EMBO reports

FMRP recruitment of β-catenin to the translation pre-initiation complex represses translation

Saviz Ehyai, Tetsuaki Miyake, Declan Williams, Jyotsna Vinayak, Mark A Bayfield, John C McDermott

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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.)

1st Editorial Decision 29 January 2018

Thank you for your patience while your manuscript was peer-reviewed at EMBO reports. I am very sorry for the unusual delay in getting back to you; we have only received the third referee report today. The full set of reports is pasted below.

As you will see, all referees acknowledge that the findings are potentially interesting and novel. However, they also raise important concerns that will need to be addressed. Reading through the comments I think that all of them make sense and should therefore be addressed.

I would thus like to invite you to revise your manuscript with the understanding that the referee concerns must be fully addressed and their suggestions taken on board. Please address all referee concerns in a complete point-by-point response. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore 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. Please contact us if a 3-months time frame is not sufficient for the revisions so that we can discuss this further. You can either publish the study as a short report or as a full article. For short reports, the revised manuscript should not exceed 27,000 characters (including spaces but excluding materials & methods and references) and 5 main plus 5 expanded view figures. The results and discussion sections must further be combined, which will help to shorten the manuscript text by eliminating some redundancy that is inevitable when discussing the same experiments twice. For a normal article there are no length limitations, but it should have more than 5 main figures and the results and discussion sections must be separate. In both cases, the entire materials and methods must be included in the main manuscript file. Supplementary figures, tables and movies can be provided as Expanded View (EV) files, and we can offer a maximum of 5 EV figures per manuscript. EV figures are embedded in the main manuscript text and expand when clicked in the html version. Additional supplementary figures will need to be included in an Appendix file. Tables can either be provided as regular tables, as EV tables or as

Datasets. Please see our guide to authors for more information.

Regarding data quantification, please specify the number "n" for how many independent experiments were performed, the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values in the respective figure legends. This information must be provided in the figure legends. Please also include scale bars in all microscopy images.

We now strongly encourage the publication of original source data with the aim of making primary data more accessible and transparent to the reader. The source data will be published in a separate source data file online along with the accepted manuscript and will be linked to the relevant figure. If you would like to use this opportunity, please submit the source data (for example scans of entire gels or blots, data points of graphs in an excel sheet, additional images, etc.) of your key experiments together with the revised manuscript. Please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure or per figure panel.

When submitting your revised manuscript, we will require:

- a complete author checklist, which you can download from our author guidelines (http://embor.embopress.org/authorguide#revision). Please insert page numbers in the checklist to indicate where in the manuscript the requested information can be found. The completed author checklist will also be part of the RPF (see below).
- a letter detailing your responses to the referee comments in Word format (.doc)
- a Microsoft Word file (.doc) of the revised manuscript text
- editable TIFF or EPS-formatted figure files in high resolution. In order to avoid delays later in the process, please read our figure guidelines before preparing your manuscript figures at: http://www.embopress.org/sites/default/files/EMBOPress_Figure_Guidelines_061115.pdf

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

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I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

REFEREE REPORTS

Referee #1:

This manuscript reports an interaction between b-catenin and FMRP, and a surprising role of b-catenin in repression of translation initiation. While I am always quite sceptical about the real significance of proteomic data (see comment below), the data presented here demonstrate quite convincingly the specificity and robustness of the b-cat-FMRP interaction. The GFP-GBP nanotrap assay gives particularly impressive results, which support a strong b-cat-FMRP interaction.
The fact that FMRP is implicated in regulation of translation, and the identification of another component of the initiation complex in the proteomic experiment lead to the logical exploration of a role of b-cat in this process.

The effects of b-catenin depletion and Wnt stimulation are striking, showing huge effects on puromycin incorporation. From these results, I find it convincing that b-catenin has an important

repressive role on translation initiation. These findings are new and of general interest.

However, what I find less convincing is the proposed mechanism, which would implicate the b-cat-FMRP interaction with the initiation complex as sole cause for this large effect on translation. Conceptually, it is hard to imagine how FMRP could be responsible/co-responsible for a two-fold change in translation. It is known to bind 4% of mRNAs in the brain, and since this is the organ affected by its mutation, which lead to a mental syndrome, it would be surprising that it would massively regulate translation in other cell types. I can see however two simple alternative explanations: a) b-cat is indeed directly responsible for massive repression of translation, but without the absolute need for FMRP. In fact, the manuscript does not present any evidence for the involvement of FMRP, thus this protein may well play little to no role in this repression. b) b-cat, with/without FMRP, is directly responsible only for PART of the repression, the rest of the effect could result from transcriptional events. In this interesting case, b-cat could have two antagonistic roles, directly repressing translation in the absence of Wnt signalling, but participating to stimulation of translation via regulation of transcription upon Wnt stimulation.

Importantly, the si experiments last 24-48 hours, which give plenty of room for complex changes in genetic program. Note that most cells do have a baseline of active Wnt signalling, which will be blocked by b-catenin deletion. Other indirect effects could be generated by b-catenin depletion, for instance by affecting cadherin cell contacts, which in turn could trigger other signals impinging on transcription and translation. As for Wnt treatments, 4 hrs give plenty of time for changes in gene activity. I agree that one would expect at least in the simplest model that Wnt activation would have the opposite effect to b-cat depletion, but as just mentioned, long term effect may be quite complex.

In any case, these are core issues, which must be absolutely solved in order to give coherence to this potentially very interesting story. One would expect to see two major additional sets of data: 1) an estimate of the extent of direct repression by b-catenin (e.g. in the presence of transcriptional inhibitor); 2) evidence that FMRP is actually involved in this b-cat-dependent repression.

I have also issues with another related statement of the proposed model, i.e. the nuclear 'sequestration' of b-cat upon Wnt stimulation. I don't find fully convincing evidence that the effect of Wnt is a simple shift of b-cat from the cytoplasm to the nucleus. Fig5: In VSMC cells, one does see a decreased in cytoplasmic b-cat, and a strong increase in nuclear b-catenin, but also an increase at the plasma membrane. One actually may wonder whether the 'cytoplasmic' signal is really cytoplasmic, or rather corresponds to the plasma membrane of flat cells. Wnt stimulation does tend to induce changes in cell shape, and the apparent cytoplasmic 'depletion' may be partly an optical artefact. In A10 cells, one only sees increased nuclear b-cat, as expected by the classical model of b-cat stabilization, without any obvious change in cytoplasmic signal. This is clearly a weak aspect of this study, which needs to be strengthened by more accurate analysis of b-cat levels in different subcellular locations.

OTHER COMMENTS:

Interaction specificity: Specificity is a recurrent issue with proteomic analysis. In the present case, I am rather puzzled to see relatively highly ranked interactions that seem obviously non physiological. In particular, one mitochondrial protein is ranks similar to FMRP, and a secreted protein, procollagen, is not far behind.

I would also feel more comfortable to see unarguable negative controls demonstrating that the plectrin (here used as a positive control) and FMRT interactions are 'uniquely' specific. For instance, it would be nice to show that b-cat does NOT co-IP/colocalizes with procollagen nor mitochondrial Mthfd1.

FigS3+5, Control b-cat/plectrin colocalization: I find worrying the fact that all bright structures in the red channel are also observed in the green channel. This is very striking is fig S5. Of course this could mean that all plectrin structures also accumulate b-cat. However, I would feel reassured to see controls for the absence of bleed-through, which may occur if the plectrin staining is particularly strong.

Page 8: "FMRP was present in five out of six biological replicates"?? I see three out of five!!

Fig4a: Cell fractionation is quite a crude method to analyse distribution of a protein such as b-cat, which may be potentially found in multiple unrelated complexes in addition to ribosomes/polysomes. However, the strong decrease of signal in fraction 2 upon FMRP depletion is another confirmation of their interaction. And their presence in the initiation complex is supported by m7GTP pulldown (panels C,D).

Referee #2:

In this report, Ehyai et al. aim to identify additional roles for beta-catenin by investigating its potential binding partners. They find that beta-catenin interacts with a number of mRNA translation factors including FMRP, and show that this interaction occurs within the context of pre-initiation complexes. Activation of the Wnt pathway results in re-localization of beta-catenin to the nucleus, and a correlative increase in global translation. The authors conclude that beta-catenin acts with FMRP as a translational repressing complex, and that Wnt activation dissociates beta-catenin from FMRP, allowing for a global increase in translation.

This manuscript presents a number of new and interesting findings, including a potential role for Wnt/beta-catenin in protein translation, and a direct interaction between two disease-implicated proteins in beta-catenin and FMRP. However, evidence demonstrating that the physical interaction between beta-catenin and FMRP directly influences these global changes in protein translation, as claimed in the title and abstract, is not established.

Specific Concerns:
1. The authors mention "initial MS/MS analysis revealed that FMRP was present in five out of six biological replicates and correspondingly absent in IgG controls," but do provide this information in the manuscript. Instead, in Figures 1 and 2, they present a set of MS/MS experiments that do not include FMRP as one of their top hits. The MS/MS data indicating FMRP should be included in the main body of the manuscript and provision of some rationale for pursuing FMRP rather than the other more abundant hits would be helpful.
2. The beta-catenin antibody used targets the N-terminal region of a ~780 amino acid protein. It is thus possible that the 1% found to associate with ribosomes (Figure 1) is just a reflection of the nascent peptide during translation. This could be addressed by repeating the MS/MS with a C-terminal antibody (preferred) or doing this in the setting of RNAase or puromycin (to dissociate nascent peptides from the ribosome).
3. The evidence linking beta catenin interaction with FMRP lacks two important controls. First, this interaction could occur indirectly through interactions with mRNA. Thus, treatment of their IP with RNAse should be done to establish whether this is a direct or indirect interaction. Second, FXR1 was among the top hits on their IP and it forms a heteromeric complex with FMRP. Does FXR1 mediate this interaction? This possibility should at least be acknowledged.
4. Authors propose that activation of Wnt causes beta-catenin to dissociate from FMRP, leading to increased translation. If this was the case, FMRP should stop associating with beta-catenin following Wnt activation. This should be directly tested by IP and co-localizaiton ICC studies. Alternatively, as FMRP is a translational repressor, and the authors have shown that FMRP can re-localize from the cytoplasm to the lamin when beta-catenin is tethered there (Figure 3E), perhaps FMRP stays associated with beta-catenin and translocates to the nucleus. This should be visible by ICC. Lastly, does tethering of beta-catenin and FMRP in Figure 3E also result in an increase or change in translation?
5. While the results of beta-catenin knockdown and Wnt signaling increasing global translation is striking, it is well known that both are primarily involved in transcriptional activation, and thus the puromycin SUNSET assays after Wnt activation should be re-performed in the presence of a pol II inhibitor to rule out any transcription-dependent effects.
Minor issues:
1. Authors should include GFP and/or FLAG only controls in Figures 3D-F, and 4D, to confirm that interactions are directly between beta-catenin and FMRP.
2. Figure 4E can be taken out or moved to supplemental.

Referee #3:

A Wnt-dependent β-catenin:FMRP interaction regulates translation.

Ehyai and colleagues show a new role of β-catenin in translational repression by the interaction with Fragile X Mental Retardation Protein (FMRP) at the level of the pre-initiation complex. Moreover, the silencing of β-catenin induces an increase of the global protein synthesis in three different cellular models (VSMC, A10, HEK293T). Finally, stimulation of the Wnt pathway induces a translocation of β-catenin into the nucleus leading to a de-repressed FMRP-dependent translation. Globally, the study reports novel and potentially very interesting data and suggest a novel role of β-catenin in regulating protein synthesis.

There are a few points that the authors need to address.
1. It is unclear the reason why FMRP was chosen for this study because it is not in the LC MS/MS dataset -in the list of the β-catenin interactors- while two partners of FMRP, namely FXR1P and CYFIP1 were listed and not further investigated.
2. The majority of the validations- have been performed using ectopically expressed proteins. The authors should perform co-localization and IP studies looking at endogenous proteins.
3. It is not clear how the block of elongation step and the inhibition of the mTOR pathway could affect the β-catenin:FMRP interaction in the pre-initiation complex.
4. In order to assess the role of β-catenin:FMRP in the translation repression the authors need to validate the deregulation of some FMRP mRNA targets in presence and absence of β-catenin or FMRP.
5. The authors should better clarify the choice of the different cell lines used in this study.
6. Fig 1: please provide the immunoblotting of β-catenin immunoprecipitation used for the LS MS/MS analysis.
7. Fig S4: The authors need to show the immunoblotting for plectin to assess its enrichment in the IP.
8. Fig 4E-F and Fig 5A-B statistical analysis is missing.
9. Fig 4E the negative control of the SUnSET assay (cells treated with cicloheximide) is missing.
Minor points
10. Fig 4A please replace siRNA FMRP with siRNA FMR1.
11. Page 8 there is a repeat of "in in" - fifth line.

1st Revision - authors' response 12 July 2018

Response to Reviewers, Ehyai et al.

Referee #1:

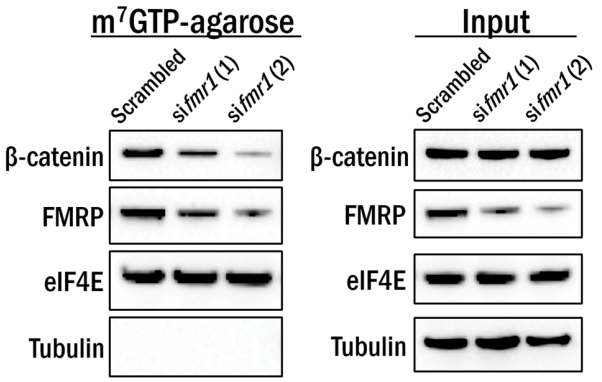
Comment/Question: *This manuscript reports an interaction between b-catenin and FMRP, and a surprising role of b-catenin in repression of translation initiation. While I am always quite sceptical about the real significance of proteomic data (see comment below), the data presented here demonstrate quite convincingly the specificity and robustness of the b-cat-FMRP interaction. The GFP-GBP nanotrap assay gives particularly impressive results, which support a strong b-cat-FMRP interaction.*
Response: The authors appreciate the positive comments. We have used proteomic methods for many years to study post translational control and protein interactions and our approach is to use it as a "discovery tool". We concur that on its own it means little without detailed biochemical and cell biological support data- which is what we strive for in determining the relevance of any interaction or PTM. In this manuscript we have done extensive analysis downstream of the MS/MS identification of the interaction between FMRP and β-catenin.

Comment/Question: *The fact that FMRP is implicated in regulation of translation, and the identification of another component of the initiation complex in the proteomic experiment lead to the logical exploration of a role of b-cat in this process.*
Response: This interaction (along with some other translational regulators) caught our attention in the data set.

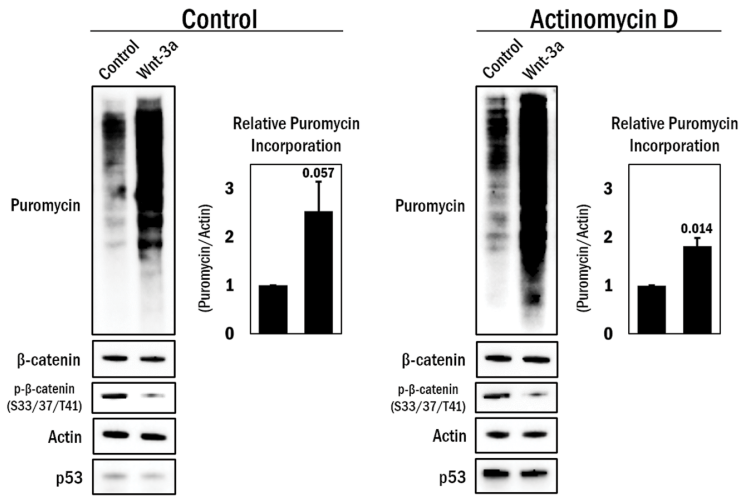
Comment/Question: *The effects of b-catenin depletion and Wnt stimulation are striking, showing huge effects on puromycin incorporation. From these results, I find it convincing that b-catenin has an important repressive role on translation initiation. These findings are new and of general interest.*
Response: This is, in our view, the major message of the paper in that β-catenin manipulation has this unexpectedly robust and previously unreported effect on overall levels of translation.

Comment/Question: *However, what I find less convincing is the proposed mechanism, which would implicate the b-cat-FMRP interaction with the initiation complex as sole cause for this large effect on translation.*
Conceptually, it is hard to imagine how FMRP could be responsible/co-responsible for a two-fold change in translation. It is known to bind 4% of mRNAs in the brain, and since this is the organ affected by its mutation, which lead to a mental syndrome, it would be surprising that it would massively regulate translation in other cell types. I can see however two simple alternative explanations: a) b-cat is indeed directly responsible for massive repression of translation, but without the absolute need for FMRP. In fact, the manuscript does not present any evidence for the involvement of FMRP, thus this protein may well play little to no role in this repression. b) b-cat, with/without FMRP, is directly responsible only for PART of the repression, the rest of the effect could result from transcriptional events. In this interesting case, b-cat could have two antagonistic roles, directly repressing translation in the absence of Wnt signalling, but participating to stimulation of translation via regulation of transcription upon Wnt stimulation.
Importantly, the si experiments last 24-48 hours, which give plenty of room for complex changes in genetic program. Note that most cells do have a baseline of active Wnt signalling, which will be blocked by b-catenin deletion. Other indirect effects could be generated by b-catenin depletion, for instance by affecting cadherin cell contacts, which in turn could trigger other signals impinging on transcription and translation. As for Wnt treatments, 4 hrs give plenty of time for changes in gene activity. I agree that one would expect at least in the simplest model that Wnt activation would have the opposite effect to b-cat depletion, but as just mentioned, long term effect may be quite complex. In any case, these are core issues, which must be absolutely solved in order to give coherence to this potentially very interesting story. One would expect to see two major additional sets of data: 1) an estimate of the extent of direct repression by b-catenin (e.g. in the presence of transcriptional inhibitor); 2) evidence that FMRP is actually involved in this b-cat-dependent repression.
Response:
We fully appreciate the point made by the reviewer here and it did prompt us to re-evaluate how we interpret these data somewhat. In considering the reviewer's point that the puromycin incorporation data indicates a more general effect of β-catenin manipulation on translation than the FMRP dependent translational control reported in neurons, we addressed this further as follows (figures below): We performed an m7GTP pulldown assay from cells in which FMRP protein was depleted by siRNA technology. Treatment with siRNA and subsequent pulldown assays indicated that β-catenin recruitment to the pre-initiation complex appears to be dependent on FMRP. We have amended the text from lines 268-271 and added Figure 4E to reflect this. Furthermore, as suggested, we also repeated the SUnSET assay with Wnt-3a in the presence of the transcriptional inhibitor, actinomycin D, in order to begin to clarify the contribution of transcriptional and translational changes to Wnt mediated proteome changes (this experiment is found in Figure EV5 and the text is in lines 360-362). In addition, we also performed another SUnSET assay with actinomycin D in combination with β-catenin silencing. Loss of β-catenin noticeably enhanced translation in the control (solvent) and transcriptional inhibition (actinomycin D) did not substantially affect this enhancement. Based on these data, we conclude that changes in translation due to depletion of β-catenin or Wnt stimulation under the conditions observed here were not substantially dependent on transcriptional events (Figure EV4 and added lines 296-305 of the text). These results, in conjunction with the polysome profiling, indicate that β-catenin interaction with the pre-initiation complex is to a large extent dependent on FMRP. However, one alternate explanation is that the mechanism of translation repression when β-catenin is recruited is not the same as the role played by FMRP in binding a specific subset of mRNA's (as occurs in neurons in Fragile X syndrome). It could also be that the depletion of FMRP results in the loss of other repressive subunits that are also required for β-catenin's translational repressor effect. The fact that a number of other translational regulatory proteins also co-precipitate with β-catenin in the MS analysis possibly supports this idea and maybe points towards an FMRP independent role of β-catenin after it is recruited. An

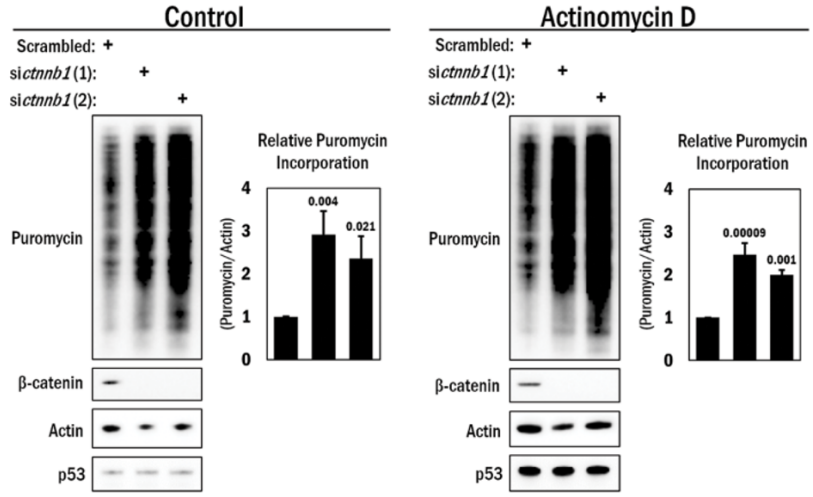
additional and, we think, highly relevant point is that the characterization of FMRP function is as yet incomplete, and two well characterized RNA motifs mediate FMRP dependent translational repression. More recently FMRP has also been implicated as a positive translational regulator of Superoxide Dismutase 1 mRNA through a novel RNA stem loop motif [1]. The actual number of direct RNA cargoes with which FMRP and the related FXR1 protein interact has been a subject of some debate and a detailed discussion of this topic is provided elsewhere [2]. Moreover it has been speculated that the FMRP/FXR1 RNA cargoes may also vary from one cell type to another. Importantly, the effects of β-catenin:FMRP/FXR1 interaction on protein synthesis, as we have documented here, may be far more profound since many of the protein products of the direct RNA binding targets of FMRP/FXR1 are involved in a myriad of cellular processes, in particular cellular signalling and gene/protein expression [2]. Thus, alteration in the protein level expression of these direct FMRP mRNA cargoes will ultimately result in a potentially vast change in the cellular proteome due to the compound effects of the direct RNA binding along with the downstream consequences on gene expression of those changes. If β-catenin participates in a FMRP/FRX1 interaction with its primary RNA cargo, then the impact on protein expression, as we have monitored by the SUnSET assay in β-catenin depleted cells, might extend much further than the sum of the direct RNA binding targets. In support of this idea is a published quantitative proteomics study using SILAC which reported that the levels of 5,023 proteins were altered in Fmr1 KO cell lines [3]. Thus the impact of alteration in the direct FMRP target RNAS (approx. 4% of the RNAS in neurons) has a potentially massive downstream effect on the proteome- possibly affecting the protein levels of a large proportion of the proteome (possibly as high as 40—50% of the proteome). This is a complex issue that awaits more detailed characterization of how β-catenin might influence FMRP target RNAs and, importantly, the downstream consequences of those translational events. So we fully appreciate the reviewer's comments and have amended the discussion to incorporate discussion of these ideas (see lines 431-456). Also, we decided to modify the title of the manuscript to more accurately reflect this in a balanced way, and it now reads "FMRP recruitment of β-catenin to the translation pre-initiation complex". We think that this reflects the main theme of the manuscript in that we think we have very solid evidence that FMRP is required for the recruitment of β-catenin by FMRP and that, once there, β-catenin influences the overall level of translation. What is perhaps less clear at this point is whether FMRP is absolutely required for the translational repression by β-catenin once it is recruited to the pre-initiation complex.



(Added as Figure 4E of manuscript): HEK 293T cells were transfected with either siRNAs targeting fmr1 (FMRP) or scrambled control and were subjected to m^7GTP-agarose pull-downs and precipitated proteins were identified by Western blot analysis. eIF4E and tubulin were shown as positive and negative controls, respectively. Total lysates were used as input.



(Added as figure EV5): A10 cells were serum deprived overnight prior to 1 hr pre-treatment with either actinomycin D (0.5 µg/mL) or its solvent (DMSO), and the cells were stimulated with Wnt-3a (100 ng/mL) or its solvent (0.1% BSA in PBS) for 4 hr. Before harvesting for Western blot analysis, the cells were further treated with 0.5 µM puromycin for 15 min. Total β-catenin and phospho-β-catenin (S33/37/T41) levels indicates effective Wnt-3a stimulation. P53 was used as a control for actinomycin D activity, and actin was used as a loading control.



(Added as Figure EV4): De-repression of β-catenin mediated translation repression is independent of transcription. A10 cells were transfected with siRNAs targeting *ctnnb1* (β-catenin) or scrambled RNA (control) and 36 hr later treated with either actinomycin D (0.5 µg/mL) or DMSO for 4 hr. Before harvesting, the cells were pulsed with 0.5 µM puromycin for 15 min, and subjected to Western blot analysis for detection of the puromycin incorporated peptides. β-catenin blots indicated efficacy of siRNA. Actin was used as a loading control, and p53 was used as a control for actinomycin D activity.

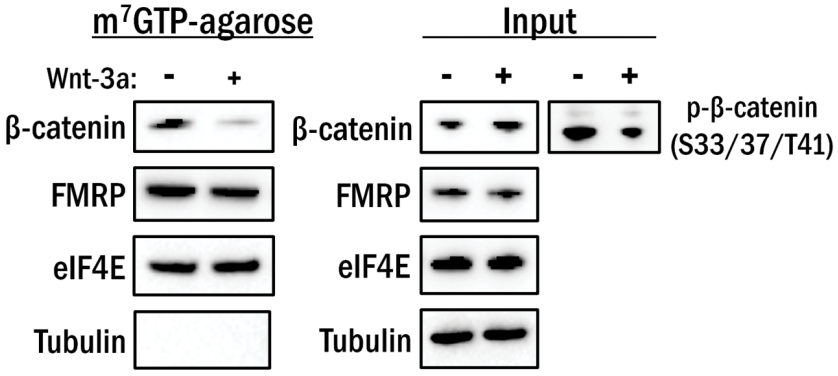
Comment/Question: *I have also issues with another related statement of the proposed model, i.e. the nuclear 'sequestration' of b-cat upon Wnt stimulation. I don't find fully convincing evidence that the effect of Wnt is a simple shift of b-cat from the cytoplasm to the nucleus. Fig5: In VSMC cells, one does see a decreased in cytoplasmic b-cat, and a strong increase in nuclear b-catenin, but also an increase at the plasma membrane. One actually may wonder whether the 'cytoplasmic' signal is really cytoplasmic, or rather corresponds to the plasma membrane of flat cells. Wnt stimulation does tend to induce changes in cell shape, and the apparent cytoplasmic 'depletion' may be partly an optical artefact. In A10 cells, one only sees increased nuclear b-cat, as expected by the classical model of b-cat stabilization, without any obvious change in cytoplasmic signal. This is clearly a*

weak aspect of this study, which needs to be strengthened by more accurate analysis of b-cat levels in different subcellular locations.

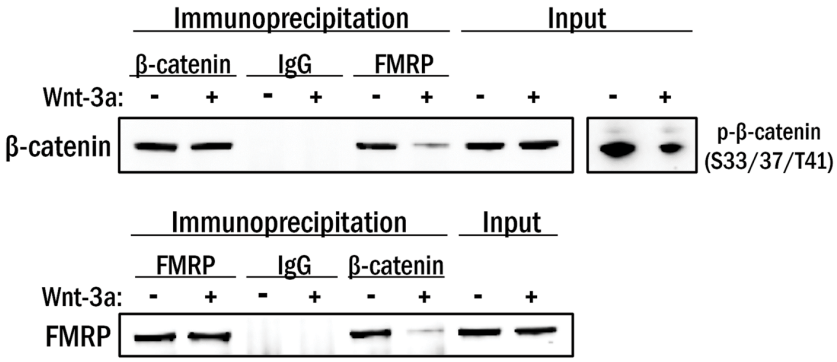
Response: The canonical Wnt signalling pathway is defined by a cytoplasmic accumulation and stabilization of β-catenin following Wnt activation, which results in a nuclear translocation of β-catenin. However, while there is increased nuclear β-catenin, the total stabilization of β-catenin protein levels due to Wnt mediated APC/GSK-3β inhibition suggests that cytoplasmic β-catenin levels are not dramatically reduced as pointed out by the reviewer, although β-catenin function and interactions may alter. The lack of change in cytoplasmic β-catenin in the confocal images may be a result of this, and has been reported by others performing similar Wnt stimulation experiments (see [4], figure 1; [5], figure 1). We have been very careful to look at the individual optical sections as well as the Z stacks using high resolution confocal microscopy. Based on these observations, we are sure that the nuclear accumulation is not an artifact of plasma membrane overlay since this is also observed in individual slices of the Z stacks in the central nuclear region of the cells.

To also begin to address this question biochemically, we determined the effect of Wnt activation on β-catenin association with the pre-initiation complex. We performed an m7GTP pulldown assay in control (0.1% BSA in PBS) cells and Wnt-3a stimulated cells (200 ng/mL, 4 hr, HEK 293T). As can be seen below, β-catenin association with the pre-initiation complex is diminished in cells treated with Wnt-3a consistent with our idea that sequestration to the nucleus depletes it at the translation machinery. In addition, confocal imaging indicated that peri-nuclear localized β-catenin was diminished with Wnt-3a treatment. The most straightforward interpretation of that is that the Wnt dependent re-location of β-catenin to the nucleus results in less recruitment to the translation machinery. However, there could also be other factors involved since the post-translational modification of β-catenin promotes considerable changes in its properties and in the proteins with which it interacts. We previously reported a p38MAPK dependent increase in an interaction with another nuclear transcription factor [6]. Therefore, we have modified the text to acknowledge that our interpretation is the most straightforward one but that there could be several other possibilities for the loss of β-catenin from the translation machinery in response to Wnt stimulation (see lines 421-430).

Finally, we performed co-immunoprecipitation assays with cells that were treated with Wnt-3a. In these experiments, the amounts of β-catenin and FMRP were similar in Wnt-3a treated and non-treated cells. However, β-catenin association with m7GTP beads and β-catenin precipitation with FMRP were both reduced in Wnt-3a stimulated cells. We have added text from lines 363-370 and figures 6C-i and 6C-ii to reflect this.



(Added as figure 6C-ii): HEK 293T cells were serum deprived for 1 hr and then stimulated with Wnt-3a (100 ng/mL) or its solvent (0.1% BSA in PBS) for 4 hr. Cell lysates were then subjected to m^7GTP-agarose pull-down analysis. eIF4E and tubulin were used as positive and negative controls, respectively, and phospho-β-catenin (S33/37/T41) Western blots were used as controls for Wnt-3a stimulation.



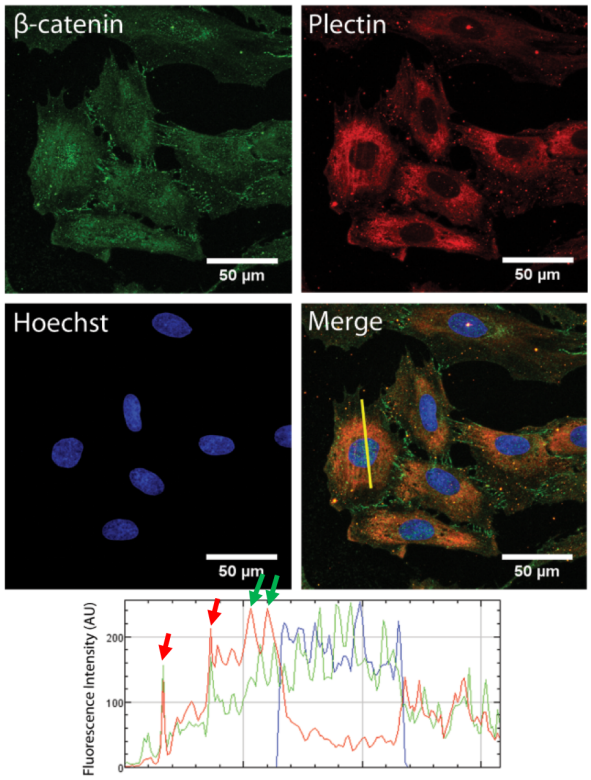
(Added as Figure 6C-i): HEK 293T cells were serum deprived for 1 hr and then stimulated with Wnt-3a (100 ng/mL) or its solvent (0.1% BSA in PBS) for 4 hr. The cells were harvested and subjected to an endogenous coIP analysis using either β-catenin or FMRP antibody. The corresponding proteins in the precipitated immunocomplex were detected by Western blot analysis. A non-programed rabbit IgG was used as a control. Efficacy of Wnt-3a treatment was assessed by inhibition of GSK3 mediated phosphorylation of β-catenin (S33/37/T41) by Wnt-3a.

Comment/Question: *Interaction specificity: Specificity is a recurrent issue with proteomic analysis. In the present case, I am rather puzzled to see relatively highly ranked interactions that seem obviously non physiological. In particular, one mitochondrial protein is ranks similar to FMRP, and a secreted protein, procollagen, is not far behind.*
Response: The reviewer is absolutely correct in this concern related to the identification of proteins that are seemingly unrelated to the bait protein in this type of affinity purification- mass spectrometry based interactome analysis. We do go to considerable lengths to subtract the non-specific interactions (ie proteins that non-specifically interact with the affinity matrix) in this approach. However, there will always be identification of interactions of questionable physiological relevance in this type of analysis for many reasons related to protein abundance and loss of cellular compartmentation in affinity purification. Therefore, our philosophy is to minimize the possibility of false positives in the AP-MS as much as possible (using appropriate negative controls to subtract away the non-specific interactors) and then rely on rigorous downstream analysis of the confirmed interactions to probe the physiological relevance of the identified interactions. We therefore agree that some of the identified interactions in the interactome analysis will not be physiologically relevant even though they are detected (proteins in affinity purification interact based on their biochemical properties *in vitro* and not based on their physiological relevance). In this study, we do however contend that subsequent detailed analysis supports the cell biological relevance. In addition, we would also point out that some of the very well known, previously characterized β-catenin interacting proteins eg α-catenin and Mediator components, are robustly identified in our analysis. To consider this point slightly differently, one major advantage of this approach is that it is truly unbiased and not based on pre-conceived notions about the known role of particular proteins. There are now many cases of proteins that were not suspected to play a role in certain cellular processes that have now been associated with new functional roles based on this type of unbiased protein interactome analysis.

Comment: *I would also feel more comfortable to see unarguable negative controls demonstrating that the plectin (here used as a positive control) and FMRP interactions are 'uniquely' specific. For instance, it would be nice to show that b-cat does NOT co-IP/colocalizes with procollagen nor mitochondrial Mthfd1.*
Response: We tested several interactions by coIP analysis that were not confirmed biochemically along with the FMRP/β-catenin which was positively confirmed. In addition we have included further analysis of the Plectin interaction (in reponse to your next comment). In showing the Plectin data we were primarily trying to illustrate the general initial approach that we use following the interactome identification by MS/MS. This has a peripheral bearing on the manuscript since the detailed downstream analysis of the FMRP/β-catenin interaction by coIP, nanotrap, immunofluorescence analysis all confirm the initial identification by MS.

Comment/Question FigS3+5*, Control b-cat/plectin colocalization: I find worrying the fact that all bright structures in the red channel are also observed in the green channel. This is very striking is*

fig S5. Of course this could mean that all plectin structures also accumulate b-cat. However, I would feel reassured to see controls for the absence of bleed-through, which may occur if the plectin staining is particularly strong.
Response: In analyzing the micrographs, we determined the two highest peaks of the red signal (green arrows) corresponding to FMRP were not matched with green peaks. However, two sharp peaks of the red signal (red arrows) are tightly matched with the corresponding green peaks. If the red signal bleeds through to the green signal, there should have been peaks of the green signal corresponding to these two highest peaks of the red signal. Therefore, it is highly unlikely that the green signal was a bleed-through signal from the red. Therefore, we concluded that matching peaks indicate a true co-localization of Plectin with β-catenin. We have added this analysis to the manuscript on lines 138-142, and amended the figure (Appendix Figure S3).



(Updated and labelled as Appendix Figure S3): Assessment of the Co-localization of β-catenin and Plectin. A10 cells were fixed and stained for β-catenin (green), Plectin (red), and nucleus (blue). Co-localization of these proteins was analyzed by an RGB scan. Green arrows indicate un-matched β-catenin and FMRP (green and red lines, respectively) and red arrows indicate matching peaks, demonstrating that the Plectin signal is independent of the β-catenin signal.

Comment/Question Page 8: *"FMRP was present in five out of six biological replicates"?? I see three out of five!!*
Response: We have amended this in the manuscript (lines 164-173), and mentioned that FXR1 was identified in this MS experiment (we also highlighted FXR1 in purple in Figure 1B).

Comment/Question Fig4a: *Cell fractionation is quite a crude method to analyse distribution of a protein such as b-cat, which may be potentially found in multiple unrelated complexes in addition to ribosomes/polysomes. However, the strong decrease of signal in fraction 2 upon FMRP depletion is another confirmation of their interaction. And their presence in the initiation complex is supported by m7GTP pulldown (panels C,D).*
Response: In addition, m7GTP pulldowns with siRNA depletion of FMRP, as shown above, can be used as another confirmation of the interaction.

Referee #2:

Comment/Question *In this report, Ehyai et al. aim to identify additional roles for beta-catenin by investigating its potential binding partners. They find that beta-catenin interacts with a number of mRNA translation factors including FMRP, and show that this interaction occurs within the context of pre-initiation complexes. Activation of the Wnt pathway results in re-localization of beta-catenin to the nucleus, and a correlative increase in global translation. The authors conclude that beta-catenin acts with FMRP as a translational repressing complex, and that Wnt activation dissociates beta-catenin from FMRP, allowing for a global increase in translation.*

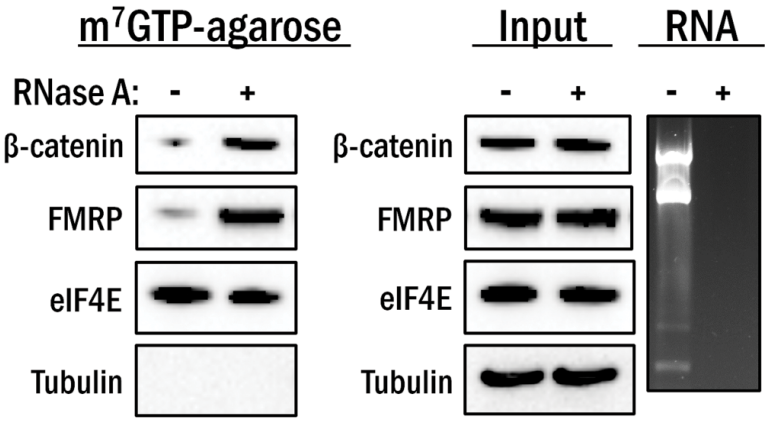
Comment/Question *This manuscript presents a number of new and interesting findings, including a potential role for Wnt/beta-catenin in protein translation, and a direct interaction between two disease-implicated proteins in beta-catenin and FMRP. However, evidence demonstrating that the physical interaction between beta-catenin and FMRP directly influences these global changes in protein translation, as claimed in the title and abstract, is not established.*
Response: We appreciate the reviewers comment and after consideration of this point we decided to modify the title of the manuscript to more accurately reflect this in a balanced way and it now reads "FMRP recruits β-catenin to the translation pre-initiation complex". We think that this better reflects the main theme of the manuscript in that we think we have very solid evidence that FMRP is required for the recruitment of β-catenin by FMRP and that, once there, β-catenin influences the overall level of translation. What is perhaps less clear at this point is whether FMRP is absolutely required for the translational repression by β-catenin once it is recruited to the pre-initiation complex, as indicated by the reviewer. We have also amended the text to incorporate this idea from lines 431-456.

Specific Concerns:

Comment/Question 1*. The authors mention "initial MS/MS analysis revealed that FMRP was present in five out of six biological replicates and correspondingly absent in IgG controls," but do provide this information in the manuscript. Instead, in Figures 1 and 2, they present a set of MS/MS experiments that do not include FMRP as one of their top hits. The MS/MS data indicating FMRP should be included in the main body of the manuscript and provision of some rationale for pursuing FMRP rather than the other more abundant hits would be helpful.*
Response: Yes indeed this is an important point that we need to clarify. Initially we did a preliminary AP/ LC-MS/MS study of β-catenin in which we included a condition of p38 MAPK activation (a previous study warranted this). In terms of the treatment (p38 activation), this experiment proved inconsequential. However, in this experiment we detected FMRP interaction with β-catenin in 5 out of 6 samples (2 control and 3 p38 activation) and it was not detected in any of the IgG controls (4). This prompted us to begin working on the FMRP /β-catenin interaction since this was a highly positive observation. However, since the p38 activation condition would be confusing (since it was irrelevant) we re-did the interactome analysis. In the repeat experimentation we again detected FMRP and FXR1, however FMRP was present in some of the IgG control condition samples. This is not uncommon because of the sensitivity of modern MS/MS and more recently we have used quantitative MS to deal with this issue. Isobaric labeling allows one to look at the enrichment of the protein between experimental and control conditions rather than a binary detection of presence or absence. However, because we set our original criteria cut off for inclusion on the list to be NO detection of the candidate protein in the IgG control we could not legitimately include FMRP in the most recent list even though the closely related FXR1 protein (very highly conserved with FMRP with the same domain structure and very high sequence identity) is there. We realize that this is not ideal but addressing again would require re-doing the complete MS data set using quantitative proteomics which is costly and labour intensive. Given the MS data that we did acquire (and the extensive downstream analysis that we have done) we are confident in the identification and interaction. We have now put a statement in the results to indicate the above discussion from lines 164-173.
The main reason we pursued the translational direction we did was because the GO analysis indicated that control of translation (and proteins associated with it) was the most statistically significant cellular process associated with the β-catenin interactome. We have highlighted this in Figure 2A by adding a box around the terms "translation" and "peptide metabolic process" to emphasize this.

Comment/Question 2*. The beta-catenin antibody used targets the N-terminal region of a ~780 amino acid protein. It is thus possible that the 1% found to associate with ribosomes (Figure 1) is just a reflection of the nascent peptide during translation. This could be addressed by repeating the MS/MS with a C-terminal antibody (preferred) or doing this in the setting of RNAase or puromycin (to dissociate nascent peptides from the ribosome).*

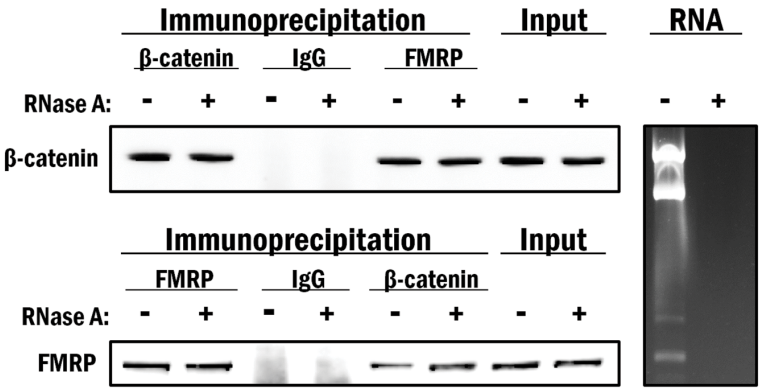
Response: To address this concern that the β-catenin:FMRP interaction may be due to nascent β-catenin peptide being detected during translation, we performed an m7GTP pulldown assay in the presence or absence of RNase A, as suggested by the reviewer. With overnight RNase A treatment at 10ug/mL during bead incubation, there is a slight increase in both FMRP and corresponding β-catenin association with the m7GTP-agarose beads. Our interpretation is that this increased association is due to the removal of free mRNAs in the cell lysate, thus promoting FMRP enrichment to the m7GTP beads, and β-catenin along with it. Importantly for the point raised, this result demonstrates that removal of RNA (by RNase A treatment) eliminates the possibility that detection of β-catenin at the ribosome reflects β-catenin peptides being translated. We have amended the text from lines 271-279 to include this experiment and our interpretation of the results, and added Figure 4F.



(Added as Figure 4F): HEK 293T cells lysates were subjected to m^7GTP-agarose pull-downs in the presence or absence of RNase A (10 µg/mL) and precipitated proteins were identified by Western blot analysis. eIF4E and tubulin were shown as positive and negative controls, respectively. Total lysates were used as input. RNA was extracted from parallel lysates and RNA content was analyzed by agarose gel electrophoresis.

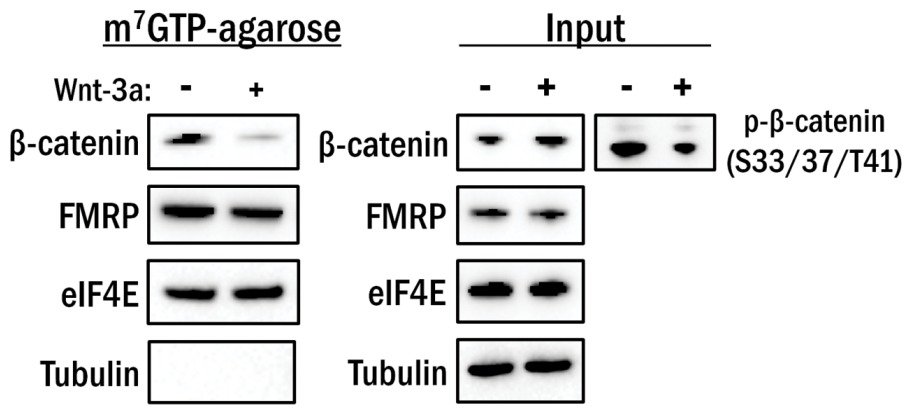
Comment/Question 3*. The evidence linking beta catenin interaction with FMRP lacks two important controls. First, this interaction could occur indirectly through interactions with mRNA. Thus, treatment of their IP with RNAse should be done to establish whether this is a direct or indirect interaction.*

Response: We have performed the requested experiment. Co-immunoprecipitation assays with RNase A treatment did not considerably change the β-catenin:FMRP interaction. HEK 293T cell lysates were treated with RNase A at 10ug/mL overnight during antibody-protein G bead incubation and analyzed by Western blot. When comparing the amount of corresponding FMRP that was present in the β-catenin IP, and vice versa, we observe similar amounts of peptide in RNase A treated or untreated lysates. Based on this information, and the GST-pulldown assays using purified FMRP and β-catenin peptides (Fig 3C-i), we can conclude that these two proteins directly interact, and this interaction does not require RNA. This experiment has been added in the text from lines 187-195, and as Figure 3C-ii.

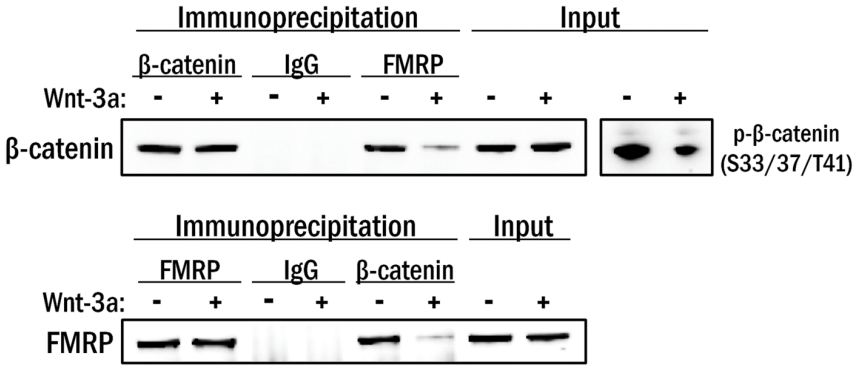


(Added as Figure 3C-ii): Interaction between FMRP and β-catenin was assessed in the absence of RNA. HEK 293T cells were subjected to an endogenous coIP analysis using either β-catenin or FMRP antibody, in the presence or absence of RNase A (10 µg/mL). The corresponding protein was detected in the precipitated immunocomplex by Western blot, and non-programmed rabbit IgG was used as a control. RNA was extracted from parallel cultured cells and total RNA content was analyzed by agarose gel electrophoresis in the presence or absence of RNase A.

Comment: *Second, FXR1 was among the top hits on their IP and it forms a heteromeric complex with FMRP. Does FXR1 mediate this interaction? This possibility should at least be acknowledged.*
Response: Yes, we fully acknowledge this point since it has been reported that FMRP and the highly related FXR1P exist in close proximity to one another in the mRNP complex and FXR1p is in our interactome list (see previous discussion). Also, the known interaction of the FMRP modulatory protein CYFIP1/2 has been highlighted in the text on lines 312-314.
Comment/Question 4. *Authors propose that activation of Wnt causes beta-catenin to dissociate from FMRP, leading to increased translation. If this was the case, FMRP should stop associating with beta-catenin following Wnt activation. This should be directly tested by IP and co-localizaiton ICC studies. Alternatively, as FMRP is a translational repressor, and the authors have shown that FMRP can re-localize from the cytoplasm to the lamin when beta-catenin is tethered there (Figure 3E), perhaps FMRP stays associated with beta-catenin and translocates to the nucleus. This should be visible by ICC. Lastly, does tethering of beta-catenin and FMRP in Figure 3E also result in an increase or change in translation?*
Response: In order to address the reviewer's comment regarding FMRP and β-catenin association following Wnt stimulation, we tested the extent of β-catenin association with the pre-initiation complex, and compared it with FMRP's association. We therefore performed an m7GTP pulldown assay in the presence or absence of Wnt-3a (100 ng/mL, 4 hr, HEK 293T). Following treatment with Wnt-3a, there is a clear reduction in β-catenin association with the pre-initiation complex, while FMRP association remains unchanged. This has been added to the text from lines 365-377 and we have added Figure 6C-ii.
(Bottom figure) Similar to above, we also performed coimmunoprecipitation assays with cells that were treated with Wnt-3a. In these experiments, the amounts of β-catenin and FMRP immunoprecipitated were similar in Wnt-3a treated and non-treated cells. However, corresponding FMRP and β-catenin, respectively, was considerably reduced in Wnt-3a stimulated cells. This can be found on lines 363-365 and in Figure 6C-i.

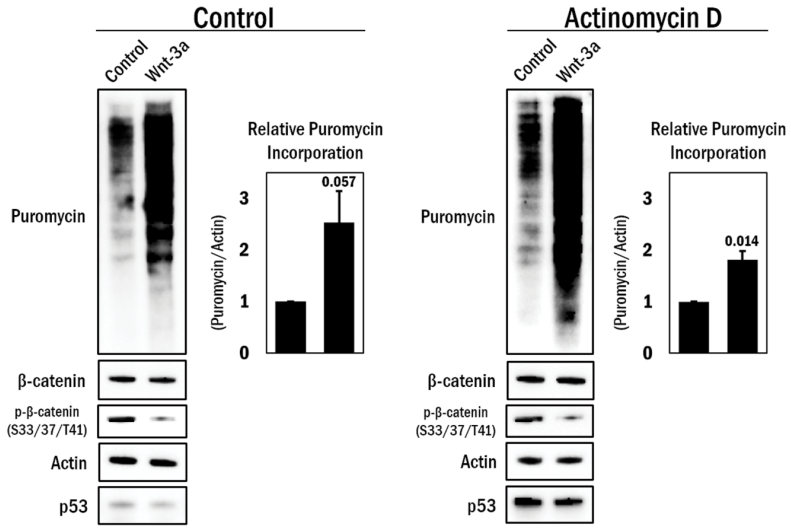


(Added as figure 6C-ii): HEK 293T cells were serum deprived for 1 hr and then stimulated with Wnt-3a (100 ng/mL) or its solvent (0.1% BSA in PBS) for 4 hr. Cell lysates were then subjected to m^7GTP-agarose pull-down analysis. eIF4E and tubulin were used as positive and negative controls, respectively, and phospho-β-catenin (S33/37/T41) Western blots were used as controls for Wnt-3a stimulation.



(Added as Figure 6C-i): HEK 293T cells were serum deprived for 1 hr and then stimulated with Wnt-3a (100 ng/mL) or its solvent (0.1% BSA in PBS) for 4 hr. The cells were harvested and subjected to an endogenous coIP analysis using either β-catenin or FMRP antibody. The corresponding proteins in the precipitated immunocomplex were detected by Western blot analysis. A non-programed rabbit IgG was used as a control. Efficacy of Wnt-3a treatment was assessed by inhibition of GSK3 mediated phosphorylation of β-catenin (S33/37/T41) by Wnt-3a.

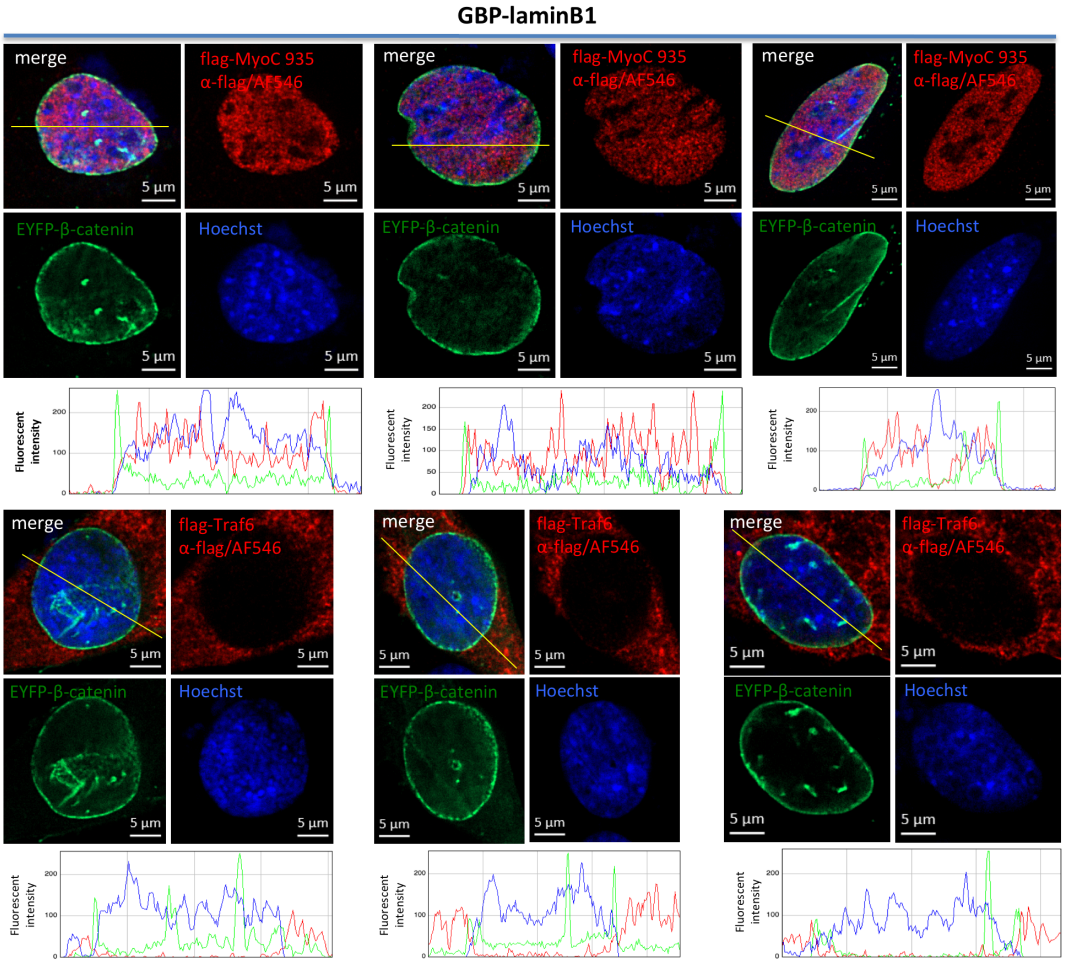
Comment/Question 5. *While the results of beta-catenin knockdown and Wnt signaling increasing global translation is striking, it is well known that both are primarily involved in transcriptional activation, and thus the puromycin SUNSET assays after Wnt activation should be re-performed in the presence of a pol II inhibitor to rule out any transcription-dependent effects.*
Response: The suggested experiment was carried out and, as can be seen below, the de-repression of translation by Wnt-3a was minimally affected by Actinomycin D treatment to inhibit transcription. These data suggest that, at least under the specific conditions of this assay, the main contribution to this effect is at the translational and not transcriptional level of control. We have added text from lines 360-362 and Figure EV5 to reflect this.

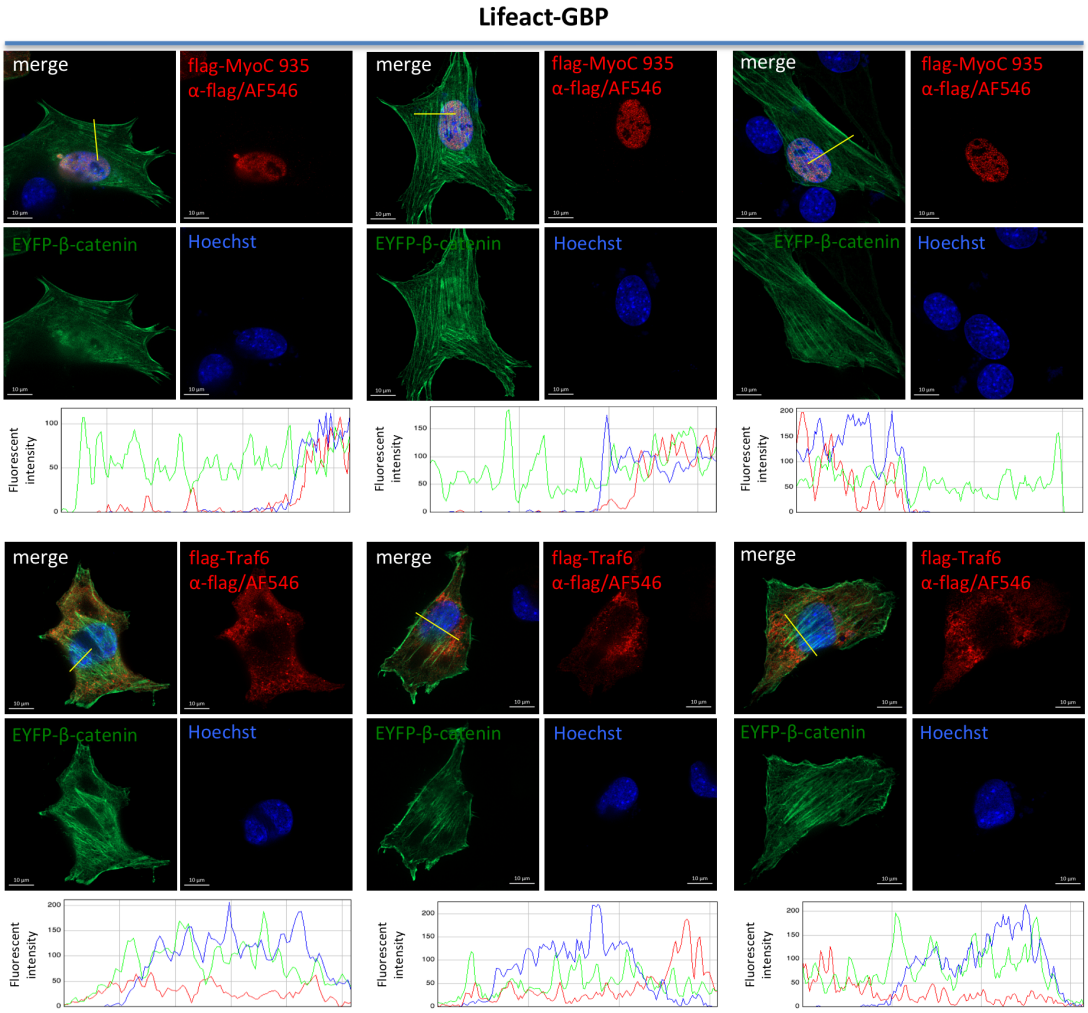


(Added as figure EV5): A10 cells were serum deprived overnight prior to 1 hr pre-treatment with either actinomycin D (0.5 μg/mL) or its solvent (DMSO), and the cells were stimulated with Wnt-3a (100 ng/mL) or its solvent (0.1% BSA in PBS) for 4 hr. Before harvesting for Western blot analysis, the cells were further treated with 0.5 μM puromycin for 15 min. Total β-catenin and phospho-β-catenin (S33/37/T41) levels indicates effective Wnt-3a stimulation. P53 was used as a control for actinomycin D activity, and actin was used as a loading control.

Minor issues:

Comment/Question 1. *Authors should include GFP and/or FLAG only controls in Figures 3D-F, and 4D, to confirm that interactions are directly between beta-catenin and FMRP.*
Response: In order to address this concern, we repeated experiments using Flag-Myocardin-935 and Flag-Traf6 to confirm that the observed interaction of EYFP-β-catenin with Flag-FMRP is not non-specific through the Flag-peptide. Both Flag-tagged proteins were, indeed, not co-localized with EYFP-β-catenin when it was nano-trapped at either the nuclear envelope or F-actin (please see figures below). Therefore, these data indicate that the interaction of EYFP-β-catenin with Flag-FMRP is not mediated by Flag-peptide. The text from lines 234-236 and Appendix Figure S6 discusses this experiment.





(Added as Appendix Figure S6): Flag peptide does not interact with neither GBP, EYFP, nor β-catenin. EYFP-β-catenin was co-expressed with Flag-Myocardin 935 (MyoC 935) or Flag-Traf6 with (top) GBP-Lamin B1 or (bottom) GBP-Lifeact. The transfected cells were subjected to immunofluorescence analysis with a Flag antibody (AF546, red) and the nucleus was visualized by DNA staining with Hoechst 33342. The micrograph shows EYFP-β-catenin (green) was trapped at anchor sites (nuclear envelope with GBP-Lamin B1 or F-actin with Lifeact-GBP, respectively). Flag-MyoC 935 and Flag-Traf6 were localized at the expected sub-cellular location such as the nucleus for MyoC 935 and cytosol for Traf6. Line scanning of the RGB shows no enrichment of the red signal on either green signal peaks.

Comment/Question 2. *Figure 4E can be taken out or moved to supplemental.*
Response: We have moved this figure to Figure EV2, as suggested.

Referee #3:

A Wnt-dependent ß-catenin:FMRP interaction regulates translation.

Comment/Question*: Ehyai and colleagues show a new role of ß-catenin in translational repression by the interaction with Fragile X Mental Retardation Protein (FMRP) at the level of the pre-initiation complex. Moreover, the silencing of ß-catenin induces an increase of the global protein synthesis in three different cellular models (VSMC, A10, HEK293T). Finally, stimulation of the Wnt pathway induces a translocation of ß-catenin into the nucleus leading to a de-repressed FMRP-dependent translation.*

Globally, the study reports novel and potentially very interesting data and suggest a novel role of ß-catenin in regulating protein synthesis.
There are a few points that the authors need to address.

Comment/Question 1. *It is unclear the reason why FMRP was chosen for this study because it is not in the LC MS/MS dataset -in the list of the ß-catenin interactors- while two partners of FMRP, namely FXR1P and CYFIP1 were listed and not further investigated.*
Response: Thanks for this comment which is appropriate. This is addressed above and clarified in the results section on lines 164-173.

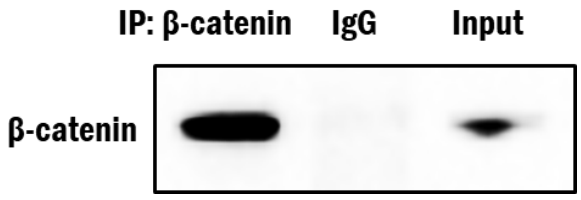
Comment/Question 2. *The majority of the validations- have been performed using ectopically expressed proteins. The authors should perform co-localization and IP studies looking at endogenous proteins.*
Response: To address this we carried out studies analyzing the endogenous proteins in primary tissue culture cells in Figure 3B-ii. Also, in our revisions, we have added two more endogenous coIP experiments (Figure 3C-ii and Figure 6C-i).

Comment/Question 3. *It is not clear how the block of elongation step and the inhibition of the mTOR pathway could affect the ß-catenin:FMRP interaction in the pre-initiation complex.*
Response: At this point we can only surmise that pharmacological interference with the overall regulation of translation interferes with the regulatory machinery at the pre-initiation complex possibly by causing a feedback inhibition due to the accumulation of partial translation products.

Comment/Question 4. *In order to assess the role of ß-catenin:FMRP in the translation repression the authors need to validate the deregulation of some FMRP mRNA targets in presence and absence of ß-catenin or FMRP.*
Response: We have begun studies using RNA-seq to detect the RNA compliment at the translation machinery (using polysome fractions) under conditions of β-catenin and FMRP knockdown but this has not been completed at this point. Obviously this is a question that we are very interested in but this analysis will take substantially longer to complete.

Comment/Question 5. *The authors should better clarify the choice of the different cell lines used in this study.*
Response: Our main purpose in this study was to assess the role of β-catenin in vascular smooth muscle cells and therefore the majority of experiments were conducted with primary vascular smooth muscle cells or with the A10 smooth muscle cell line. We used the A10 cells when it was not possible to achieve the scale required for the experimentation by primary cultures. Lastly, when we wanted to use a neutral cell background for overexpression of exogenous proteins and for basic biochemical assays we used a generic HEK cell line. HEK were also used for the polysome experiments as the polysome fractionation procedure was optimized for this cell line. We have now included a statement to in the methods section to reflect these choices, from lines 558-566.

Comment/Question 6. *Fig 1: please provide the immunoblotting of ß-catenin immunoprecipitation used for the LS MS/MS analysis.*
Response: Prior to this analysis we carried out several β-catenin IPs which were published in Molecular and Cellular Biology [6], and the same procedure was used in this experiment. We also detected substantial levels of β-catenin peptides in the MS analysis (and not in the control IgG) indicating the efficacy of the original IP. Below, we have provide a representative β-catenin IP that we performed in A10 cells in response to this comment (although it is not from the LC MS/MS samples), which indicates immunoprecipitation of the β-catenin protein compared to an IgG control. This representative Western blot has been added as Appendix Figure S1 and in the text on line 105.

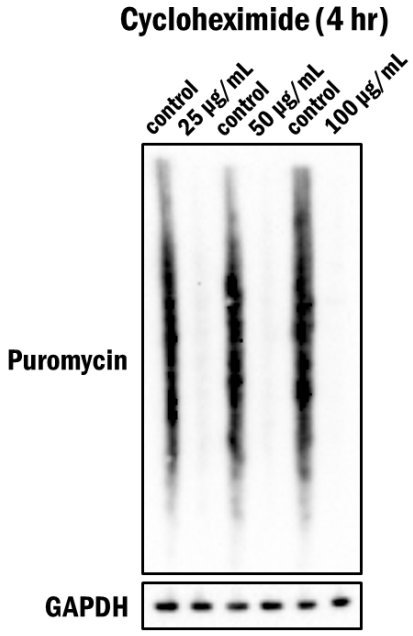


(Added as Appendix Figure S1): Specificity of the antibody used for affinity purification of β-catenin. Representative β-catenin immunoprecipitation used for LC MS/MS analysis. Non-programmed rabbit IgG was used as a control.

Comment/Question 7. *Fig S4: The authors need to show the immunoblotting for plectin to assess its enrichment in the IP.*
Response: We added some additional analysis of Plectin to further verify it. However, despite trying various approaches we did not manage to perform a convincing Plectin immunoblot. The protein is approximately 500kDa and, unfortunately, we could not get the western blot to work for this protein, and so have removed the blot and added additional verification by microscopy. The added text is on lines 138-142 and Appendix Figure S3 has been amended.

Comment/Question 8. *Fig 4E-F and Fig 5A-B statistical analysis is missing.*
Response: We have performed relevant statistical analyses for all quantified images, and have indicated the p-values above the error bars in the bar graphs.

Comment/Question 9. *Fig 4E the negative control of the SUnSET assay (cells treated with cicloheximide) is missing.*
Response: We agree with the reviewer and have performed the negative control of the SUnSET assay. HEK cells were treated with either ddH_2O (control) or 25 µg/mL, 50 µg/mL, and 100 µg/mL cycloheximide for 4 hours prior to the addition of 0.5 µM puromycin for 15 minutes and subsequent harvesting and visualization. Cells were immunoblotted with puromycin antibody and GAPDH (as a loading control). This has been added to the text on lines 284-288 and Figure EV3.



(Added as Figure EV3): HEK 293T cells were treated with indicated concentrations of cycloheximide or solvent (water) for 4 hr prior to incubation with 0.5 µM puromycin for 15 min. Cell lysates were subjected to Western blot analysis for detection of incorporated puromycin. GAPDH was used as a loading control.

Minor points
Comment/Question 10. *Fig 4A please replace siRNA FMRP with siRNA FMR1.*
Response: Thank you for pointing this out, FMRP has been changed to FMR1.

Comment/Question 11. *Page 8 there is a repeat of "in in" - fifth line.*
Response: We have removed the repeat.

References:
1. Bechara EG, Didiot MC, Melko M, Davidovic L, Bensaid M, Martin P, Castets M, Pognonec P, Khandjian EW, Moine H (2009) A novel function for fragile X mental retardation protein in translational activation. *PLoS biology* **7:** e1000016
2. Miyashiro KY, Beckel-Mitchener A, Purk TP, Becker KG, Barret T, Liu L, Carbonetto S, Weiler IJ, Greenough WT, Eberwine J (2003) RNA cargoes associating with FMRP reveal deficits in cellular functioning in Fmr1 null mice. *Neuron* **37:** 417-431
3. Matic K, Eninger T, Bardoni B, Davidovic L, Macek B (2014) Quantitative phosphoproteomics of murine Fmr1-KO cell lines provides new insights into FMRP-dependent signal transduction mechanisms. *Journal of proteome research* **13:** 4388-4397
4. Klapholz-Brown Z, Walmsley GG, Nusse YM, Nusse R, Brown PO (2007) Transcriptional program induced by Wnt protein in human fibroblasts suggests mechanisms for cell cooperativity in defining tissue microenvironments. *PloS one* **2:** e945
5. Huang J, Guo X, Li W, Zhang H (2017) Activation of Wnt/β-catenin signalling via GSK3 inhibitors direct differentiation of human adipose stem cells into functional hepatocytes. *Scientific reports* **7:** 40716
6. Ehyai S, Dionyssiou MG, Gordon JW, Williams D, Siu KW, McDermott JC (2015) A p38 Mitogen-Activated Protein Kinase-Regulated Myocyte Enhancer Factor 2-beta-Catenin Interaction Enhances Canonical Wnt Signaling. *Mol Cell Biol* **36:** 330-346

2nd Editorial Decision 10 September 2018

Thank you for the submission of your revised manuscript. We have now received the full set of referee reports and I am happy to tell you that all referees support its publication now. Referee 3 asks for a few more experiments but neither referee 1 nor referee 2 agree that these are essential. All other concerns must be addressed.

A few other changes are also needed.

- Each figure must be uploaded as a single file and must fit on one page. This also applies to the EV figures.

- Please add a data availability section at the end of the materials and methods that includes the accession number of the proteomics data.

- Please provide up to 5 keywords

- Several blots are over-contrasted, please provide better images

- The source data need to be provided as one file per figure, please send us new files.

EMBO press papers are accompanied online by A) a short (1-2 sentences) summary of the findings and their significance, B) 2-3 bullet points highlighting key results and C) a synopsis image that is 550x200-400 pixels large (the height is variable). You can either show a model or key data in the synopsis image. Please note that text needs to be readable at the final size. Please send us this information along with the revised manuscript.

I would like to suggest a few minor changes to the abstract that needs to be written in present tense. Please let me know whether you agree with the following:

Canonical Wnt/β-catenin signalling is an essential regulator of various cellular functions throughout development and adulthood. Aberrant Wnt/β-catenin signalling also contributes to various pathologies including cancer, necessitating an understanding of cell context dependent mechanisms regulating this pathway. Since protein-protein interactions underpin β-catenin function and localization, we sought to identify novel β-catenin interacting partners by affinity purification coupled with tandem mass spectrometry in vascular smooth muscle cells (VSMCs), where β-catenin

is involved in both physiological and pathological control of cell proliferation. Here, we report novel components of the VSMC β-catenin interactome. Bioinformatic analyses of the protein networks imply potentially novel functions for β-catenin, particularly in mRNA translation, and we confirm a direct interaction between β-catenin and the fragile X mental retardation protein (FMRP). Biochemical studies reveal a basal recruitment of β-catenin to the messenger ribonucleoprotein and translational pre-initiation complex, fulfilling a translational repressor function. Wnt stimulation antagonizes this function, in part, by sequestering β-catenin away from the pre-initiation complex. In conclusion, we present evidence that β-catenin fulfils a previously unrecognized function in translational repression.

I am looking forward to receiving the final version of your manuscript as soon as possible.

REFEREE REPORTS

Referee #1:

The revised manuscript has significantly improved. All the key issues have been satisfactorily adressed. The additional data increase the confidence in these interesting (and provocative) results.

Referee #2:

The authors have adequately addressed my concerns and the manuscript is significantly improved. Moreover, they have provided an outstanding example of how to respond to a reviewers comments. Hopefully they won't mind if I show it to my own trainees after their paper is accepted for publication.

Referee #3:

The revised manuscript by Ehyai and colleagues has improved and provides additional evidence for a role of the β-catenin:FMRP complex in regulating translational initiation.

Important points that need to be taken into consideration by the authors.

1) As mentioned in my previous report, in order to assess the role of β-catenin:FMRP in the translation repression the authors need to validate the deregulation of some FMRP mRNA targets in presence and absence of β-catenin and FMRP. While the role of FMRP as repressor of translation is not novel, the interaction with β-catenin in repressing translational it is and requires validation.

2) There are no attempts to investigate the mechanism of sequestration of cytoplasmic β-catenin into the nucleus and the de-repression of translation. I appreciate that the authors do speculate in the discussion about a few possibilities but one or two simple experiments would provide an added value to the work.

3) Some of the images - including puromycin treated samples- are not of high quality. It might be the resolution of the PDF.

4) Surprisingly the authors did not discuss the literature on FMRP regulating translational initiation. Several papers from different labs have clearly helped dissecting the mechanism of FMRP-mediated translational regulation at the level of initiation. Those findings need to be taken into consideration in the discussion.

Cross-comments from referee 2:

While I think the points and experiments raised by reviewer #3 are reasonable, I feel that the authors have taken significant steps to address the reviewer concerns. Clearly there will always be additional

experiments to do- and I suspect they will do them and publish them as a separate manuscript. However, I think there is enough in the manuscript to justify its publication in EMBO reports without further experimentation.

It would be reasonable for them to provide a better image (point #3) and to cite published work on the roles of FMRP in initiation.

Cross-comments from referee 1:

I agree with reviewer 2: the data presented are sufficiently compelling for publication. Surely, the experiments proposed by reviewer 3 make sense, but they seem to me beyond what can be asked at this stage. There is an inflating trend to ask for more and more data. The effect can be positive (solidifying confidence in the conclusions), but this too often results in diluting and even obscuring the core message of the study. I consider a great quality of EMBO Reports to publish interesting and compelling new discoveries, while remaining reasonable in the amount of data presented.

I would accept the manuscript as it is.

The only point which may be worth improving is the last one (point 4), including a sentence and a couple of references would not harm.

2nd Revision - authors' response 12 September 2018

Comment: Thank you for the submission of your revised manuscript. We have now received the full set of referee reports and I am happy to tell you that all referees support its publication now. Referee 3 asks for a few more experiments but neither referee 1 nor referee 2 agree that these are essential. All other concerns must be addressed.

Response: *We have addressed the non-experimental concerns of reviewer 3 (comments 3 and 4), which were also suggested in the cross-comments of reviewers 1 and 2. The specific changes are indicated below.*

Comment: A few other changes are also needed.
- Each figure must be uploaded as a single file and must fit on one page. This also applies to the EV figures.

Response: *We have made the changes, each figure is now on its own page.*

Comment: - Please add a data availability section at the end of the materials and methods that includes the accession number of the proteomics data.

Response: *We have added this information as requested on lines 710-712, in the materials and methods section.*

Comment: - Please provide up to 5 keywords

Response: *Our 5 keywords are the following: mRNA translation, β-catenin, Wnt signalling, FMRP, and pre-initiation complex*

Comment: - Several blots are over-contrasted, please provide better images

Response: *We have reduced the contrast of the blots in Figs 3C ii, 6C i, and 6C ii.*

Comment: - The source data need to be provided as one file per figure, please send us new files.

Response: *We have now separated all source data TIFF and Excel files so that each figure has an individual source data file.*

Comment: EMBO press papers are accompanied online by A) a short (1-2 sentences) summary of the findings and their significance, B) 2-3 bullet points highlighting key results and C) a synopsis image that is 550x200-400 pixels large (the height is variable). You can either show a model or key data in the synopsis image. Please note that text needs to be readable at the final size. Please send us this information along with the revised manuscript.

Response: *We have uploaded the file "synopsis text" that contains text and bullet points, and the file "synopsis image" that contains a schematic aimed at a graphical representation of the main findings reported in the manuscript.*

Comment: I would like to suggest a few minor changes to the abstract that needs to be written in present tense. Please let me know whether you agree with the following:

Canonical Wnt/β-catenin signalling is an essential regulator of various cellular functions throughout development and adulthood. Aberrant Wnt/β-catenin signalling also contributes to various pathologies including cancer, necessitating an understanding of cell context dependent mechanisms regulating this pathway. Since protein-protein interactions underpin β-catenin function and localization, we sought to identify novel β-catenin interacting partners by affinity purification coupled with tandem mass spectrometry in vascular smooth muscle cells (VSMCs), where β-catenin is involved in both physiological and pathological control of cell proliferation. Here, we report novel components of the VSMC β-catenin interactome. Bioinformatic analyses of the protein networks imply potentially novel functions for β-catenin, particularly in mRNA translation, and we confirm a direct interaction between β-catenin and the fragile X mental retardation protein (FMRP). Biochemical studies reveal a basal recruitment of β-catenin to the messenger ribonucleoprotein and translational pre-initiation complex, fulfilling a translational repressor function. Wnt stimulation antagonizes this function, in part, by sequestering β-catenin away from the pre-initiation complex. In conclusion, we present evidence that β-catenin fulfils a previously unrecognized function in translational repression.

Response: *We agree with your changes and thank you for making the edits. This revised abstract has been updated in the manuscript text file.*

Referee #3:

The revised manuscript by Ehyai and colleagues has improved and provides additional evidence for a role of the β-catenin:FMRP complex in regulating translational initiation.

Important points that need to be taken into consideration by the authors.

1) As mentioned in my previous report, in order to assess the role of β-catenin:FMRP in the translation repression the authors need to validate the deregulation of some FMRP mRNA targets in presence and absence of β-catenin and FMRP. While the role of FMRP as repressor of translation is not novel, the interaction with β-catenin in repressing translational it is and requires validation.

2) There are no attempts to investigate the mechanism of sequestration of cytoplasmic β-catenin into the nucleus and the de-repression of translation. I appreciate that the authors do speculate in the discussion about a few possibilities but one or two simple experiments would provide an added value to the work.

Comment: 3) Some of the images - including puromycin treated samples- are not of high quality. It might be the resolution of the PDF.

Response: *We have double checked the TIFF files that were uploaded in the most recent submission. The images are all 600 DPI (the journal requires a minimum of 300 DPI), and are of high quality when magnified. The reduced quality may have resulted from the compression of the combined PDF that is created for review purposes. As far as we can determine the TIFF files for the figure mentioned by the reviewer are of high quality.*

Comment: 4) Surprisingly the authors did not discuss the literature on FMRP regulating

translational initiation. Several papers from different labs have clearly helped dissecting the mechanism of FMRP-mediated translational regulation at the level of initiation. Those findings need to be taken into consideration in the discussion.

Response: *We appreciate this comment and have now referred to the literature addressing the role of FMRP on regulating translation initiation in the discussion. The following sentences have been added on lines 455-460 of the text with appropriate references: "A more general mechanism of FMRP mediated translational repression has been implicated in a study in which an FMRP/CYFIP1 complex was shown to bind the translation initiation factor eIF4E [28]. Interestingly, we also identified CYFIP1 in the β-catenin interactome. In addition, FMRP has also been characterized to directly associate with polyribosomes causing reversible ribosome stalling in order to regulate translation [47]."*

References added:

28. Napoli I, Mercaldo V, Boyl PP, Eleuteri B, Zalfa F, De Rubeis S, Di Marino D, Mohr E, Massimi M, Falconi M, *et al.* (2008) The Fragile X Syndrome Protein Represses Activity-Dependent Translation through CYFIP1, a New 4E-BP. *Cell* **134:** 1042-1054

47. Darnell JC, Van Driesche SJ, Zhang C, Hung KYS, Mele A, Fraser CE, Stone EF, Chen C, Fak JJ, Chi SW (2011) FMRP stalls ribosomal translocation on mRNAs linked to synaptic function and autism. *Cell* **146:** 247-261

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: John Mcdermott
Journal Submitted to: EMBO Reports
Manuscript Number: EMBOR-2017-45536

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Three biological replicates were used for cell culture assays and within each assay, each data point represents the mean of triplicate values in the experiments (see individual figure legends for details). For AP-LC-MS/MS experiments, five biological replicates for control and experimental conditions were used. Based on empirical evidence working with these datasets, these n sizes are sufficient for meaningful conclusions to be made.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	NA
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	NA
For animal studies, include a statement about randomization even if no randomization was used.	NA
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	NA
4.b. For animal studies, include a statement about blinding even if no blinding was done	NA
5. For every figure, are statistical tests justified as appropriate?	The means of experiments with two conditions were compared using a T-test (independent samples, two-tailed). The means of experiments with three conditions were compared using a one-way ANOVA, followed by a Tukey post-hoc test.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Replicates were tested for a normal distribution using the Shapiro-Wilk calculation. All conditions were normally distributed, and thus parametric tests were performed, as described above.
Is there an estimate of variation within each group of data?	NA
Is the variance similar between the groups that are being statistically compared?	NA

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C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Catalog numbers are provided for each antibody used.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Catalog numbers for purchased cell lines are listed, and primary cell line preparation protocols are cited. Mycoplasma contamination was tested using Hoechst staining.

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	NA
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	NA
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	NA

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD010421.
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	The raw data set is provided in the source data section as an Excel file.
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	NA