factor required for this transport. At this point the study comes up with a surprising observation which is that the g-quartets of PSD-95 and CamkII solely act as localization elements but do not affect mRNA turnover or translation. This is puzzling, since one would expect that the localization elements directly affect the dendritic fate of localized transcripts. Hence, the authors have to investigate in further detail how the observed g-quartet dependent localization affects the fate of targeted transcripts in terms of turnover and spatially restricted protein synthesis. Moreover, the authors have to provide some more insight into involved trans-acting factors modulating g-quartet dependent RNA localization. Only this would provide a significant step forward in the understanding of how spatially restricted protein synthesis in neurons could be regulated by g-quartets and potential trans-acting factors like FMRP.

Additional aspects to be addressed:

1) Instead of using deletion mutants in Fig.2B/C and supplemental Fig. S4, the authors should use point mutants in which the formation of g-quartets is abrogated.
2) How is dendritic localization of mRNAs validated versus axonal localization?
3) The authors need to normalize their image analyses, since the lack of dendritic localization could be due to different expression levels and exposure times. Thus, the authors should attempt to normalize to some sort of dendritic immune-staining, for instance via Cy5/Cy7. Moreover, fluorescence intensities in dendrites need to be quantified in Fig.S4, since also g-quartet lacking transcripts are found in dendrites (see magnification).
4) Data shown inFig.3C need to be quantified.
5) The competition studies in Fig.5A need more data points.
6) How to explain the obvious difference in K+ versus Li+ sensitivity in FMRP binding when comparing PSD-95 to CamkII?
7) The colocalization in Fig.6A needs to be quantified and validated by immunostaining for endogenous FMRP and other potential trans-acting factors factors.
8) The authors propose that that removal of the g-quartets in reporter transcripts does not affect translation nor turnover of mRNAs (Fig. S5). These studies need a better explanation of how qRT-PCRs studies were normalized and require decay analyses. Moreover, the authors have to also analyze reporter transcripts with q-quartets in a non-natural 3'-UTR.
9) Finally the authors should attempt to determine how mutation of q-quartets affects spatially restricted protein synthesis in dendrites in cortical neurons.

Referee #3 (Remarks to the Author):

The authors extend previous work in this very interesting and timely manuscript identifying G-quartet RNA motifs in a series of dendritically localized mRNAs, and further characterize its role in two prominent mRNAs, e.g. CaMK2a and PSD95. Interestingly, PSD95 mRNA has three G-quartets whereas CaMK2a mRNA has only one. Using a previously uncharacterized GFP-based localization system developed by the Ellenberg lab for cell lines, they provide evidence that both mRNAs indeed localize to dendrites of cortical neurons. Furthermore, they extend previous work by the Bassell lab

using DHPG stimulation to show that the G-quartet-driven localization increases upon metabotropic glutamate receptor stimulation. Finally, the authors provide interesting evidence that FMRP participates in this localization process, but it not essential arguing for more than a more than one trans-acting factor model.

Overall, the manuscript reports important new findings that would be relevant for a general audience. In the remainder, I would like to point out a few issues that need to be addressed before publication:

1. Most data are shown for cortical neurons that are significantly less polarized than hippocampal neurons. The manuscript states that both neurons were used, however, that only cortical neurons are shown. However, some of the neurons shown look very much like hippocampal neurons. It would be important to state for each experiment which type of neurons were used. Along those lines, I would suggest that at least some of the key findings are reproduced in hippocampal neurons, since dendritic localization is much better established in those cells.

2. The authors make a strong point about the lambda system being better than the MS2 system (data not shown). Many labs have tried the lambda system for localization experiments, but failed. Therefore, it would be important to demonstrate why it is better and support this with data. For sure, the video provided does not (yet) support this statement.

3. An essential part of this manuscript is Figure 2 providing evidence that PSD95 and CaMK2a mRNAs indeed localize into dendrites of cortical neurons. I suggest rearranging this essential figure as follows: panel A can stay (alternatively, it would fit better to Figure 1 or go to Supplements); revised panel B could have the cartoon containing the constructs shown (however, significantly more experimental detail is necessary in the figure legend and/or the material and method section for the various mRNAs, e.g. PSD837deltaG, PSDGq, etc.: what are the exact regions deleted/added) followed by examples of neurons as shown in old panel C. It would be essential to provide phase contrast pictures for these neurons to assess cell integrity. Otherwise, the observed localization patterns could results from dying or dead neurons. In addition, DAPI staining could be provided to show nuclear integrity of the cells shown. Most importantly, this revised panel should include high magnification insets of selected dendrites to compare dendritic localization of the three constructs. Revised panel C would then be the quantification of panel B. For this figure, it would be essential to provide (graphical) evidence on the previously identified dendritic localization elements in CaMK2a mRNA and their relative position to each other as defined by Mori et al., 2000, Blichenberg et al., 2001 and Huang et al, 2003.

4. I am not sure that I understand the experiment in Fig. 3B. According to the experiment shown in Fig. 2, I guess there is GFP as well as RFP (transfection control) expressed. However, the overview pictures are green only, but the high magnification insets contain red and green. It would be better to separate the two channels for the insets, since I am currently unable to compare localization patterns in green for the two top pictures. More importantly, I am not sure that the data for the various G constructs shown in Fig. 2B and Fig. 3C are consistent. Is the quantification shown in Fig. 2B for the original data in Fig. 3C? It seems to me as if there were significant discrepancies between the data shown and the quantifications. The results (page 9) states: "Thus, a very stable 4-layer G-quartet was a better DTE than a comparatively less stable 3-layer G-quartet,...". However, the graph in Fig. 2B says that G3-A > G4-A. Along those lines, the examples in Fig. 3C are overexposed and signal intensity should be reduced together with the inclusion of high magnification insets.

5. Fig. 5 is currently the weak spot of this manuscript. In comparison of the neurons used in this study (Figs. 2, 3), both cells are dying or dead indicated by the unusually large overexpression artifacts representing varicosity-like enlargements of the dendrites. It would be very important for this study to demonstrate convincingly that FMRP (labeled by mCherry) indeed colocalizes with the two respective RNAs (labeled by GFP). Furthermore, experiments shown in panel B are insufficiently explained in the figure legend and the results section preventing the reader to understand the experiment. The results section (page 10) states: "and only the mGluR-triggered transport of the RNA bearing Camk2a was found diminished (Fig. 5B). However, the figure symbols state Camk2a (wt) versus Camk2a (KO). Could it be that both graphs are wrongly labeled and show FMRP (wt) versus FMRP (KO)? But then what about the mentioned stimulation? If it is NOT mislabled, I strongly suggest including the data from FMRP KO neurons cited as data not

shown.

Additional points:
- I understand that the authors have previously established G quartets as possible FMRP binding motifs. However, the Darnell lab has recently provided evidence that FMRP might recognize other RNA structures than G quartets. It would be very important for the general audience to refer to this work and also provide a short discussion on alternative FMRP binding motifs.
- The mentioned bio-informatic GRSDB tool (Kostadinov et al, 2006) has been instrumental for this manuscript. It would be very helpful to provide the direct link in the Material and methods section. I googled the side, found the link but it did not work for me. Is it not yet activated? This might be very useful for the community!
- Generally, the field relates to Camk2a mRNA as CaMIIa or CaMIIalpha. I suggest sticking to this terminology.
- Results, page 7: "Using this system we could visualize very efficiently the localization of a reporter mRNA bearing the well established 54-nt dendritic targeting element (DTE) "zipcode" of the fl-actin mRNA (Kislauskis et al, 1994) in the dendrites of living (Supplementary Fig. 3) or fixed neurons (data not shown)." I think that the two references got mixed up? The fixed cells are shown in Suppl. Fig. 3 and the videos are not shown?
- Figure 2 and figure legend: Panels B and C have been inverted compared to the corresponding figure legend (contains two panels B, but no panel C).
- Page 19: thice G-guartet sequence instead of G-quartet.

1st Revision - authors' response

(see following pages)

Referee #1 (Remarks to the Author):

In this manuscript Subramanian and colleagues describe the identification of G(uanine)-quartet structures as cis-acting dendritic-localization elements (DTEs) present within neuronal mRNAs. The Moine group and others previously identified G-quartets as binding sites for Fragile-X Mental Retardation protein (FMRP), a factor involved in activity-dependent mRNA transport and translation in neurons, and here test the hypothesis that these structures could act as mRNA localization signals. Indeed, the authors demonstrate that G-quartet consensus sequences are over-represented in transcripts transported into dendrites when compared to a random pool of mRNAs. The authors select two mRNAs, PSD-95 and CamKIIa, for detailed molecular analysis. Using various biochemical assays the authors demonstrate that these two transcripts indeed harbor G-quartet structures in their 3'UTRs. They go on to test for a functional requirement of these sequences using a previously described GFP-based mRNA

tracking assay, coupled to welcome validation of the key results by in situ hybridization. Deleting the G-quartet forming sequences within the 3'UTR resulted in both cases in loss of dendritic targeting. Moreover, isolated G-quartet forming sequences could confer dendritic localization to heterologous sequences, providing strong evidence that G-quartet structures can act as dendritic-localization elements. The authors continue by showing that FMRP binds to PSD-95 and CamKII mRNAs and colocalizes with these transcripts in dendrites. However, absence of FMRP does not interfere with dendritic localization of mRNAs at steady-state and has only a minor role in activity induced transport.

The identification of G-quartet structures as dendritic localization elements is an important contribution towards a better understanding of the molecular mechanisms that control mRNA transport in neurons. The experiments demonstrating the presence and importance of G-quartets are sound and well controlled and the results presented clearly support the drawn conclusions. However, I have major concerns regarding the section of the manuscript describing the role of FMRP as well as with the overall structure and novelty of this manuscript. I shall outline these in a point-by-point manner.

1. The role of FMRP is over-represented in the current version of the manuscript. Two figures and much of the text (particularly the discussion) are devoted to the role of FMRP, despite the fact that this protein appears to be of only minor importance for G-quartet mediated transport. What is more is that some of the key observations and conclusions drawn from the FMRP experiments are not novel. Two previous papers (Dictenberg et al., 2008 and Kao et al., 2010) have studied mRNA transport in FMRP deficient neurons. These studies present data showing that FMRP colocalizes with relevant mRNAs (here confirmed in Fig. 5a) and that loss of FMRP leads to decreased mRNA transport in stimulated but not unstimulated neurons (here reproduced in Fig. 5b). Although replication of published results is important for the field, the reduced novelty of these sections counts substantially against the overall suitability for publication of this manuscript in EMBO Journal. Moreover, I believe that placing the FMRP data in such a prominent position unnecessarily diverts attention from the strong point of the paper - the identification of a novel dendritic targeting element.

As recommended here above, the FMRP data have been removed (and mentioned as data not shown). The term "G-quartet" was replaced by its synonym "G-quadruplex" which seems more in use among structuralists

2. Discrepancies between the current study and previous findings are not appropriately discussed. The effect of the G-quartets for mRNA localization reported here is very striking. Deleting exclusively the G-quartet forming sequence virtually eliminates dendritic localization, whereas the same sequence can mediate strong localization on its own. In particular in the case of CamKIIa these results are at odds with previous results of other groups that have found DTEs in other parts of the 3'UTR. For example, Huang et al. showed in 2003 that the cytoplasmic polyadenylation element (CPE) present in the CamKIIa 3'UTR is sufficient for dendritic targeting. This CPE is still present in the CamK2a3112∆G construct that does not show any dendritic localization in the present study, in apparent contradiction to the aforementioned paper. The authors of the current manuscript appear to control their mapping experiments very well, and their conclusions therefore seem sound. However,the reader should be made more aware of previously identified DTEs present within the used transcripts and interpretation should be offered to why these might have only little to no effect in the current study.

The presence of CPE elements nearby the G-quadruplex in CamkIIa RNA is now well stated in the manuscript: "*To demonstrate their role as dendritic targeting elements (DTE) and evaluate the contribution of additional nearby cis-acting elements (in particular the CPE element, two copies of which are close to the Gq structure in CamkIIa mRNA (Fig. 1B) and were proposed to be involved in localization (Huang et al, 2003)), the Gq forming sequences alone (as defined in Fig. 1B) were tested out of their natural 3'-UTR context. Gq structure formation was confirmed in these RNAs (Fig 1A, PSDGq, CaMKIIaGq) and dendritic targeting was found comparable to the one of the full-length UTRs (Fig 2, Sup Fig 4) indicating an essential role of the Gq in localization.*"

Our data concerning the CaMKIIa mRNA seem indeed in apparent contradiction with those of Huang et al., however they do not absolutely invalidate them because:

- the two studies were done in different brain regions (cortex vs hippocampus) where trans-acting factor availability maybe different,

- the 3' context in which the CaMKIIa 3'UTR was tested in the two studies is different. In Huang et al, a fragment of CaMKIIA 3'UTR (170nt) was inserted 400 nt upstream of a polyA tail in the ORF of Sindbis virus. In our study the full length UTR (3112 nt) was present in front of SV40 polyA signal in plasmid vector. As the CPE element acts on polyadenylation rate, its contribution may be different in the two studies.

- in the deletion construct CaMKIIaΔG, the deletion may disrupt the secondary structure of the CPE preventing to see its DTE activity.

- the minimal CaMKIIaGq does not restaures full DTE activity (55% compare to 82% full length CamKIIa 3'UTR), therefore leaving space for a role of CPEs.

So to make it short we proposed that *"For CaMKIIa mRNA this suggests a minor contribution of CPEs in cortex (this study) compared to hippocampus (Huang et al, 2003). Alternatively, Gq and CPEs could act in concert in CaMKIIa mRNA, as the efficiency of transport is not totally recovered in CaMKIIa Gq and Gq deletion in CaMKIIa3112ΔG may have perturbed CPEs folding masking their effect."*

3. On page 9 the authors note that "...a very stable 4-layer G-quartet was a better DTE than a comparable less stable 3-layer G-quartet...". However, in Figure 2b it is shown that constructs with G4-quartets localize in 60+-5% (G4-A) and 35+-4% (G4-T) of cells, whereas G3-A containing constructs localize in 80+-5% of cells. Also in Fig. 3c G3-A containing constructs appear to localize at least as efficient as G4-A containing ones. Such discrepancies between text and experimental data are not acceptable.

These errors in text were corrected.

4. The manuscript would greatly benefit from careful editing. There are multiple mistakes in spelling and grammar and it would be advisable to have the manuscript corrected by a native English speaker. Other parts are mixed up (for example legend to figure 2) and require proofreading. In addition, the composition of the figures does not always follow a clear logic (Fig. 3a is mentioned in the text before Fig. 2, Fig. 3c reports visually on the same data summarized in Fig. 2b), and it is not clear where the results end and the discussion begins.

Editing was performed and figures were recomposed

Overall, this manuscript contains important findings about the role of the G-quartet in neuronal mRNA targeting. However, in the absence of information about the factor responsible for binding this feature and mediating localization, this manuscript is, in my opinion, more suitable for publication in another journal (possibly in a shorter format). If they had this factor the authors would probably wish to send the manuscript even higher, but that does not mean that in its current form it is acceptable for EMBO Journal.

Referee #2 (Remarks to the Author):

In their study Subramanian et al. identify g-quartets in the 3'-UTRs of dendritically localized mRNAs and validate their role as dendritic

targeting elements (DTEs) promoting the transport of reporter transcripts. The study embarks on the established association of FMRP with g-quartets and hypothesizes that these act as cis-elements promoting dendritic localization of mRNAs. The biochemical studies validating g-quartets in CamkII and PSD-95 3'-UTRs convincingly demonstrate potassium sensitivity. Using a lambda-tagging approach, the authors demonstrate that reporter transcripts comprising the g-quartet are localized to dendrites whereas they observe a striking restriction to the cell body upon q-quartet deletion. These findings are supported by FISH studies and the observation that g-quartet dependent localization of transcripts is DHPG-responsive. Finally, the authors demonstrate that FMRP associates with the CamkII as well as PSD-95 3'-UTRs in a g-quartet and potassium-dependent manner. Although exogenous FMRP co-localizes with reporter transcripts, the protein appears to be dispensable for g-quartet dependent dendritic localization of reporter transcripts.

The presented study provides evidence for g-quartet dependent dendritic localization of mRNAs but fails to identify a key trans-acting factor required for this transport. At this point the study comes up with a surprising observation which is that the g-quartets of PSD-95 and CamkII solely act as localization elements but do not affect mRNA turnover or translation. This is puzzling, since one would expect that the localization elements directly affect the dendritic fate of localized transcripts. Hence, the authors have to investigate in further detail how the observed g-quartet dependent localization affects the fate of targeted transcripts in terms of turnover and spatially restricted protein synthesis. Moreover, the authors have to provide some more insight into involved trans-acting factors modulating g-quartet dependent RNA localization. Only this would provide a significant step forward in the understanding of how spatially restricted protein synthesis in neurons could be regulated by g-quartets and potential trans-acting factors like FMRP.

Not seeing an impact of the localization element on global mRNA turnover is not surprising: Huang et al (2003) reporting on the CPE elements or Kislauskis et al. (1994) on the Zipcode did not find either an influence of the disruption of the localization element on RNA steady states nor on general translation (effects on RNA stability were seen later on by deprivating the proteins that binds to these elements, which is not the same).
We completely agree on investigating the function of the G-quadruplex in local translation regulation. However this study goes beyond the scope of this short report.

Additional aspects to be addressed:

1) Instead of using deletion mutants in Fig.2B/C and supplemental Fig. S4, the authors should use point mutants in which the formation of g-quartets is abrogated.

A G-quadruplex **cannot** be disrupted by point mutation (the Gq structure is very stable and requires multiple mutations)… Recapitulation of the function with a "generic" G-quadruplex where the intervening nucleotides are different and in a non-natural context is for us the best possible validation.

2) How is dendritic localization of mRNAs validated versus axonal localization?

We do not absolutely exclude that axonal localization occurs, however the fact that the reporter RNAs are localized in all visible neuronal processes underlines that dendritic localization is occurring.

3) The authors need to normalize their image analyses, since the lack of dendritic localization could be due to different expression levels and exposure times. Thus, the authors should attempt to normalize to some sort of dendritic immune-staining, for instance via Cy5/Cy7. Moreover, fluorescence intensities in dendrites need to be quantified in Fig.S4, since also g-quartet lacking transcripts are found in dendrites (see magnification).

The exact same settings were used for each image (camera aperture, exposure time, under the control of Metamorph). Expression of reporter mRNA is indeed controlled as explained by RFP protein (red chanel). Furthermore the results obtained with the Lambda-GFP visualization system was confirmed in parallel by the use of a different technique : Fluorescent in situ hybridization of a DNA probe (data presented in the supplementary figure 4). In Fig. S4 there is no signal in dendrites for the Gq lacking transcripts.

4) Data shown inFig.3C need to be quantified.

The quantification of data shown in Fig. 3C (now 3D) was shown in Fig. 2A. The quantifications are now presented directly beside the pictures.

5) The competition studies in Fig.5A need more data points.

Data not presented here

6) How to explain the obvious difference in K+ versus Li+ sensitivity in FMRP binding when comparing PSD-95 to CamkII?

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7) The colocalization in Fig.6A needs to be quantified and validated by immunostaining for endogenous FMRP and other potential trans-acting factors factors.

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8) The authors propose that that removal of the g-quartets in reporter transcripts does not affect translation nor turnover of mRNAs (Fig. S5). These studies need a better explanation of how qRT-PCRs studies were normalized and require decay analyses. Moreover, the authors have to also analyze reporter transcripts with q-quartets in a non-natural 3'-UTR.

Due to lack of space all the details of qRT-PCRs are given in supplementary material.
Reporter transcripts have indeed been tested in a non-natural 3'UTR context, reviewer must have missed it. Decay analyses had been performed to detect possible differential degradation of the reporter mRNAs with or without the G-quadruplex (10µg/ml actinomycin D treatment 2 and 6h prior RNA extraction) as no significant difference was observed between the RNA levels, data are reported as data not shown).

9) Finally the authors should attempt to determine how mutation of q-quartets affects spatially restricted protein synthesis in dendrites in cortical neurons.

Does not apply here anymore

Referee #3 (Remarks to the Author):

The authors extend previous work in this very interesting and timely manuscript identifying G-quartet RNA motifs in a series of dendritically localized mRNAs, and further characterize its role in two prominent mRNAs, e.g. CaMK2a and PSD95. Interestingly, PSD95 mRNA has three G-quartets whereas CaMK2a mRNA has only one. Using a previously uncharacterized GFP-based localization system developed by the Ellenberg lab for cell lines, they provide evidence that both mRNAs indeed localize to dendrites of cortical neurons. Furthermore, they extend previous work by the Bassell lab using DHPG stimulation to show that the G-quartet-driven localization increases upon metabotropic glutamate receptor stimulation. Finally, the authors provide interesting evidence that FMRP participates in this localization process, but it not essential arguing for more than a more than one trans-acting factor model.

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The neuronal type is now well indicated in the legends of figures. Still, all images presented in manuscript are cortical neurons. (mouse hippocampal neurons are more challenging to obtain due to small amount available)

2. The authors make a strong point about the lambda system being better than the MS2 system (data not shown). Many labs have tried the lambda system for localization experiments, but failed. Therefore, it would be important to demonstrate why it is better and support this with data. For sure, the video provided does not (yet) support this statement.

This statement has been removed

3. An essential part of this manuscript is Figure 2 providing evidence that PSD95 and CaMK2a mRNAs indeed localize into dendrites of cortical neurons. I suggest rearranging this essential figure as follows: panel A can stay (alternatively, it would fit better to Figure 1 or go to Supplements); revised panel B could have the cartoon containing the constructs shown (however, significantly more experimental detail is necessary in the figure legend and/or the material and method section for the various mRNAs, e.g. PSD837deltaG, PSDGq, etc.: what are the exact regions deleted/added) followed by examples of neurons as shown in old panel C. It would be essential to provide phase contrast pictures for these neurons to assess cell integrity. Otherwise, the observed localization patterns could results from dying or dead neurons. In addition, DAPI staining could be provided to show nuclear integrity of the cells shown. Most importantly, this revised panel should include high magnification insets of selected dendrites to compare dendritic localization of the three constructs. Revised panel C would then be the

quantification of panel B. For this figure, it would be essential to provide (graphical) evidence on the previously identified dendritic localization elements in CaMK2a mRNA and their relative position to each other as defined by Mori et al., 2000, Blichenberg et al., 2001 and Huang et al, 2003.

To bring clarity the whole secondary structure of the 3' UTR regions bearing the G-quadruplex is now depicted in Fig. 1B, (previously as supplementary data) the "Gq" minimal region is now well shown, together with the previously identified dendritic localization elements. Additional details on constructions are also provided in supplementary materials. High magnifications is now provided for the depicted neurons as requested. Dapi staining is provided for figure 3B and for figure S4 (FISH experiments done with the same constructs and which show the same localization results). Live imaging (fig 3A and sup movie) showing some of the fastest transport speed measured so far (1µm/sec) is obviously attesting that those neurons are not dying. Phase contrast pictures was not made for all constructions but can be provided upon request.

4. I am not sure that I understand the experiment in Fig. 3B. According to the experiment shown in Fig. 2, I guess there is GFP as well as RFP (transfection control) expressed. However, the overview pictures are green only, but the high magnification insets contain red and green. It would be better to separate the two channels for the insets, since I am currently unable to compare localization patterns in green for the two top pictures. More importantly, I am not sure that the data for the various G constructs shown in Fig. 2B and Fig. 3C are consistent. Is the quantification shown in Fig. 2B for the original data in Fig. 3C? It seems to me as if there were significant discrepancies between the data shown and the quantifications. The results (page 9) states: "Thus, a very stable 4-layer G-quartet was a better DTE than a comparatively less stable 3-layer G-quartet,...". However, the graph in Fig. 2B says that G3-A > G4-A. Along those lines, the examples in Fig. 3C are overexposed

and signal intensity should be reduced together with the inclusion of high magnification insets.

Corrections were made in text, pictures were not overexposed. Separation of color channels is now provided as well as high magnifications for Fig. 2C as requested.

5. Fig. 5 is currently the weak spot of this manuscript. In comparison of the neurons used in this study (Figs. 2, 3), both cells are dying or dead indicated by the unusually large overexpression artifacts representing varicosity-like enlargements of the dendrites. It would be very important for this study to demonstrate convincingly that FMRP (labeled by mCherry) indeed colocalizes with the two respective RNAs (labeled by GFP).

Furthermore, experiments shown in panel B are insufficiently explained in the figure legend and the results section preventing the reader to understand the experiment. The results section (page 10) states: "and only the mGluR-triggered transport of the RNA bearing Camk2a was found diminished (Fig. 5B). However, the figure symbols state Camk2a (wt) versus Camk2a (KO). Could it be that both graphs are wrongly labeled and show FMRP (wt) versus FMRP (KO)? But then what about the mentioned stimulation? If it is NOT mislabled, I strongly suggest including the

data from FMRP KO neurons cited as data not shown.

These data have been removed for the present manuscript

Additional points:

- I understand that the authors have previously established G quartets as possible FMRP binding motifs. However, the Darnell lab has recently provided evidence that FMRP might recognize other RNA structures than G quartets. It would be very important for the general audience to refer to this work and also provide a short discussion on alternative FMRP binding motifs.

The part about FMRP has been removed, this does not apply anymore

- The mentioned bio-informatic GRSDB tool (Kostadinov et al, 2006) has been instrumental for this manuscript. It would be very helpful to provide the direct link in the Material and methods section. I googled the side, found the link but it did not work for me. Is it not yet activated? This might be very useful for the community!

The link to the program QGRS-mapper (on the new GRSDB-2 site) has been added in supplementary legend of table 1, a more recent reference Kikin et al. 2008, replaces Kostadinov et al, 2006

- Generally, the field relates to Camk2a mRNA as CaMIIa or CaMIIalpha. I suggest sticking to this terminology.

This was corrected

- Results, page 7: "Using this system we could visualize very efficiently the localization of a reporter mRNA bearing the well established 54-nt dendritic targeting element (DTE) "zipcode" of the ß-actin mRNA (Kislauskis et al, 1994) in the dendrites of living (Supplementary Fig. 3) or fixed neurons (data not shown)." I think that the two references got mixed up? The fixed cells are shown in Suppl. Fig. 3 and the videos are not shown?

This is in fact correct, the picture depicted in Fig. 3 was taken on living cells

- Figure 2 and figure legend: Panels B and C have been inverted compared to the corresponding figure legend (contains two panels B, but no panel C).

This was corrected

- Page 19: thice G-guartet sequence instead of G-quartet.

This was corrected

1st Editorial Decision

Thank you for the submission of your manuscript to EMBO reports. We have now received the enclosed reports from referees 1 and 2.

As you will see, the referees agree that the study is potentially suitable for publication in EMBO reports. They do raise a few points though, which need to be addressed before the manuscript can be considered for publication in our journal.

Referee 1 indicates that it needs to be explained more clearly which experiments have been performed in hippocampal versus cortical neurons. The referee also suggests that the data on the role of FMRP in Gq-dependent mRNA localization should be included in the supplementary information and that these results should be discussed in more detail in the main manuscript file. Referee 2 points out a missing negative control and suggests to replace the lambda-studies in the main manuscript file with the FISH studies currently presented in supplementary figure S4.

Given these evaluations and the constructive referee comments, I would like to give you the opportunity to revise your manuscript, with the understanding that the referee concerns must be fully addressed and their suggestions (as detailed above and in their reports) taken on board. Acceptance of the manuscript will depend on a positive outcome of a second round of review and I should also remind you that it is EMBO reports policy to allow a single round of revision only and that, therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions. Also, the length of the revised manuscript may not exceed 27,500 characters (including spaces) and, including the maximum of 5 figures, the paper must ultimately fit onto optimally six, and maximally seven, pages of the journal. I also would like to add that the supplementary figures should ideally directly relate to one of the main figures in the manuscript and that therefore not more than 5 supplementary figures should be included. Given that the current manuscript has only three figures, up to two figures could be moved from the supplementary information to the main manuscript file. EMBO reports also recently decided that the results and discussion section should always be combined. I would therefore like to ask you to combine both sections. This may help in reducing the overall length of the article as it may eliminate some redundancy that is inevitable when discussing the same experiments twice.

When submitting your revised manuscript, please include:

A Microsoft Word file of the manuscript text, editable high resolution TIFF or EPS-formatted figure files, a separate PDF file of any Supplementary information (in its final format) and a letter detailing your responses to the referee comments. Please also include a two sentence-summary of the manuscript that will appear online on our webpage in case of acceptance of the study for publication.

We would also welcome the submission of cover suggestions, or motifs to be used by our Graphics Illustrator in designing a cover.

As part of the EMBO publication's Transparent Editorial Process, EMBO reports publishes online a Review Process File to accompany accepted manuscripts. This File will be published in conjunction with your paper and will include the referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript.

You are able to opt out of this by letting the editorial office know (emboreports@embo.org). If you do opt out, the Review Process File link will point to the following statement: "No Review Process File is available with this article, as the authors have chosen not to make the review process public in this case."

I look forward to seeing a revised version of your manuscript when it is ready.

Yours sincerely

Editor
EMBO Reports

REFEREE REPORTS

Referee #1 (Remarks to the Author):

In this work Subramanian et al. report the discovery of G-quadruplex (Gq) structures as dendritic targeting elements (DTEs). Previous work by the same lab and others has identified G-quadruplex structures as binding sites for Fragile X Mental Retardation protein (FMRP), a factor regulating neuronal RNAs at various levels. Based on this observation the authors hypothesized that Gq structures could act to regulate mRNA localization in neurons. Indeed, they found that Gq structures are overrepresented in the 3'UTRs of 33 mRNAs known to localize to dendrites compared to a random mRNA pool. Two well-known examples of localizing mRNAs, PSD-95 and CamKIIa, were retained for further analysis. Using various biochemical assays the authors convincingly demonstrate that Gq structures indeed form in the PSD-95 and CamKIIa 3'UTRs. They went on to test the role of Gq forming sequences for mRNA localization by using a previously described GFP-based RNA tracking assay. Whereas deletion of Gq forming sequences abolishes the ability of PSD-95 and CamKIIa 3'UTR to confer dendritic transport to reporter RNAs, heterologous sequences can be targeted to dendrites by adding a Gq forming sequence. These results are confirmed by independent analysis of mRNA localization by fluorescent in situ hybridization.
Overall, this manuscript presents convincing evidence that Gq structures can act as DTEs in neuronal mRNAs. The identification of novel cis-acting elements that contribute to mRNA localization is an important contribution towards a better understanding of the mechanisms that drive mRNA localization and of interest to a broader audience. However, I feel that there are a few issues with the current version of the manuscript that have to be resolved before publication.

Major points:
1. Whereas all data presented in the manuscript were obtained using cultured cortical neurons, some results have been confirmed in hippocampal neurons (mentioned on page 6). It should be made clear which experiments have also been performed in hippocampal neurons. This is very important as the authors speculate more than once that mRNA transport could be differentially regulated in cortex and hippocampus. For example, they propose that the CPE present in CamKIIa 3'UTR could be of greater importance in hippocampal neurons compared to cortical neurons. Given that they have tested their full-length 3'UTR plasmids also in hippocampal neurons, I wonder whether they also tested their deletion mutants?

2. A previous version of this manuscript contained data from experiments investigating the role of FMRP in Gq dependent mRNA localization. These experiments have been removed from the current manuscript, since they failed to provide evidence for an important role of FMRP and overlapped with already published results. However, I think they should be shown in the supplemental material as they present welcome validation of published results that are of interest to the field. Should the authors have a strong reason not to include these data, they should better indicate which specific experiments/observations of Dictenberg et al. they are referring to as showing that FMRP does not mediate Gq-dependent RNA localization (e.g. on page 10). At the moment the statement is not explicit enough, and one wonders again if the results may be specific to different types of neurons, experimental differences between the two labs, and whether constitutive or activity-dependent localization is being referred to. In addition, the absence of a strong effect of FMRP, compared to the loss of a Gq, could be discussed in some more detail. In order to comply with space restrictions, these thoughts on FMRP could substitute the speculation on the mechanism that brings about mRNA localization, as this is not the prime focus of this work.

Minor points:
3. The manuscript would benefit greatly from proofreading by a native English speaker. I do not believe that perfect English is a requirement for publication, but removing the mistakes in spelling and grammar would help the reader follow the manuscript.

4. page 7: "....dendritic targeting was found comparable to the one of the full-length UTRs (Fig2,

Sup Fig S4) indicating an essential role of the Gq in localization." I believe these experiments show that Gq structures are sufficient, rather that essential, for dendritic localization. (That they are indeed also essential was shown by the deletion mutants.) Also it would be helpful to refer in the text to which heterologous RNA was used for the sufficiency test.

5. page 9: "The first mechanism is a priori excluded for neurons due to their size." However, the dsRed protein can clearly diffuse throughout the neuron, even when presumably synthesized exclusively in the cell body. Diffusive properties for protein and RNA will likely be very different, yet I feel that the above statement is too strong.

6. p. 3.: Do the authors mean that very few RNAs contain a UUUUAU sequence or similar (which would seem surprising given the UA rich nature of 3'UTRs), or that in few cases has such an element been shown to be important for localization?

Referee #2 (Remarks to the Author):

In their study Subramanian et al. identify g-quartets in the 3'-UTRs of dendritically localized mRNAs and validate their role as dendritic targeting elements (DTEs) promoting the transport of reporter transcripts. The authors identify g-quartets in CamkII and PSD-95 3'-UTRs and demonstrate potassium sensitivity. Using a lambda-tagging approach as well as FISH, the authors demonstrate that reporter transcripts comprising the g-quartet are localized to dendrites whereas they observe a striking restriction to the cell body upon q-quartet deletion. Moreover, these findings are supported by the observation that g-quartet dependent localization of transcripts is DHPG-responsive.
Taken together, the presented study convincingly demonstrates g-quartet-dependent dendritic localization of mRNAs and comes up with the intriguing hypothesis that these DTEs could have a potential spatial restriction in 3'-UTRs and direct ion-sensitivity of complex formation and thus mRNA localization. These findings provide novel insides important for the field. In contrast to the previously submitted manuscript now focus on mRNA localization and have addressed most of my previous comments.

Minor comments:

1) Fig.S3 Validation of the lamda-GFP/B-box assay actually requires a negative control lacking a DTS but including B-boxes. This transcript should result in restriction of the transcript in the cell-body, but should lead to a cytoplasmic accumulation of the GFP-lambda. Along these lines I feel that the authors should include the FISH studies presented in S4 instead of the lambda-studies in the main manuscript.
2) As previously explained the authors should not exclude the potential role of g-quartets in axonal mRNA localization by claiming to have identified DTEs. I feel the term NTE (neurite targeting element) is more appropriate.
3) Is there a mis-labeling of supplemental figures? Fig.S2 on p6 should be Fig.S1 whereas S1A/B on p5 should be S2A/B to my understanding.

2nd Revision - authors' response

Please find hereafter the revised version of our manuscript entitled "Gquadruplex RNA structure as a signal for mRNA targeting in neurites" by M. Subramanian, F. Rage, R. Tabet, E. Flatter, J.L. Mandel, and H. Moine.

Here follows the point by point response to the referee comments:
Referee 1

1. Whereas all data presented in the manuscript were obtained using cultured cortical neurons, some results have been confirmed in hippocampal neurons (mentioned on page 6). It should be made clear which experiments have also been performed in hippocampal neurons. This is very important as the authors speculate more than once that mRNA transport could be differentially regulated in cortex and hippocampus. For example, they propose that the CPE present in CamKIIa 3'UTR could be of greater importance in hippocampal neurons compared to cortical neurons. Given that they have tested their full-length 3'UTR plasmids also in hippocampal neurons, I wonder whether they also tested their deletion mutants?

As written in first version of manuscript the full-length 3'UTR (PSD-95 and CaMKIIa) plasmids have been tested in both cortical and hippocampal neurons. As there was no apparent difference in the localization of these RNAs between cortical and hippocampal neurons the study was continued only with cortical neurons (working with mouse hippocampal neurons being more challenging than with cortical neurons as there is much less material available). The deletion mutants and other constructs were not tested in hippocampal neurons. We cannot therefore extrapolate our conclusions to all types of neurons. It is clearly stated throughout the text that the study was performed in cortical neurons (including in abstract). The mention that some experiments had been done in hippocampal neurons has now been removed to avoid confusion.

2. A previous version of this manuscript contained data from experiments investigating the role of FMRP in Gq dependent mRNA localization. These experiments have been removed from the current manuscript, since they failed to provide evidence for an important role of FMRP and overlapped with already published results. However, I think they should be shown in the supplemental material as they present welcome validation of published results that are of interest to the field. Should the authors have a strong reason not to include these data, they should better indicate which specific experiments/observations of Dictenberg et al. they are referring to as showing that FMRP does not mediate Gq-dependent RNA localization (e.g. on page 10). At the moment the statement is not explicit enough, and one wonders again if the results may be specific to different types of neurons, experimental differences between the two labs, and whether constitutive or activitydependent localization is being referred to. In addition, the absence of a strong effect of FMRP, compared to the loss of a Gq, could be discussed in some more detail. In order to comply with space restrictions, these thoughts on FMRP could substitute the speculation on the mechanism that brings about mRNA localization, as this is not the prime focus of this work.

The data concerning the role of FMRP in Gq dependent mRNA localization have been placed in the supplemental material as requested. The FMRP binding data have also been added as they complement these data. The absence of strong effect of FMRP on transport compared to the loss of the Gq is now discussed in more details (see p. 9).

3. The manuscript would benefit greatly from proofreading by a native English speaker. I do not believe that perfect English is a requirement for publication, but removing the mistakes in spelling and grammar would help the reader follow the manuscript.

Proofreading has been performed by native english speaker as requested

4. page 7: "....dendritic targeting was found comparable to the one of the fulllength UTRs (Fig2, Sup Fig S4) indicating an essential role of the Gq in localization." I believe these experiments show that Gq structures are sufficient, rather that essential, for dendritic localization. (That they are indeed also essential was shown by the deletion mutants.) Also it would be helpful to refer in the text to which heterologous RNA was used for the sufficiency test.

Page 7: the term "essential" was replaced by "sufficient"

The nature of the heterologous RNA used for the sufficiency test is now described in text.
"The Gq forming sequences only (Fig 1B and C) were tested out of their natural 3'-UTR context in the heterologous 3'UTR sequence of pDsRed-Mono-4BB in front of the SV40 polyadenylation signal."

5. page 9: "The first mechanism is a priori excluded for neurons due to their size." However, the dsRed protein can clearly diffuse throughout the neuron, even when presumably synthesized exclusively in the cell body. Diffusive properties for protein and RNA will likely be very different, yet I feel that the above statement is too strong.

This statement was removed, so was the other speculations on the possible mechanisms involved in the localization as suggested in point 2 to make room for discussion of the FMRP effect.

6. p. 3.: Do the authors mean that very few RNAs contain a UUUUAU sequence or similar (which would seem surprising given the UA rich nature of 3'UTRs), or that in few cases has such an element been shown to be important for localization?

Indeed in few cases has UUUUAU sequence been shown to be important for localization.
Correction was done.
"However, these elements have been shown to be important for localization in very few mRNAs, thus, for a majority of mRNAs localized in dendrites, the cis-acting signal(s) involved remains unknown."

Referee 2

1. Fig.S3 Validation of the lamda-GFP/B-box assay actually requires a negative control lacking a DTS but including B-boxes. This transcript should result in restriction of the transcript in the cell-body, but should lead to a cytoplasmic accumulation of the GFP-lambda. Along these lines I feel that the authors should include the FISH studies presented in S4 instead of the lambda-studies in the main manuscript.

The control "B-boxes" lacking a dendritic targeting signal was included in Fig.S3 as requested. Fish pictures from S4 were also included in figure 2. However, the GFPLambda data were also kept in fig2 because they are important to maintain a cohesion of the data throughout the paper. Indeed the FISH data intended to validate the GFP-Lambda approach. The GFP-Lambda data were then used for the quantifications of all constructions analyzed in the paper. For the Gquadruplex variants (Fig 3) the FISH was not performed and only GFPLambda data are available. It would seem odd to present FISH data for one part of the constructs and GFP-Lambda for the rest.

Unless you recommend otherwise, we feel there is space to accommodate both FISH and GFP-Lambda data in fig 2. (Fish data were maintained also in Sup FigS4 where DAPI and merges are presented).

2. As previously explained the authors should not exclude the potential role of g-quartets in axonal mRNA localization by claiming to have identified DTEs. I feel the term NTE (neurite targeting element) is more appropriate.

The terms "dendrite" and "dendritic" were replaced throughout the paper by "neurite" and "neuritic", including in title.

3. Is there a mis-labeling of supplemental figures? Fig.S2 on p6 should be Fig.S1 whereas S1A/B on p5 should be S2A/B to my understanding.

Yes indeed. Figure number was corrected.

We hope that these revisions will fully address all the requested points.

2nd Editorial Decision

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

At the end of this email I include important information about how to proceed. Please ensure that you take the time to read the information and complete and return the necessary forms to allow us to publish your manuscript as quickly as possible.

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If you do NOT want this File to be published, please inform the editorial office within 2 days, if you have not done so already, otherwise the File will be published by default [contact: emboreports@embo.org]. If you do opt out, the Review Process File link will point to the following statement: "No Review Process File is available with this article, as the authors have chosen not to make the review process public in this case."

Thank you again for your contribution to EMBO reports and congratulations on a successful publication. Please consider us again in the future for your most exciting work.

Yours sincerely,

Editor
EMBO Reports

