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DSCR1 interacts with FMRP and is required for spine morphogenesis and local protein synthesis

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 17 May 2012

Thank you for submitting your manuscript to the EMBO Journal. I am sorry for the delay in getting back to you with a decision, but I have now received the comments back from the two referees that I contacted to review your paper.

As you can see below, both referees find that the paper very interesting and suitable for publication in the EMBO Journal. However both of the referees also find that further revisions are needed for publication here to strengthen the key findings of the paper. In some ways the paper almost contain too much data and too many separable stories. I recognize that a lot of work has gone into this study already and I would like to work with you to streamline what experiments are needed for publication here. It would be good if you could send me a detailed point-by-point response and let me know what you can easily address. We can then discuss further what is needed.

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

Thank you for submitting your interesting study to the EMBO Journal.

Yours sincerely,

Editor
The EMBO Journal

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REFEREE REPORTS:

Referee #1 (Remarks to the Author):

This study makes an important contribution to research on mental retardation, and the mechanisms that relate Down Syndrome and Fragile X to deficits in spines, in translation, and in learning & memory. The main advance is the evdence for intersecting pathways involving FMRP (Fragile X) and DSCR1 (Down Syndrome), in the regulation of spine morphology and local protein synthesis in dendrites.

However, the paper is too ambitious, and the last sections regarding the mechanisms relating BDNF to translation and spine growth (Fig. 6 and 7) are not compelling. These parts should either be strengthened with new and more conclusive data, or substantially toned down in order for the paper to provide a qualitatively more homogeneous set of conclusive findings.

1) A main weakness concerns the claim that phosphorylated DSCR1 would activate calcineurin, in contrast to non-Pi-DSCR1, which inhibits CaN. The second part is supported by clear data, but the first part is not. The data (Fig. 7) seem to show that when it is phosphorylated, DSCR1 does not inhibit CaN anymore. But they do not show, as claimed repeatedly in the text, that Pi-DSCR1 activates CaN.

2) Generally, the evidence relating DSCR1 to CaN and then Pi-cofilin and spine function (respectively local translation) is plausible, but not completely compelling. Most of the assays are rather complex, and parallel pathways plus additional pathway components should not be considered as ruled out. In the opinion of this reviewer the paper makes a strong case up to the data shown in Fig. 5. These results are sufficiently important to justify publication, and the additional evidence, although intriguing, is not of the same caliber.

3) The relative roles of translation and actin regulation in the control of spine morphology and function are difficult to tease apart. The authors should discuss critically the extent to which, based on their findings, both phenomena may be regulated by a shared FMRP and DSCR1 pathway. This more critical treatment of the data should also include reference to recent studies that have exposed possible intersections and trade-offs between local F-actin regulation, and local regulation of translation at spines.

Referee #2 (Remarks to the Author):

The manuscript by Wang et al entitled 'DSCR1 interacts with FMRP and is required for spine morphogenesis and local protein synthesis' reports a novel signaling mechanism that links, DSCR1, implicated in Down's Syndrome, and FMRP, whose deficiency is the cause of fragile x syndrome. Such a link between the two major causes of intellectual disability represents a potentially very exciting finding. The data suggest an interesting and provocative model whereby, DSCR1, a calcineurin inhibitor, known to regulate actin dynamics in spines, can also regulate FMRP, a translational repressor involved in local protein synthesis necessary for dendritic spine development and plasticity. Using a comprehensive battery of in vitro and in vivo approaches and different mouse models, the authors find that these two proteins genetically and physically interact in a common signaling pathway and that DSCR1 regulates local protein synthesis. The mechanism for the link between pathways is suggested by the finding that DSCR1 interacts preferentially with phosphorylated FMRP, which is well known know to inhibit translation. Since DSCR1 is a calcineurin inhibitor, and the authors show here that calcineurin activity is needed to dephosphorylate FMRP, the implication is that DSCR1 helps maintain FMRP and bound mRNAs in an inhibitory complex. Despite the enthusiasm for many of the data presented, there are a number of technical and interpretive issues that are concerning, and so some of the key conclusions are not substantiated or solidified. Another issue is that there seem to be separate stories that don't weave together into a cohesive model, but these issues are addressable. The major issues associated with

each figure, and general comments are discussed below.

Figure 1. In vitro data using DSCR1 shRNA, Fmr1 siRNA or overexpression of DSCR1, and double manipulations on cultured neurons is shown that demonstrates an interaction between DSCR and FMRP to regulate spine morphology. An interesting finding is that increased spine number in Fmr1 silenced neurons is rescued by DSCR overexpression, but worsened by DSCR1 knockdown. The immature spine morphology in Fmr1 silenced neurons is rescued by DSCR1 overexpression. Where are the appropriate negative controls for these experiments? Typically, a scrambled sequence is used, and there should be a separate negative control for each of the experimental paradigms e.g. siRNA, shRNA, overexpression. Can some of this control data be added to the supplement? Presumably, the "control" shown refers to untreated neurons, but this is not acceptable, because there will always be off-target effects. It is often customary to rescue at least a couple of the knockdown phenotypes by expression of construct that is insensitive to siRNA or shRNA. I recognize that rescue experiments can themselves be problematic due to overexpression effects. Yet, the conclusions are solidified by evidence of rescue.
Minor comment: the fluorophore/label used to identify the transfected neuron, the spine morphology in each case needs to be labeled and described in the figure, methods and legends.
Figure 1B,C requires a one-way ANOVA, not a two-way.

Figure 2 describes the spine analyses in vivo. Of interest, the data show that the DSCR1 transgenic mice have increased spine head size which can be rescued in compound mice that are Fmr1 heterozygous. The assumption is that removing one copy of Fmr1 was sufficient to rescue the increased spine size observed in the DSCR1 transgenic mice. However, the Fmr1 gene is on the X chromosome and only one allele is expressed in each cell. In the female heterozygous Fmr1 mice examined, each neuron will either have the normal amount of FMRP or no FMRP, rather than the 50% suggested by the authors. Therefore, many of the neurons examined likely had full FMRP. Did the authors perform immunostaining for FMRP, to select the neurons for spine analysis that lacked FMRP? If not, there should be a bimodal distribution, where some neurons have no rescue and others have full rescue. The authors need to discuss this issue.

Figure 4A-C. A major conclusion is that DSCR1 interacts with phosphorylated FMRP, however, there are some technical issues and questions about the experiments and data. The authors claim that DSCR1 does not interact with total FMRP in IPs with FMRP antibody and that this antibody is biased to unphosphorylated form (Figure S8). The FMRP antibody used for IP needs to be stated. One commonly used anti-FMRP monoclonal antibody (IC3) recognizes two epitopes on FMRP distinct from phosphorylation sites and are known in the field to be unbiased to phosphorylation of FMRP. Another FMRP monoclonal that is commonly used for IPs is 7G1. It is surprising that IP of endogenous FMRP doesn't co-IP DSCR1. The authors need to test a couple of FMRP specific antibodies that are commercially available, and examine for an endogenous FMRP-DSCR1 interaction. Does an IP with S499D, the FMRP phospho-mimic, show increased levels of DSCR1? This would strengthen the authors' claim.
Minor comment: In panel B, the efficiency of FLAG-IP with WT and S499A should be demonstrated and should be uniform for both constructs.

Fig. 4C shows FRET interaction between phosphorylated FMRP and DSCR1 using double label IF in fixed neurons. I find this data not very convincing, since FRET is not practical with antibodies, because the distance between the fluorochrome and the antigen is spatially beyond the FRET range of less than 10nm. Can the authors cite some uses of this method? The authors should support this data with standard quantitative colocalization analysis. In addition to showing single spines, where it is hard to interpret colocalization, the authors should show larger dendritic regions to quantitatively assess colocalization.

Figure 5 and S9 show imaging experiments with a Dendra CaMKIIa 3'UTR reporter in cultured neurons to demonstrate the requirement of DSCR and FMRP for BDNF induced local protein synthesis. It is not mentioned if the construct used would be membrane bound so it is surprising that: A) Dendra2 accumulates and remains in spines (as opposed to equilibrating with dendrites). B) Dendra2 from surrounding regions does not diffuse in the field that was photo-converted. In 30 minutes it would be a lot of time for equilibration of a freely diffusing protein within the cell. The authors should discuss this issue and provide their interpretation. Most studies using fluorescent reporters for local translation employ a diffusion-restricted reporter, as done initially by the

Schuman lab, also with the 3'UTR of CaMKIIa mRNA (Aakalu et al, Neuron 2003).

Also in Fig. 5 and S9, for almost all conditions analyzed, there is no change in the intensity in the dendrites, which implies that there is no basal translation in dendrites. This is surprising owing to the high neuronal activity of the culture. The authors should comment. Related to this point, the flat line observed without BDNF implies that there is no basal translation occurring in dendrites or spines. Also related to this issue, DSCR1 overexpression is reported to mimic the effect of BDNF, but since DSCR1 is also present in dendrites and spine, it would be expected that there is some basal translation in dendrites as well.
Minor: the diagram explaining what in out and spine mean should be in Fig 5 as well.

Fig. 5 needs to show the Dendra green fluorescence signal (in heat map) before photoconversion for each row (experimental condition). This will allow the reader to assess the recovery.

For Fig. 5, it is unclear why the green fluorescence signal seems so high at time zero, immediately after photoconversion. This implies inefficiency photoconversion. This is another reason to show the green signal before and after photoconversion, and then assess the recovery due to new protein synthesis.

Figure 6 shows that BDNF decreases FMRP-phosph through Calcineurin. It is essential to normalize the levels of phospho-FMRP in a dephosphorylation event to the total FMRP levels. Total FMRP levels should be measured in immunofluoresence and IP to ensure there is no FMRP degradation involved and should be used in the figure instead of total IgG heavy chain and in addition to tubulin (Fig 6 b,d,f). Have the authors used a PP2A inhibitor, okadiac acid (5 nM) in addition to CsA? The data do not show that Calcineurin directly dephosphosphorylates FMRP, in contrast to a previous study which showed that PP2A directly dephosporylates FMRP (Narayanan et al 2007). The authors should indicate the possibility that CanN may affect FMRP indirectly, perhaps through PP2A.

Other comments:

The authors show poor scholarship in their discussion of previous work on FMRP phosphorylation. The first paper to described the phosphorylation of serine 499 and its role in translation regulation was not cited (Ceman et al, HMG 2003). The authors do cite the second paper by Narayanan et al (J. Neuroscience 2007), however, they do not discuss that this paper was the first report to characterize a phospho-specific FMRP antibody and demonstrate mGluR mediated and PP2A dependent phosphorylation as a switch to remove translational inhibition and activate translation. There has since been a series of papers from several labs that characterize this molecular mechanism and genetic models, none of which were cited, and need to be cited and discussed (Muddashetty et al, Molecular Cell 2011; Nalavadi et al J. Neuroscience 2012; Niere et al J. Neuroscience 2012; Coffee et al, HMG 2012).

The authors show that BDNF stimulation of local protein synthesis (using a CaMKIIa 3'UTR reporter) is absent or worsened in FMRP deficient neurons. The authors should note that a similar result was observed by Muddashetty et al (J. Neurosci 2007) for endogenous CaMKII protein in response to mGluR stimulation of synapses from Fmr1 KO mice.

In describing the spine phenotypes in Fmr1 KO mice, the authors describe in vivo studies, however, there have been prior in vitro studies to show excess spines having an immature phenotype (Swanger et al Mol Brain 2011; Antar et al Mol Cell Neurosci 2006).

The authors should soften their claims that local translation was directly visualized in dendritic spines, because they are not using a diffusion restricted reporter, the maturation time is many minutes, and the protein could move from the dendrite to the spine. They could state that the data are consistent with local translation in the spine, but acknowledge the caveats.

In general, the authors need to clarify the full extent and rigor of morphologic analysis, by specifying how many neurons were analyzed, how many dendrites, the size of RO1, the number of

experiments.

Fig. S9: It is interesting that DSCR transgene appears to partially rescue the Fmr1 phenotype by restoring stimulus induced translation, even if only transient. This could be commented on. In general, the authors talk more in Discussion about rescuing DSCR phenotypes by modulating Fmr1, but they should also discuss how Fmr1 phenotypes are modulated by DSCR1.

Fig. S8A,B: phospho-FMRP and total FMRP are mixed up in legend

Fig. S9: BNDF should be BDNF

Fig. S10: DSCT should be DSCR1

Does DSCR associate with FMRP target mRNAs e.g. CaMKIIa?

1st Revision - authors' response 01 June 2012

2nd Editorial Decision 15 June 2012

Thank you for submitting your revised manuscript to the EMBO Journal. I asked the original referee #1 to take a look at the revised version and I have now received the report.

As you can see referee #1 appreciates that the manuscript has improved and supports publication in the EMBO Journal. There are however a few issues that have to be addressed before acceptance here. 1) The discussion concerning the role of actin regulation and local translation needs to be improved. 2) A more specific point-by-point response needs to be provided. Please indicate clearly in the rebuttal letter what has been addressed and how. In addition to points raised by referee #1 below please also including what the negative controls were for the knock down experiments (ref 2 point #1). Once we get these last issues resolved then we will proceed with the acceptance of the paper for publication here.

Yours sincerely

Editor
The EMBO Journal

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REFEREE REPORTS:

Referee #1 (Remarks to the Author):

The manuscript has been improved by toning down some of the claims and by providing a more critical discussion of the possible limitations involved in some of the assays.

However, the authors addressed many of the concerns in a very cursory manner, and it is generally difficult to ascertain which parts of the manuscript have been modified and how. This should be specified, at least to some extent in a modified rebuttal letter. Furthermore, the discussion of the relative roles of actin regulation and local translation in the results amounts to one generic sentence in the discussion - that should be improved.
Finally, some important concerns raised by Reviewer 2 were not addressed properly - the authors should address in the manuscript the concerns expressed in points 2) (bi-modal distribution?) and 6) of that Reviewer. They should also specificy in their rebuttal which of the references requested by Rev2 (points 9)- 11)) they included ("most relevant papers") and which ones they did not.

2nd Revision - authors' response 18 June 2012

