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Decreased synthesis of ribosomal proteins in tauopathy revealed by non-canonical amino acid labelling

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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 17th Dec 2018

Thank you for submitting your manuscript to The EMBO Journal. Your study has now been seen by two referees and their comments are provided below.

Both referees appreciate the approach used and the insights gained into the effects of tau pathology on protein synthesis. However, they also both find that the analysis has to be extended in order to consider publication here. Their concerns raised are clearly outlined below in their referee reports. Should you be able to address the concerns raised then we are interested in considering a revised version.

Please let me know if we need to discuss any specific issues further.

REFEREE REPORTS:

Referee #1:

This is an interesting study addressing the question of whether overexpression of tau-species associated with FTD affects de novo protein synthesis. To this end the authors perform and optimize non-canonical amino acid labelling in tau transgenic (K3 and Tg4510) mice in vivo. AHA labelling leads to the incorporation of azidohomolalanin (AHA) instead of methionine in newly synthesized proteins. Using click chemistry the azide moiety can be either flourescently labelled (FUNCAT technology) or linked to biotin for downstream proteomics analysis. This way Evans et al show that the occurrence of pathological tau isoforms (positive for AT8) is associated with a decrease in protein synthesis, as assessed by FUNCAT staining and subsequent quantification in WB and brain sections. The proteomics analysis identifies several distinct sets of proteins, in particular ribosomal proteins, that are specifically affected in K3 versus WT mice. Lastly, the authors show that newly synthesized ribosomal protein RPL23 is significantly downregulated in brain sections with high AT8 immunoreactivity. They further show that also in frontal cortex sections of FTD patients

RPL23 is significantly decreased.

Although potentially interesting several questions need to be addressed.

Major concerns.

1) The authors state in the discussion that overexpression of pathological tau species leads to a reduction in specific clusters of proteins. The question arises whether overexpression of any aggregation prone protein would lead to similar results or in as much the data set is specific for pathological tau. While they conduct a detailed analysis for K3 mice, the analysis for Tg4510 is very limited (only Figure 2C) and does not include proteomics data. It would greatly strengthen the paper to show also proteomics data from an independent transgenic line such as Tg4510 exhibiting tau pathology. Are similar gene sets affected in the two independent lines?
2) They authors need to specifically assess the de novo protein synthesis e.g.by FUNCAT-PLA of RPL23 in an independent tau line.

3) Figure 1: the authors only analyze AT8 positive tau. Do their findings also hold true for other pathological tau species? Please also include a negative control, e.g. FUNCAT data from brain regions that are largely unaffected by tau pathology in both K3 and Tg4510 mice. Please also indicate how much protein was loaded per lane in the WBs.

4) Figure 2A: the magnitude of changes appears rather small in the volcano blot. Please add a table indicating the fold-changes for all significantly affected proteins and highlight the clusters depicted in Fig3C. I would also find it informative to highlight a few proteins directly in the volcano blot.

5) Regarding all panels showing IHC and quantitative analysis: please indicate in more detail how exactly quantification was done, including e.g. thresholding of background, identification of IHC positive area/neuron counts. Was the analysis performed blind to genotype?

6) Figure 5A, B: the authors need to show a negative control such as PLA analysis without primary Anti-RPL23 antibody.

Minor concern:
7) Figure 5C: is there a mislabeling of the panels? Upper panel is FTD and lower is control?

Referee #2:

COMMENTS EVAN ET AL. MANUSCRIPT

Summary
In the present manuscript, Evans et al. made use of in vivo AHA labelling together with FUNCAT or BONCAT-MS to study the nascent proteome of the K3 transgenic mouse model of tauopathy. The authors report that FUNCAT labelling in brain slices revealed a decrease in protein synthesis in K3 mice compared to WT mice. They also report a correlation between decreased protein synthesis and the presence of hyperphosphorylated tau in K3 mice. Using BONCAT-MS, the authors found 248 of the 763 commonly identified proteins exhibited altered synthesis rates. 47 proteins showed increased synthesis rates and 191 showed decreased synthesis rates, among these were 4 protein constituents of the ribosome. To verify their mass spec findings, the authors performed immunolabelling and FUNCAT-PLA for one candidate ribosomal protein RPL23 and found that total RPL23. as well as newly synthesized RPL23, was decreased in neurons of K3 mice compared to WT mice. In addition, they also found decreased RPL23 expression in brain slices from human FTD patients compared to healthy controls.

The topic is of general interest for the field, and novel is the application of the in vivo AHA labeling in a mouse model of neurological disorders. However, there are several major issues that, in our view, would substantially improve the manuscript:

Major concerns:
1) Focus on ribosomal proteins
The authors strongly focus their analysis and discussion on ribosomes, even though only 18 out of the 80 ribosomal proteins (<25%) were identified in their proteomics data and only 4 ribosomal proteins (<5%) showed significantly decreased synthesis rates in K3 versus WT mice. The low number of regulated ribosomal proteins is also reflected by the fact that ribosomal terms do not appear in the GO enrichment analysis. Based on their proteomics data, it is not clear why the authors only focus on RPL23 for all down-stream experiments. Instead, the authors should discuss in more detail the protein groups that were significantly enriched in the GO analysis. Interesting candidates might actually be proteins which show a regulation opposite to the main trend, for example, the protein with increased synthesis rates in K3 mice. The authors should comment on the identity and function of these proteins. If the authors still decide to analyze ribosomal proteins in K3 mice, a second complementary method should be used to validate the effect on protein synthesis (polysome profiling, puromycylation staining, ...) and more candidates should be tested.
2) Proteomics data:
The analysis of the nascent proteome by BONCAT in combination with MS revealed that 191 proteins exhibited significantly decreased synthesis rates in K3 versus WT mice and the majority of these proteins were less that 2-fold down-regulated. This does not reflect the massive decrease seen by FUNCAT (Figure 2A). The nascent proteomes purified by BONCAT should be validated on a protein gel to confirm the FUNCAT results. One possible explanation for the difference seen by FUNCAT and mass spec is that many of the proteins that show decreased synthesis in K3 mice contribute to the lower FUNCAT signal but cannot be detected by MS due to their low abundance. The authors should provide a list with all identified proteins in K3 and WT mice, respectively, as well as in the PBS control. Those proteins that are exclusively detected in nascent proteomes of either K3 or WT mice might also be interesting candidates. In addition, it would be helpful to present the numbers of identified proteins as a Venn diagram in Figure 3.
By focusing only on the differentially abundant nascent proteome, the authors assume that the total brain proteome does not differ between K3 and WT mice. The authors should confirm whether this is true by measuring the total proteomes and evaluate the changes observed in the nascent proteomes relative to their respective "background" (total proteomes).

3) Imaging
As expected from a transgenic mouse line with a mutated microtubule-associated protein (K3) and as shown by the authors themselves, the neurons from K3 mice show deficits in the cytoskeleton organization (Fig. 3B) and reduction of MAP2 level (Fig.4B). Therefore MAP2 staining should not be used for normalization purposes between K3 and WT mice (e.g. Figure 4). The authors must use another staining that is unaffected in the two conditions.
In Figure 4 and 5, slices were obtained from three animals/patient samples each. Please also specify the number of fields of view (FOV) that were imaged per sample and their selection criteria. In general, multiple FOV should be imaged per sample and image acquisition should be performed blind to the genotype and blind to the channel of interest (e.g. PLA channel in Figure 4).
The images presented in Figure 2A are not representative for the quantification depicted on the right. Please describe in more detail how the images were processed and how the quantification was performed.

Minor concerns:
1) Considering the biological variability seen in Figure 1C, the authors should provide a measure for the biological variation observed in the proteomics data (e.g. a correlation matrix comparing each sample with each other sample).
2) The BONCAT samples were prepared from the whole hemisphere (minus cerebellum), while the imaging data mainly comes from cortex. Please comment.
3) In Figure 1C and 1D, please specify in the figure legend which tissue was used for the western blot.
4) the scatter plots in Figure 2B and Figure 4A (lower plot) show the same negative correlation between the FUNCAT and AT8 signal. Why are the values in the two plots so different?
5) It is not a good idea to combine PLA techniques and total staining using the same antibody. The authors should repeat the FUNCAT-PLA and the total RPL23 staining in separate experiments.
6) AT8 signal is observed in nucleoli of K3 mice (as well as WT) neurons in Figure 4A, but not in Figure 2B. Please discuss this observation.
7) In Figure 1EV and Figure 2EV the authors display protein regulations which are statistically not

significant at the 5% confidence interval ("p>0.05"). Such a presentation is not meaningful. Instead, the authors should give the exact p-values or only show proteins that are significantly regulated at a defined confidence level.

Further comments:
1) The authors should provide a list of all regulated proteins, their fold changes and p-values as a supplementary table.
2) The authors should provide a list of all identified proteins in each sample type (K3, WT and PBS control) as a supplementary table.
3) All MS data should be uploaded to PRIDE and should be publically available upon publication.
4) In the methods section, some information on the BONCAT-MS experiments is missing. Please specify the number of animals that were used for the BONCAT - MS experiments and the number of technical replicates (injections per sample). Please also specify the composition of the loading buffer and which trapping and analytical columns were used. The full parameter set of the LC-MS methods should also be provided as a supplementary table.
5) In Figure 2EV, the authors should also label RPLP0. The yellow and green shade should be removed.
6) Are both images in Figure 5C recorded from similar cortical regions?
7) The manuscript contains several typos in the main text as well as in the figures, some of which are listed below.
o Fig.2A: images for amygdala are inverted
o Fig.2B and 2C: y-axis label "FUNCAT Tau" instead of "FUNCAT"
o Fig.4C: MAP2 data points for 5-month K3 mice are misplaced
o Fig.5C: The labels "Control" and "FTD" are probably inverted.
o Method section: The anti-RPL23 is 16086-1-AP

1st Revision - authors' response 13th Feb 2019

Please see next page.

We would like to thank you and the reviewers for the thorough review of our manuscript. We have done the following major revisions:

- We validated our proteomic results in K3 mice at both 2 and 5 months of age for all regulated ribosomal subunits (RPL23, RPLPO, RPL19 and RPS16) as well as for histone H4 which showed increased synthesis in K3 mice, and synapsin I which had unaltered synthesis;
- We demonstrated that ribosomal protein synthesis is also altered in rTg4510 mice, an independent tau transgenic mouse strain;
- We demonstrated that the total abundance of RPL23 and histone H4 is altered in K3 and rTg4510 mice, as well as human FTD-brains, in accordance with the alterations in synthesis observed in previous experiments;
- We revealed that protein synthesis is decreased in the presence of FTD-tau in the HEK293 cell system.

We believe that by examining the effect of tau on protein synthesis and its machinery using a wide range of methods in two independent tau transgenic mouse strains (K3 and rTg4510), human FTD brains and HEK293 cells, we have robustly demonstrated that pathological tau interferes with the processes of protein synthesis and alters the synthesis of specific sets of proteins.

Please find our point-by-point response below:

Reviewer 1:

Major concerns:
1) The authors state in the discussion that overexpression of pathological tau species leads to a reduction in specific clusters of proteins. The question arises whether overexpression of any aggregation prone protein would lead to similar results or in as much the data set is specific for pathological tau. While they conduct a detailed analysis for K3 mice, the analysis for Tg4510 is very limited (only Figure 2C) and does not

include proteomics data. It would greatly strengthen the paper to show also proteomics data from an independent transgenic line such as Tg4510 exhibiting tau pathology. Are similar gene sets affected in the two independent lines?

We agree that it would be interesting to determine whether overexpression of any other aggregation-prone proteins would lead to comparable changes in *de novo* protein synthesis. We expect that this would depend on whether the protein in question accumulates mainly intra- or extracellularly, and also on the protein itself and where it is normally localized in the cell. Our results are consistent with the emerging realisation in the field that aggregation-prone proteins found in neurodegenerative disorders may alter ribosomal protein levels and the dynamics of translation. Two examples of these are α-synuclein and SMN (survival motor neuron) proteins. Our work represents the first robust demonstration that tau also acts in a similar way and we are the first to observe decreased synthesis of ribosomal proteins. We have added this to the Discussion on page 8.

We used *de novo* proteomic analysis to identify proteins which were altered in synthesis by pathological tau in the K3 mice. However, as pointed out by this reviewer, it was necessary to confirm if these changes were found more broadly in tauopathies or if they were only found in the K3 strain. We therefore validated a number of candidate proteins in two independent tau transgenic strains (K3 and rTg4510) along with human FTD brains.

Using two complementary techniques, FUNCAT-PLA and BONCAT followed by western blotting, we examined the *de novo* synthesis of 5 candidate proteins (RPL23, RPLP0, RPS16, RPL19 and histone H4, the latter showing increased synthesis) in both the K3 and rTg4510 tau transgenic mouse strains. We observed similar changes in both strains compared to wild-type controls, indicating that, as the reviewer postulated, similar sets of proteins are altered in synthesis across these two independent tau lines (Figure 4 and EV 3). This demonstrates that pathological tau alters the synthesis of specific sets of proteins in tauopathy.

To examine whether the effects of tau on protein synthesis were specific to tau carrying the FTD mutation K369I, we transfected HEK293 cells with plasmids expressing either hTau-GFP or K369I hTau-EGFP, using GFP as a control, and examined protein synthesis via FUNCAT. We found that protein synthesis was significantly reduced in cells expressing K369I hTau-EGFP compared to those expressing either hTau-EGFP or EGFP (Figure EV 1).

2) The authors need to specifically assess the de novo protein synthesis e.g. by FUNCAT-PLA of RPL23 in an independent tau line.

Please see our response to Point 1. In the revised manuscript, *de novo* synthesis of five candidate proteins, including RPL23, was assessed in both rTg4510 and K3 mice.

3) Figure 1: the authors only analyze AT8 positive tau. Do their findings also hold true for other pathological tau species? Please also include a negative control, e.g. FUNCAT data from brain regions that are largely unaffected by tau pathology in both K3 and Tg4510 mice. Please also indicate how much protein was loaded per lane in the WBs.

We initially examined AT8 staining as this epitope is strongly phosphorylated in the K3 mice. However, in response to the reviewer's suggestion, we have additionally probed with the AT180 antibody as this epitope is also hyperphosphorylated in these mice. Although less AT180 signal was observed in 5 month-old K3 mice compared to AT8, a negative correlation between AT180 and FUNCAT was also observed, supporting the finding obtained with AT8 (Figure 2 C).

In regards to the reviewer's second point regarding our region of interest analysis, we have now included the striatum, an area that lacks AT8 positivity in the K3 mice. Based on the FUNCAT signal, we observed no significant difference in synthesis between K3 and WT mice, confirming the presence of an inverse correlation between the levels of tau pathology and protein synthesis as found in tau-positive brain areas (Figure 2 A).

Regarding western blotting in both the initial and revised manuscript, BONCAT purification was performed prior to SDS-PAGE and therefore only newly synthesised proteins were loaded onto the blot. We have elaborated on this in the methodology of the revised manuscript on page 10.

4) Figure 2A: the magnitude of changes appears rather small in the volcano blot. Please add a table indicating the fold-changes for all significantly affected proteins and highlight the clusters depicted in Fig3C. I would also find it informative to highlight a few proteins directly in the volcano blot.

We have made the changes suggested by the reviewer. Regarding the magnitude of the fold changes observed in our *de novo* proteomic analysis of K3 compared to WT mice, the average absolute fold change of the significantly altered proteins was 1.6 fold. As noted by reviewer 2, the size of this fold change does not match that seen in our FUNCAT analysis. The most likely reason for this is that the SWATH-MS analysis was performed on BONCAT-purified whole hemisphere lysates. This lysate would therefore contain *de novo* synthesised proteins from all types of cells, including neurons with and without pathological tau, diluting the effect of pathological tau on protein synthesis.

5) Regarding all panels showing IHC and quantitative analysis: please indicate in more detail how exactly quantification was done, including e.g. thresholding of background, identification of IHC positive area/neuron counts. Was the analysis performed blind to genotype?

This information has been added to the revised manuscript. All analysis was performed blinded to genotype.

6) Figure 5A, B: the authors need to show a negative control such as PLA analysis without primary Anti-RPL23 antibody.

In the revised manuscript we have included images taken from a PBS-treated negative controls.

Minor concerns:
7) Figure 5C: is there a mislabelling of the panels? Upper panel is FTD and lower is

control?

This has been corrected.

Reviewer 2:

Major concerns:
1) Focus on ribosomal proteins: The authors strongly focus their analysis and discussion on ribosomes, even though only 18 out of the 80 ribosomal proteins (<25%) were identified in their proteomics data and only 4 ribosomal proteins (<5%) showed significantly decreased synthesis rates in K3 versus WT mice. The low number of regulated ribosomal proteins is also reflected by the fact that ribosomal terms do not appear in the GO enrichment analysis. Based on their proteomics data, it is not clear why the authors only focus on RPL23 for all down-stream experiments. Instead, the authors should discuss in more detail the protein groups that were significantly enriched in the GO analysis. Interesting candidates might actually be proteins which show a regulation opposite to the main trend, for example, the protein with increased synthesis rates in K3 mice. The authors should comment on the identity and function of these proteins. If the authors still decide to analyze ribosomal proteins in K3 mice, a second complementary method should be used to validate the effect on protein synthesis (polysome profiling, puromycylation staining, ...) and more candidates should be tested.

Our *de novo* proteomic analysis revealed a large number of proteins with altered synthesis in K3 compared to WT mice. GO and network analysis revealed that many of these proteins were involved in pathways and neuronal processes that were already well known to be altered in tauopathies (e.g. microtubule and cytoskeleton regulation, mitochondrial function and endocytosis). However, many of these categories have been subject of intensive investigation (albeit in the context of the total proteome), whereas less is known about ribosomal proteins. We focused on these in light of the emerging evidence in the field that ribosomes play an important role in neurodegeneration and that an alteration to ribosomal protein levels is a potential mechanism that might explain the observed decrease in protein synthesis in mouse models of tauopathy.

We identified and validated four ribosomal proteins that were significantly decreased in synthesis in K3 mice compared to WT controls. Given the tight regulation and importance of ribosomal biogenesis, deregulation of even such a seemingly low number of ribosomal proteins is likely to be consequential for the cell. We have addressed this point in our discussion.

We do, however, agree with the reviewer that additional validation of our proteomic analysis would strengthen our data. We therefore examined the synthesis of the ribosomal proteins RPL23, RPLP0, RPL19, and RPS16. As the reviewer suggested, we also examined the synthesis of a protein that showed increased synthesis in the K3 mice, histone H4. We further included synapsin I in the analysis, as this protein exhibited no change in synthesis between K3 and WT mice. Our proteomic data were validated by either BONCAT purification followed by western blotting, or FUNCAT-PLA. This analysis was performed in both K3 and rTg4510 mice (Figure 4 and EV 3). We also examined total levels of RPL23 and histone H4 in K3 and rTg4510 mice, together with human FTD brains. We believe that this examination using a variety of

different methods validates our finding that tau alters the synthesis of ribosomal proteins.

2) Proteomics data:
The analysis of the nascent proteome by BONCAT in combination with MS revealed that 191 proteins exhibited significantly decreased synthesis rates in K3 versus WT mice and the majority of these proteins were less that 2-fold down-regulated. This does not reflect the massive decrease seen by FUNCAT (Figure 2A). The nascent proteomes purified by BONCAT should be validated on a protein gel to confirm the FUNCAT results. One possible explanation for the difference seen by FUNCAT and mass spec is that many of the proteins that show decreased synthesis in K3 mice contribute to the lower FUNCAT signal but cannot be detected by MS due to their low abundance. The authors should provide a list with all identified proteins in K3 and WT mice, respectively, as well as in the PBS control. Those proteins that are exclusively detected in nascent proteomes of either K3 or WT mice might also be interesting candidates. In addition, it would be helpful to present the numbers of identified proteins as a Venn diagram in Figure 3.

By focusing only on the differentially abundant nascent proteome, the authors assume that the total brain proteome does not differ between K3 and WT mice. The authors should confirm whether this is true by measuring the total proteomes and evaluate the changes observed in the nascent proteomes relative to their respective "background" (total proteomes).

As this reviewer correctly points out, the decrease in protein synthesis observed via immunohistochemistry and FUNCAT is larger compared to that observed using BONCAT in combination with SWATH-MS. We believe that a likely explanation for this is that the FUNCAT analysis was performed in brain regions with high tau pathology whereas BONCAT purification was performed on whole brain lysates (minus the cerebellum). This lysate would therefore contain *de novo* synthesised proteins from all types of cells, including neurons with and without pathological tau, diluting the effect of tau on protein synthesis. As suggested by this reviewer, we analysed these BONCAT-purified whole hemisphere lysates via western blotting, with total protein stains showing a similar fold-change, supporting our explanation (Figure 4). We do, however, not discount the possibility that proteins undetected by our SWATH-MS analysis may also contribute to the observed difference in FUNCAT signal. We added a statement about the inherent difference between our FUNCAT and BONCAT results to the discussion in the revised manuscript.

As requested, we have included a list of proteins identified in K3 and WT mice in the acquisition of the 1D IDA ion library, and a Venn diagram of proteins that were uniquely identified in each mouse strain (Figure EV 3B). Proteins uniquely identified in one group via 1D IDA LC MS/MS were still quantified across all 10 samples in our SWATH analysis. Also as requested, we have included a list of all peptides that were identified in our PBS-treated controls and excluded from subsequent analysis.

We agree that it is likely that there will be differences between the total proteomes of K3 and WT mice, and we do not discount that these differences could play important roles in tauopathies. In the revised manuscript we have therefore examined the total abundance of RPL23 and histone H4 in K3 and rTg45410 mice and human

FTD-brains and also discussed possible differences between the *de novo* and total proteome.

3) Imaging
As expected from a transgenic mouse line with a mutated microtubule-associated protein (K3) and as shown by the authors themselves, the neurons from K3 mice show deficits in the cytoskeleton organization (Fig. 3B) and reduction of MAP2 level (Fig.4B). Therefore MAP2 staining should not be used for normalization purposes between K3 and WT mice (e.g. Figure 4). The authors must use another staining that is unaffected in the two conditions.

In Figure 4 and 5, slices were obtained from three animals/patient samples each. Please also specify the number of fields of view (FOV) that were imaged per sample and their selection criteria. In general, multiple FOV should be imaged per sample and image acquisition should be performed blind to the genotype and blind to the channel of interest (e.g. PLA channel in Figure 4).

The images presented in Figure 2A are not representative for the quantification depicted on the right. Please describe in more detail how the images were processed and how the quantification was performed.

In regards to the use of MAP2 as a neuronal marker, we would like to point out that MAP2 was not used to normalise staining because, as pointed out by this reviewer, this would indeed be erroneous. Instead, MAP2 was only used to draw regions of interest around individual neurons. Although MAP2 intensity was decreased in the K3 mice compared to WT controls, we were still able to consistently define clear regions of interest for individual neurons as the brightness and contrast of the MAP2 stain was adapted such that each neuron was clearly visible in each image, regardless of genotype. The validity of this approach was confirmed as there was no significant difference between K3 and WT mice in the average areas of neuronal ROIs drawn using MAP2, indicating no alteration in MAP2 distribution between K3 and WT mice (Figure EV 4B). The drawing of regions of interest, together with all analysis, was performed blinded to genotype. We expanded the description of how the images were prepared and quantified in the methodology section of the revised manuscript.

In regards to the images presented in Figure 2, the monochromatic FUNCAT images were originally obtained by thresholding for FUNCAT-positive areas. However, as pointed out by this reviewer, given that the median FUNCAT intensity was used for quantification, a thresholded image is not representative of its quantification. Therefore in the revised manuscript, we present non-thresholded images.

Minor concerns:
1) Considering the biological variability seen in Figure 1C, the authors should provide a measure for the biological variation observed in the proteomics data (e.g. a correlation matrix comparing each sample with each other sample).

We included this information in Figure 2 EV.

2) The BONCAT samples were prepared from the whole hemisphere (minus cerebellum), while the imaging data mainly comes from cortex. Please comment.

When performing immunohistochemical stainings, we chose to examine the effect of tau on protein synthesis mainly in layer 2/3 of the cortex as this region showed the most robust tau pathology. We had also chosen this region as it is structurally well defined, enabling us to consistently take images in the same brain region across multiple sections and samples. Although the changes we observed using BONCAT were similar to those found by immunohistochemistry, the differences were more apparent in the immunohistochemical analysis. We have commented on this in the discussion of the revised manuscript.

3) In Figure 1C and 1D, please specify in the figure legend which tissue was used for the western blot.

A whole hemisphere (without cerebellum) was used for the BONCAT purification in the optimisation of AHA labelling. We have now included this information in the figure legend.

4) the scatter plots in Figure 2B and Figure 4A (lower plot) show the same negative correlation between the FUNCAT and AT8 signal. Why are the values in the two plots so different?

We believe that the fluorescence intensity values between these two figures should not be quantitatively compared. For all experiments in this study, comparisons were only made between samples which were prepared simultaneously. This is because different materials were used between experiments if required as part of the design of the analysis. For example, in Figure 2, AT8 was detected using the Alexa488 anti-mouse antibody. This, however, was not possible in Figure 4 (now Figure 5 and Figure EV 4 in the revised manuscript) as the Alexa488 anti-rabbit antibody was used to detect RPL23. We therefore used the Alexa405 anti-mouse antibody to detect AT8, resulting in this difference in staining intensity.

5) It is not a good idea to combine PLA techniques and total staining using the same antibody. The authors should repeat the FUNCAT-PLA and the total RPL23 staining in separate experiments.

In the FUNCAT-PLA reaction, the secondary antibody used to detect either RPL23 or histone H4 was added after the FUNCAT-PLA signal had been obtained. The intention of this particular experiment was only to confirm the specificity of the FUNCAT-PLA signal and the total antibody stain was not used for quantification.

6) AT8 signal is observed in nucleoli of K3 mice (as well as WT) neurons in Figure 4A (now Figure EV5A), but not in Figure 2B. Please discuss this observation.

Although there are some reports on nuclear staining of tau, we believe that the discrepancy you refer to may be due to non-specific binding of the secondary antibody as this staining was only observed when using an Alexa405-labelled anti-mouse antibody. This non-specific binding does not affect the interpretation of the results.

7) In Figure 1EV and Figure 2EV the authors display protein regulations which are statistically not significant at the 5% confidence interval ("p>0.05"). Such a presentation is not meaningful. Instead, the authors should give the exact p-values or only show proteins that are significantly regulated at a defined confidence level.

We agree and have altered the figure to only label proteins with a $p \leq 0.05$. We have also included a graph of the fold changes of all identified ribosomal subunits, highlighting those with a $p \leq 0.05$ in Figure EV 2.

Further comments:
1) The authors should provide a list of all regulated proteins, their fold changes and p-values as a supplementary table.

This has been provided.

2) The authors should provide a list of all identified proteins in each sample type (K3, WT and PBS control) as a supplementary table.

This has been provided.

3) All MS data should be uploaded to PRIDE and should be publically available upon publication.

Thank you, yes, this will be made publicly available after upon publication of our manuscript.

4) In the methods section, some information on the BONCAT-MS experiments is missing. Please specify the number of animals that were used for the BONCAT - MS experiments and the number of technical replicates (injections per sample). Please also specify the composition of the loading buffer and which trapping and analytical columns were used. The full parameter set of the LC-MS methods should also be provided as a supplementary table.

We have included these added details in the revised manuscript.

5) In Figure 2EV, the authors should also label RPLP0. The yellow and green shade should be removed.

This shading has been removed as requested. RPLP0 forms part of the lateral stalk of the ribosome and currently there is no published structures of RPLP0 complexed with the 60s and 40s ribosomal subunits. For this reason, RPLP0 is not shown in Figure EV 2.

6) Are both images in Figure 5C recorded from similar cortical regions?

They are.

7) The manuscript contains several typos in the main text as well as in the figures, some of which are listed below.
o Fig.2A: images for amygdala are inverted
o Fig.2B and 2C: y-axis label "FUNCAT Tau" instead of "FUNCAT"
o Fig.4C: MAP2 data points for 5-month K3 mice are misplaced
o Fig.5C: The labels "Control" and "FTD" are probably inverted.
o Method section: The anti-RPL23 is 16086-1-AP

We thoroughly corrected the manuscript for typos for the revised version of the manuscript.

We hope that with our intensive revisions, our manuscript is now suitable for publication in the EMBO Journal.

2nd Editorial Decision 13th Mar 2019

Thank you for submitting your revised manuscript to The EMBO Journal.

Your study has now been re-reviewed by the two referees and their comments are provided below. Both referees appreciate the introduced changes. They raise a number of constructive points that shouldn't involve too much additional work to sort out. Let me know if we need to discuss any of them in further.

When you re-submit would you also take care of the following editorial issues

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REFEREE REPORTS:

Referee #1:

The revised manuscript is much improved. The authors have adequately adressed all my concerns.

Despite this I would like the authors to address one additional point regarding the correlation analysis presented in Fig. 2.They have used two different types of correlation analysis, Spearman´s and Peearson´s correlation for very similar data sets obtained from either K3 or rTg4510 mice. Please comment why this was done.

Referee #2:

Evans et al. made use of in vivo AHA labelling together with FUNCAT or BONCAT-MS to study the nascent proteome of the K3 transgenic mouse model of tauopathy.
In their revised manuscript, the authors present more validation experiments (for four down-regulated ribosomal proteins, one unchanged protein (synapsin I) and one up-regulated protein (histone H4)) to support their mass spec results. These new data improve the quality of the manuscript. There are still a few points that should be addressed by the authors.

1. Increased synthesis/ mass spec signal for histone H4
It is surprising, that histone H4 shows the strongest increase in protein synthesis (highest fold change in mass spec signal) in K3 compared to WT, as histones are generally considered as "static" proteins in the cell. It is assumed that the amount of histones (approximated by the mass spec signal of histones) is proportional to the amount of DNA in a sample, which is proportional to the number of cells in a sample. For this reason, histones were even suggested to be used as "proteomic ruler" to estimate the number of cells in a sample (Wiśniewski et al, Mol Cell Proteomics, 2014). As such it possible that the synthesis rates of histone H4 was constant in the K3 and WT mice, but that more cells were used as input for the K3 sample compared to the WT sample leading to relative higher histone signals? As the protein synthesis rates were decreased in K3 mice (see FUNCAT data), it seems plausible that the total protein amount per cell is decreased in K3 mice and that more cells comprise the input of 250 µg total protein. In that case, the protein intensities could be quantified relative to the histone signal in the respective sample to correct for different numbers of cells in the starting material. By doing so, the quantified proteome would shift towards less synthesis in the K3 mice, which would be consistent with the FUNCAT data.
To rule out the possibility that different numbers of cells were used as input, the authors should report the protein concentrations measured by BCA assay and should also measure the amount of genomic DNA in the inputs.
Has the increased synthesis/ amount of histones described in the context of neurodegenerative disease been observed/reported in the literature? The authors mention that acetylation pattern of histones H4 have been shown to be altered in AD (Lu et al, 2015). Histones are heavily modified. Importantly, differences in the modification of a protein can lead to its mis-quantification, because only the unmodified peptides are typically identified. Could this be the case for histone H4?

The authors used immunolabeling to validate their proteomics data and showed that the histone signal is increased in K3 mice. However, the histone signal does not co-localize with the DAPI stain indicating that the antibody they used is likely not specific for histone H4 and thus should probably not be trusted.

2. Discussion section
The authors need to use much more discretion in their interpretation of the data in the discussion. The authors state that "[they] identified impaired ribosomal protein synthesis as a novel pathomechanism of tau". In fact, the data only shows reduced protein synthesis in the investigated transgenic mouse models of tauopathy. The authors do not present mechanistic data thus no such claims should be made.
They also claim that "[their] results further support the emerging hypothesis that tau interacts with the translational machinery and that this interaction is altered in disease". However, in the current manuscript, the authors do not present evidence for a direct or indirect interaction between tau and the translational machinery. Thus, this statement should be removed.
The authors found "[...] 190 proteins that were significantly decreased in synthesis and 57 proteins that were significantly increased in synthesis in K3 mice compared to their WT littermates." and concluded that "[these] results suggest that, rather than decreasing protein synthesis globally, pathological tau alters the synthesis of specific proteins". Almost one third of the quantified proteins (247 of 762) showed a significant regulation in the K3 mice compared to WT mice. As such, this indicates a global effect rather than a specific regulation of individual proteins. In addition, as the authors describe, the effect of pathological tau is likely underestimated/diluted in the BONCAT results, as the whole brain (minus cerebellum) was used. In contrast, a massive and global decrease in protein synthesis is seen in the FUNCAT data for regions that show high levels of pathological tau.

Minor concerns:
• In the result section, the authors state that "[their] analysis revealed clusters formed by proteins associated with mitochondrial dysfunction, tau interactions, endocytosis and ribosomal formation". We consider the term ribosomal formation misleading, as none of the ribosome biogenesis factors are in the cluster. Often in the manuscript, the authors seem to conclude from the low synthesis rate of 4 ribosomal proteins that ribosome biogenesis is impaired. Direct evidence should be provided before such statement.
• In total, 723 proteins were identified and used for generation of the ion library (venn diagram in Figure EV2), but a higher number of proteins (762 proteins) were quantified in the SWATH data. Please explain the discrepancy.
• For image representation, different color combinations should be used. The current color palette makes it very difficult to evaluate individual signals and to estimate co-localization. Perhaps use grey for the MAP2 signal?
• Please describe in more detail the analysis of protein synthesis in transfected HEK cells. In Fig. EV1 the FUNCAT signal is generally low, both in transfected and un-transfected cells, suggesting that the effect is not due to the expression of K369I hTau, as the authors conclude.

2nd Revision - authors' response 12th Apr 2019

The authors performed all requested editorial changes.

Referee #1:

(1) I would like the authors to address one additional point regarding the correlation analysis presented in Fig. 2.They have used two different types of correlation analysis, Spearman´s and Pearson´s correlation for very similar data sets obtained from either K3 or rTg4510 mice. Please comment why this was done.

Response: We used these two different types of correlation analysis depending on the normality of the data distribution. The widely used Pearson's correlation assumes that the data is normally distributed, and therefore was only used when the data passed normality checks. When data did not pass normality checks, we used a Spearman's correlation instead.

Referee #2:

(1) Increased synthesis/ mass spec signal for histone H4
It is surprising, that histone H4 shows the strongest increase in protein synthesis (highest fold change in mass spec signal) in K3 compared to WT, as histones are generally considered as "static" proteins in the cell. It is assumed that the amount of histones (approximated by the mass spec signal of histones) is proportional to the amount of DNA in a sample, which is proportional to the number of cells in a sample. For this reason, histones were even suggested to be used as "proteomic ruler" to estimate the number of cells in a sample (Wiśniewski et al, Mol Cell Proteomics, 2014). As such it possible that the synthesis rates of histone H4 was constant in the K3 and WT mice, but that more cells were used as input for the K3 sample compared to the WT sample leading to relative higher histone signals? As the protein synthesis rates were decreased in K3 mice (see FUNCAT data), it seems plausible that the total protein amount per cell is decreased in K3 mice and that more cells comprise the input of 250 µg total protein. In that case, the protein intensities could be quantified relative to the histone signal in the respective sample to correct for different numbers of cells in the starting material. By doing so, the quantified proteome would shift towards less synthesis in the K3 mice, which would be consistent with the FUNCAT data.
To rule out the possibility that different numbers of cells were used as input, the authors should report the protein concentrations measured by BCA assay and should also measure the amount of genomic DNA in the inputs.

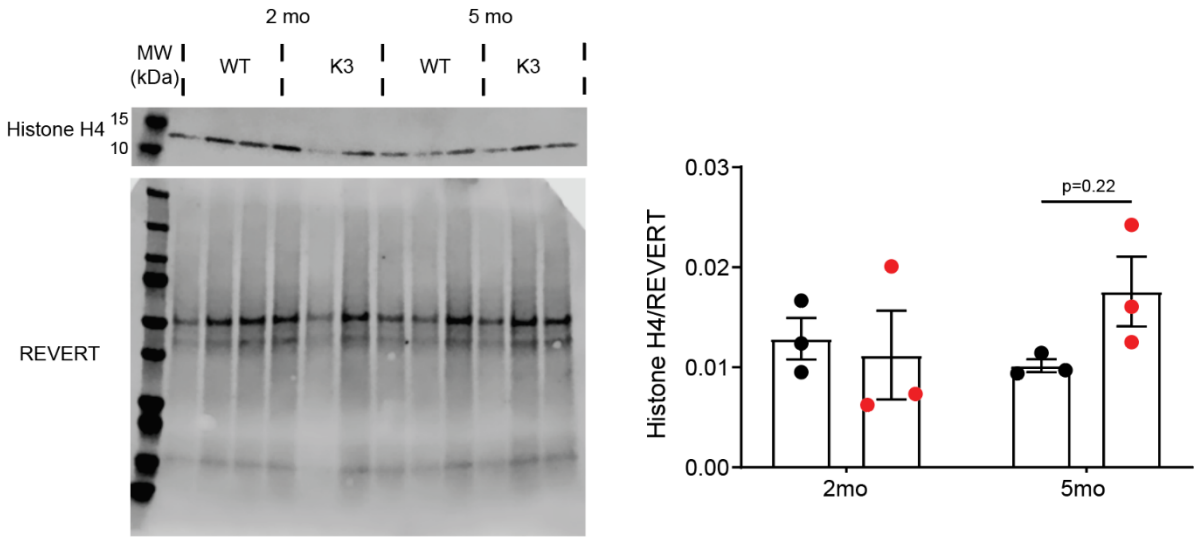
Response: The K3 strain is a mouse strain that does not show overt neurodegeneration, and there is only very limited loss of cerebellar basket cells (van Eersel *et al*, 2010) and neurons in the substantia nigra (Ittner *et al*, 2008), both of which are tiny neuronal populations that do not affect the total cell number. We were therefore initially also surprised by the observed increase in the *de novo* synthesized and total levels of histone H4 and contemplated the explanation proposed by the reviewer for this observation. However, our *de novo* proteomic analysis also revealed that the synthesis of another histone protein, histone H3, was in fact decreased. In the revised manuscript, we have validated this finding using BONCAT-WB in both K3 and rTg4510 mice. This would argue against the hypothesis that the observed increase in histone H4 synthesis in the K3 mice is simply because we had used more cells in the analysis.

(2) Has the increased synthesis/ amount of histones described in the context of neurodegenerative disease been observed/reported in the literature? The authors mention that acetylation pattern of histones H4 have been shown to be altered in AD (Lu et al, 2015). Histones are heavily modified. Importantly, differences in the modification of a protein can lead to its mis-quantification, because only the unmodified peptides are typically identified. Could this be the case for histone H4?

More generally, not only histones, but also ribosomal proteins have been widely considered to be static house-keeping proteins (Zhou *et al*, 2010; Wiśniewski *et al*, 2014; Li *et al*, 2011). However, our results, together with recent studies (Garcia-Esparcia *et al*, 2015; Narayan *et al*, 2015), suggest that this rule does not apply in several forms of neurodegeneration. Indeed, it has been previously observed that total histone H4 levels are increased in post-mortem AD brains compared to healthy controls (Narayan et al., 2015). We have added this information to the discussion in the revised manuscript.

(3) The authors used immunolabeling to validate their proteomics data and showed that the histone signal is increased in K3 mice. However, the histone signal does not co-localize with the DAPI stain indicating that the antibody they used is likely not specific for histone H4 and thus should probably not be trusted.

Response: In the updated manuscript we have increased the contrast and magnification of our histone H4 IHC images (Figures 5 and EV4). These images do now more clearly show that in WT mice and healthy human control brains, the vast majority of staining is confined to the nucleus. We believe that there is an additional cytoplasmic staining in the K3 and rTg4510 mice as well as in human FTD brains. This suggests an interesting partial mislocalisation of histone H4 in primary tauopathies, which will be subject to further investigation in future studies. We have also analysed the total levels of histone H4 by western blotting and were able to detect a trend towards increases in histone H4 in 5 month-old K3 mice compared to age-matched wild-type littermate controls; however, this increase did not reach significance.



Discussion section

(4) The authors need to use much more discretion in their interpretation of the data in the discussion. The authors state that "[they] identified impaired ribosomal protein synthesis as a novel pathomechanism of tau". In fact, the data only shows reduced protein synthesis in the investigated transgenic mouse models of tauopathy. The authors do not present mechanistic data thus no such claims should be made.

Response: In the revised version of our manuscript, we have toned down our statements as requested. We have instead stated that our findings identified a potential novel pathomechanism of tau. We agree with the reviewer that this will need further investigation in future studies to determine if impaired ribosomal protein synthesis drives tau pathology.

(5) They also claim that "[their] results further support the emerging hypothesis that tau interacts with the translational machinery and that this interaction is altered in disease". However, in the current manuscript, the authors do not present evidence for a direct or indirect interaction between tau and the translational machinery. Thus, this statement should be removed.

Response: While tau has been previously demonstrated to interact with some components in the translational machinery, we agree that our study does not provide further evidence for the existence of this interaction. We have therefore clarified our statements as requested, stating that our results support the emerging hypothesis that tau affects components of the translational machinery.

(6) The authors found "[...] 190 proteins that were significantly decreased in synthesis and 57 proteins that were significantly increased in synthesis in K3 mice compared to their WT littermates." and concluded that "[these] results suggest that, rather than decreasing protein synthesis globally, pathological tau alters the synthesis of specific proteins". Almost one third of the quantified proteins (247 of 762) showed a significant regulation in the K3 mice compared to WT mice. As such, this indicates a global effect rather than a specific regulation of individual proteins. In addition, as the authors describe, the effect of pathological tau is likely underestimated/diluted in the BONCAT results, as the whole brain (minus cerebellum) was used. In contrast, a massive and global decrease in protein synthesis is seen in the FUNCAT data for regions that show high levels of pathological tau.

Response: We would like to kindly disagree with the reviewer's assessment of our results. As pointed out by the reviewer, our FUNCAT analysis demonstrated that on average, protein synthesis is decreased in the presence of pathological tau. However, by using FUNCAT it is not possible to determine if this decrease is due to certain proteins being altered in synthesis or just a global decrease in synthesis rates. This was one of the principle reasons why we performed the BONCAT-SWATH-MS analysis. Our *de novo* proteomic analysis revealed that between K3 and WT, approximately 68% (515 of 762 quantified proteins) were not significantly altered in synthesis. The

average fold-change for these proteins was 1.014. This would suggest that the expression of FTD-mutant tau does not decrease (or indeed alter) the synthesis for the majority of proteins. Therefore, our results suggest that instead of having a random effect upon the *de novo* proteome, tau alters the synthesis of specific sets of proteins. This is further supported by our STRING network analysis, in which for ≈69% of the significantly regulated proteins, strong evidence of interaction (STRING score ≥0.7) was observed with at least one other significantly regulated protein, suggesting that tau likely alters the synthesis of proteins associated with distinct pathways. We have discussed this in the revised manuscript.

Minor concerns:

(7) In the result section, the authors state that "[their] analysis revealed clusters formed by proteins associated with mitochondrial dysfunction, tau interactions, endocytosis and ribosomal formation". We consider the term ribosomal formation misleading, as none of the ribosome biogenesis factors are in the cluster. Often in the manuscript, the authors seem to conclude from the low synthesis rate of 4 ribosomal proteins that ribosome biogenesis is impaired. Direct evidence should be provided before such statement.

Response: We have changed “ribosomal formation” or “ribosomes” in this sentence and clarified our statements of ribosomal biogenesis. We have also discussed the possibility that the decreased synthesis of RPL23, RPL19, RPLP0 and RPS16 may alter the subunit composition of ribosomes in tauopathy.

(8) In total, 723 proteins were identified and used for generation of the ion library (venn diagram in Figure EV2), but a higher number of proteins (762 proteins) were quantified in the SWATH data. Please explain the discrepancy.

Response: The differences pointed out by the reviewer are commonly observed in SWATH-MS analysis. The reason for this is that the database used for identifying proteins from the fragment-ion library acquisition is larger than the database used for the SWATH-MS analysis. Therefore, the false discovery rate is higher for proteins identified from the fragment-acquisition, meaning that less proteins are able to be identified with confidence. We have removed figure EV 2B from the manuscript as mapping the peptides identified from the fragment-ion library to proteins is rarely done with SWATH-MS analysis and we believe that the inclusion of this data is misleading.

(9) For image representation, different color combinations should be used. The current color palette makes it very difficult to evaluate individual signals and to estimate co-localization. Perhaps use grey for the MAP2 signal?

Response: We agree with the reviewer that this colour scheme may better illustrate our results and have used it in the revised manuscript.

(10) Please describe in more detail the analysis of protein synthesis in transfected HEK cells. In Fig. EV1 the FUNCAT signal is generally low, both in transfected and un-transfected cells, suggesting that the effect is not due to the expression of K369I hTau, as the authors conclude.

Response: We repeated this experiment, ensuring that all treatment groups had the same confluency when treated with AHA. We have also normalised the FUNCAT signal to the EGFP signal present in each cell. We have described our analysis method in more detail in the manuscript as suggested.

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

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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?
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  - 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?	Power analysis was performed using the means and standand deviation for cortical FUNCAT signal from K3 and WT mice. With an α of 0.05 and a required power of 80%, an n value of 3 for each group was determined to be suitable. This was also the case when using means and standard deviations of total protein stains obtained for BONCAT purifcation of K3 and WT, followed by western blotting
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	Based off our previous power calculation, a n value of 3 was chosen as a minimum number of biological replicates for all experiments. In order to increase the confidence of our proteomics results, an n of 5 was used.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No data was excluded from this study.
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.	WT and K3 mice were injected with AHA in an alternating manner. This was also the case for mouse perfusion.
For animal studies, include a statement about randomization even if no randomization was used.	See above
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.	Image analysis was performed blinded to genotype and sample ID. This was achieved using an in house blinding script.
4.b. For animal studies, include a statement about blinding even if no blinding was done	See above
5. For every figure, are statistical tests justified as appropriate?	Yes
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Where appropriate, normality was tested using the D'Agostino & Pearson normality test and the Shapiro-Wilk normality test.
Is there an estimate of variation within each group of data?	Standard Error of the Mean is given for all bar graphs

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Is the variance similar between the groups that are being statistically compared?	Yes

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7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	This has been done

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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.	This has been done
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	This has been done
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.	We have complied with these guidelines

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	This has been done
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.	This has been done
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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	All proteomic data will be publically avaliable upon publication, or upon request.
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