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PP4-dependent HDAC3 dephosphorylation discriminates between axonal regeneration and regenerative failure

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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 27th Nov 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.

As you can see from their comments the referees find the analysis interesting. However, referee #1 also raises many relevant concerns regarding the quality of the data presented and also with the way the experiments are described. Referee #2 also raise a number of good points that should be addressed.

Should you be able to address the raised concerns in a good manner then I would like to invite you to submit a revised version. I should add that it is EMBO Journal policy to allow only a single major round of revision, and that acceptance of your manuscript will therefore depend on the completeness of your responses in this revised version.

REFEREE REPORTS:

Referee #1:

Hervera et al report that HDAC3 activity limits regenerative capacity in sensory neurons. The authors performed a small-scale pharmacological screen with small molecules targeting key epigenetic enzymes in DRG neurons and show that HDAC3 inhibitor enhances neurite outgrowth on both permissive and inhibitory substrates. They then provide evidence that HDAC3 activity is turned off by calcium dependent dephosphorylation that is induced by peripheral but not central

spinal injury. Bioinformatics analysis of H3K9ac-ChIPseq and RNAseq implicates HDAC3 in multiple regenerative pathways. Finally, they show that genetic or pharmacological inhibition of HDAC3 improves sensory axon regeneration after spinal cord injury. This study as presented is significant, as it demonstrates a pro-regenerative phenotype induced by HDAC3 inhibition in the injured spinal cord. However, the overall quality of the data presented dampens the enthusiasm and lessen the impact. The lack of clarity in how experiments are being done and quantified renders interpretations and conclusions difficult to assess.

Major Points:

Figure 1:
Figure1A. Why was 69A not screened at the same concentrations as the rest? 100 and 200mM only seems inadequate. The data for all drugs listed in Table 1, since listed, should be included in a supplementary figure. Especially since it is stated that EZH2 inhibition also increased neurite outgrowth by more than 2 fold, similarly to HDAC3 inhibition.
Figure 1D: Both 233 and 963 increase H3K9Ac (Supp 1) but have no effect on growth in vitro. This casts some doubt on the role of this pathway. This should be discussed.
Figure 1F: Based on the methods, this experiment was completed using virus. But in the methods it also seems like it may have been done using transfection - either way this should be explained more clearly. Furthermore, a 12 hours time point for this experiment seems very short. HDAC3 needs to be expressed, have its epigenetic effects and those targets have to either be transcribed or turned over. This whole process is likely to take several hours and thus growth phenotypes would take even longer to be observed. If virus and not transfection were used for this experiment, the time line required would be even longer. Thus, the readout of this experiment of 12 in culture doesn't seem feasible.

Figure 6 seems entirely redundant to Figure 5
6A: can be incorporated into another figure, such as 7.
6B: Except for the denoting of "validated genes", this is a repeat of Figure 5F that does not add anything and should be removed. Simply explaining that the qPCR is of HDAC3 genes from 5F is sufficient.
6C: This schematic doesn't add anything and should be removed.

Figure 7 & 8
In all of the SCI images, it is not visually apparent where the injury site is and how it was determined given that GFAP is present throughout the image. Images showing more rostral-caudal regions should make the injury site more apparent as the glial scar borders should be more apparent. This would also allow for measurement of the scar size. This would be interesting since HDAC3 has been implicated in the oligodendrocyte and astrocyte lineage fate switch (Zhang et al 2016) and in inflammatory response of primary microglia (Xia et al 2017). Given that HDAC3 inhibition (Figure 8) appears to have larger effects than the HDAC3 mutant expression in neurons (Figure 7), measuring the scar size would be interesting.

Overall, there is a consistent and frustrating lack of clarity in how experiments are being done and quantified. This makes interpretation and conclusions difficult to assess. Notable examples include:
Fig 1: It is unclear how the authors are quantifying neurite length. Additional explanation as to how they are measuring it and what this metric is (length per neuron, length per initiating neuron, longest axon, etc.) needs to be provided.
Figure 2A-C) Additional information on the quantification is necessary to understand how it was completed. Was pHDAC3 normalized to total HDAC3? In any case overall intensity of pHDAC3 is insufficient, number of neurons having pHDAC3 signal would be more appropriate.
Figure 8 E: How was the quantification carried out and is only neuronal H3K9Ac being analyzed? Visually, it does not look as though the neurons have increased H3K9Ac, but instead that more non-neuronal cells are increasing their H3K9Ac. As above, number of neurons having H3K9ac signal would be more appropriate.

Minor Points:

In all applicable experiments, were transfection/transduction efficiencies assessed? - these needs to be reported.

Fig 1B: The Vehicle PDL/Lam has quite a bit of growth for 12 hours - this is comparable to other reports at 20-24 hours in culture
Scale bars are different between B and F, might be more useful to show same scale to compare the effect of HDAC3 inhibition vs HDAC3 expression.

Figure 4:
A-B: The main text indicates method of transmission as electroporation, should it be nucleofection?
B and H: The images appear to have been cropped at different sizes and then overlayed onto black boxes, presumably to present them all at the same size. Crop originals to the same size instead.

Figure 3 reports measuring calcium levels in whole DRG after SNA or DCA. Since dissection of the tissue represents in itself an axotomy and thus leads to calcium influx, it is unclear how this experiment can work. Is the DRG tissue homogenate used or is a cellular suspension after dissociation used in the assay? What about other cells beyond neurons? Calcium transients also occur in satellite glial cells in models of pain. More details on the methods would be required. Furthermore, how and why would the neuron maintain elevated calcium for 8 h after SNA as reported? Can the neuron even survive this elevated calcium? Maybe other reviewers with more calcium regulation expertise could comment on this point.

Referee #2:

The paper describes a very extensive study that demonstrates a role for HDAC3 in the regenerative response of sensory neurons. The work uses a combination of inhibitors, mutants, genetic and promoter binding screens to make a comprehensive case for HDAC3 as a controller of regeneration. Because of its scope the study is a significant step forwards not only in control of regeneration by histone modification but also in methodology for regeneration studies.
The study is rather complete. Inevitably it is not possible to describe everything in detail in a paper of limited length, but the methods section is mostly good and comprehensive.
1. There are instances where a short term treatment delivered shortly after axotomy has led to long-term changes. This implies that the key events happen at the time that the treatments are operative lead to fairly permanent effects. It would be useful to explain at some points why a particular treatment time was chosen, and to comment on its long-term effects. Examples are the calcium chelation experiment and the RGFP966 injection experiment. It this latter experiment it is not clear how the treatment was delivered- was it a single injection? The assay is 5 weeks later.
2. In the in vivo experiments with genetic and pharmacological inhibition of HDAC3 with assessment of regeneration, if any tissue is available, it would be useful to check for upregulation of a couple of RAGs.
3. In the various diagrams and bar graphs with gene expression and interaction data, it would help readers if known RAGs were identified by colour or backfill.

1st Revision - authors' response 5th Feb 2019

We would like to thank the reviewers for their useful comments that helped us to strengthen our manuscript.

Referee #1:

Hervera et al report that HDAC3 activity limits regenerative capacity in sensory neurons. The authors performed a small-scale pharmacological screen with small molecules targeting key epigenetic enzymes in DRG neurons and show that HDAC3 inhibitor enhances neurite outgrowth on both permissive and inhibitory substrates. They then provide evidence that HDAC3 activity is turned off by calcium dependent dephosphorylation that is induced by peripheral but not central spinal injury. Bioinformatics analysis of H3K9ac-ChIPseq and RNAseq implicates HDAC3 in multiple regenerative pathways. Finally, they show that genetic or pharmacological inhibition of HDAC3 improves sensory axon regeneration after spinal cord injury. This study as presented is significant, as it demonstrates a pro-regenerative phenotype induced by HDAC3 inhibition in the

injured spinal cord. However, the overall quality of the data presented dampens the enthusiasm and lessen the impact. The lack of clarity in how experiments are being done and quantified renders interpretations and conclusions difficult to assess.
We would like to thank this reviewer very much as their comments have allowed to improve our manuscript and to clarify several point.

Major Points:

Figure 1:
Figure1A. Why was 69A not screened at the same concentrations as the rest? 100 and 200mM only seems inadequate. The data for all drugs listed in Table 1, since listed, should be included in a supplementary figure. Especially since it is stated that EZH2 inhibition also increased neurite outgrowth by more than 2 fold, similarly to HDAC3 inhibition.
We have now added a supplementary figure panel as requested (Supp Figure 1A). All drug concentrations were screened based upon the IC50 of the compound. 69A was tested at 100, 200 and 500 nM (not mM as erroneously reported, we apologize for this typo in the previously submitted version). Since 500nM induced cell death, we show the outgrowth data for 100 and 200nM only.

Figure 1D: Both 233 and 963 increase H3K9Ac (Supp 1) but have no effect on growth in vitro. This casts some doubt on the role of this pathway. This should be discussed.
Thanks for this interesting point. This has now briefly been discussed in the resubmitted version (see highlighted text in discussion). Histone deacetylases do control the level of histone acetylation at several overlapping sites. Therefore inhibiting HDAC activity will always lead to increases in histone acetylation at multiple sites compared to vehicle. However, the biological effect of the inhibition of specific HDACs or classes of HDACs are very heterogeneous and at times very different from one another both in terms of gene expression and cell phenotype. These include diverse effects on a variety of cellular processes, such as cell cycle regulation (Telles and Seto, 2012), stem cell differentiation (Hezroni et al, 2011), development (Reichert et al, 2012), and memory and brain function (Guan et al, 2009; Sailaja et al, 2012).
In fact, individual HDACs differently influence the epigenetic and gene regulatory environment by interacting with a different array of transcription factors, histone modifying enzymes and additional epigenetic modifiers such as REST and NCOR among others. A screening with HDAC inhibitors is therefore useful as it is difficult to predict the impact on a specific cell phenotype a priori. Our data suggest that HDAC3 inhibition shapes a favourable transcriptional environment that allows regenerative growth while other HDAC inhibitors tested here do not.

Figure 6 seems entirely redundant to Figure 5
6A: can be incorporated into another figure, such as 7.
6B: Except for the denoting of "validated genes", this is a repeat of Figure 5F that does not add anything and should be removed. Simply explaining that the qPCR is of HDAC3 genes from 5F is sufficient.
6C: This schematic doesn't add anything and should be removed.
Good points raised here, thank you. We agree with Reviewer#1 that some information was redundant in these figures. Following Reviewer#1 comments, 6A has been incorporated in Extended View (EV) Figure 4, which shows ex vivo growth after 966 or AAV-HDAC3mut. In our view this is more appropriate than placing it in Figure 7, which shows *in vivo* axonal regeneration. We agree that the suggested changes will simplify data analysis and consolidate the data. Additionally, panel 5F has been replaced with 6B that contains similar information. Lastly, and as also suggested by Reviewer #1, panel 6C has been removed.

Figure 7 & 8
In all of the SCI images, it is not visually apparent where the injury site is and how it was determined given that GFAP is present throughout the image. Images showing more rostral-caudal regions should make the injury site more apparent as the glial scar borders should be more apparent. This would also allow for measurement of the scar size. This would be interesting since HDAC3 has been implicated in the oligodendrocyte and astrocyte lineage fate switch (Zhang et al 2016) and in inflammatory response of primary microglia (Xia et al 2017). Given that HDAC3 inhibition (Figure

8) appears to have larger effects than the HDAC3 mutant expression in neurons (Figure 7), measuring the scar size would be interesting.
We have now provided revised micrographs to allow a better detection of the borders of the lesion. The borders have been better delineated in Figure 7 and 8 and we consider that they are now clearly visible.
We agree with Reviewer #1 that the scar should be measured. We had indeed measured the scar size following pharmacological inhibition of HDAC3 to find no difference compared to vehicle. Similarly we quantified CD11b immunolabelling that shows no difference between treatment and vehicle. This data was already present in the previous submission and it has now been revised to better depict the scar borders, EV Fig 5.

Overall, there is a consistent and frustrating lack of clarity in how experiments are being done and quantified. This makes interpretation and conclusions difficult to assess. Notable examples include:
Fig 1: It is unclear how the authors are quantifying neurite length. Additional explanation as to how they are measuring it and what this metric is (length per neuron, length per initiating neuron, longest axon, etc.) needs to be provided.
Figure 1F: Based on the methods, this experiment was completed using virus. But in the methods it also seems like it may have been done using transfection - either way this should be explained more clearly. Furthermore, a 12 hours time point for this experiment seems very short. HDAC3 needs to be expressed, have its epigenetic effects and those targets have to either be transcribed or turned over. This whole process is likely to take several hours and thus growth phenotypes would take even longer to be observed. If virus and not transfection were used for this experiment, the time line required would be even longer. Thus, the readout of this experiment of 12 in culture doesn't seem feasible.
Thanks for raising these points. We are grateful as this gives us a chance to indicate timing in culture in the figure legends for each experiment accurately. Apologies for having failed to provide these details!

Indeed Figure 1F reflects neurite outgrowth after viral transduction 36 hours after plating and viral delivery. This has now been amended in the resubmitted version.
Total neurite length was measured and divided by the number of analysed cells. This results in average neurite length per cell. This has now been added in the corresponding methods section where we had included an explanation of neurite length experiments.

Figure 2A-C) Additional information on the quantification is necessary to understand how it was completed. Was pHDAC3 normalized to total HDAC3? In any case overall intensity of pHDAC3 is insufficient, number of neurons having pHDAC3 signal would be more appropriate.
pHDAC3 and HDAC3 antibodies that work well for immunofluorescence are both generated in rabbit and a double immunostaining is not possible. Quantification was carried out by measuring the mean pixel density on selected ROIs (25-30 per sample delimiting the nucleus of neurons ($Tuj1^+$), after background subtraction. Both the mean intensity levels and the percentage of positive cells have been analysed. We have however independently measured expression of HDAC3 (EV Fig 2) and of pHDAC3 including in nuclear vs cytoplasmic extracts (Fig 2). HDAC3 is constitutively phosphorylated in sham/control DRG neurons and it is the axotomy that (via PP4/2) leads to reduced levels of pHDAC3. We have now added the number of neurons having pHDAC3 signal after SNA above the threshold of the lowest detectable signal in sham. No changes in the number of pHDAC3 neurons were found in laminectomy/control and central injury (DCA). All together, we believe we have very strong data supporting changes of pHDAC3 selectively after SNA as shown by multiple lines of evidence with several independent experiments.

Figure 8 E: How was the quantification carried out and is only neuronal H3K9Ac being analyzed? Visually, it does not look as though the neurons have increased H3K9Ac, but instead that more non-neuronal cells are increasing their H3K9Ac. As above, number of neurons having H3K9ac signal would be more appropriate.
Similarly to the point above, quantification was carried out by measuring the mean pixel density on selected ROIs (25-30 per sample for neurons; 45-60 per sample for non-neuronal cells) delimiting the nucleus of neurons ($Tuj1^+$) or non-neuronal (small nucleus from $Tuj1^-$ cells), after background subtraction. We have now added both the mean intensity levels and the percentage of positive cells from both neuronal and non-neuronal cells, the threshold

H3K9ac signal was set at the average intensity on the control group (vehicle) for each cell type. Surprisingly, we do not observe changes neither in the percentage of H3K9ac^{+} nor in the average intensity of non-neuronal cells after 966 treatment as compared to control. We believe this could be due to the higher basal levels of H3K9ac in non-neuronal cells. A complementary explanations might have to do with the unique and distinct epigenetic environments of neuronal vs non-neuronal cells, making them differentially susceptible to HDAC3 inhibition.

Minor Points:

In all applicable experiments, were transfection/transduction efficiencies assessed? - these needs to be reported.
Following Reviewer#1 suggestion has now been added. Please see table here below for the Reviewer's immediate appreciation. This table has been also added in the methods of the resubmitted version of the manuscript.

Viruses	In vitro/In vivo	Efficiency	Related data
AAV-GFP	In vitro	83.28% ± 6.93	Fig1-F; Supp Fig1-C
AAV-HDAC3	In vitro	87.69% ± 5.23	Fig1-F
AAV-HDAC3mut	In vitro	86.65% ± 5.79	Fig1-F; Supp Fig1-C
AAV-V5	In vivo	72.51% ± 8.19	Fig7-A, C, D; Supp Fig8-A
AAV-HDAC3mut	In vivo	71.46% ± 7.92	Fig7-B, E, F; Supp Fig8-A, B

Plasmids/siRNA	In vitro/In vivo	Efficiency	Related data
GFP/scr siRNA	In vitro	36.57% ± 3.34	Fig4-A
GFP/PP4c siRNA	In vitro	32.22% ± 1.92	Fig4-B

Fig 1B: The Vehicle PDL/Lam has quite a bit of growth for 12 hours - this is comparable to other reports at 20-24 hours in culture
This reviewer is correct, this is indeed 24 hours in culture. Please see the response to the comment above where we have addressed the time in culture of each experiment.

Scale bars are different between B and F, might be more useful to show same scale to compare the effect of HDAC3 inhibition vs HDAC3 expression.
This has now been amended in the resubmitted version of the manuscript.

Figure 4:
A-B: The main text indicates method of transmission as electroporation, should it be nucleofection?
Correct, nucleofection is a form of electroporation. Following Reviewer#1 comments, we have now amended as suggested as it is a more specific terminology.

B and H: The images appear to have been cropped at different sizes and then overlayed onto black boxes, presumably to present them all at the same size. Crop originals to the same size instead.
These images have not been cropped at different sizes, they have been simply cropped and rotated and black boxes have been overlaid for stylistic/aesthetic purposes. We have respected the size and kept it comparable.
Figure 3 reports measuring calcium levels in whole DRG after SNA or DCA. Since dissection of the tissue represents in itself an axotomy and thus leads to calcium influx, it is unclear how this experiment can work. Is the DRG tissue homogenate used or is a cellular suspension after dissociation used in the assay? What about other cells beyond neurons? Calcium transients also occur in satellite glial cells in models of pain. More details on the methods would be required. Furthermore, how and why would the neuron maintain elevated calcium for 8 h after SNA as reported? Can the neuron even survive this elevated calcium? Maybe other reviewers with more calcium regulation expertise could comment on this point.
Thank you for allowing this clarification. The methodology has been described in the paragraph "Ex vivo calcium assay", which has now been clarified. In order to minimize the

quick calcium influx responses caused by dissection, each dissected DRG was immediately transferred into pre-chilled Eppendorf tube on dry ice and flash frozen per collection. DRG homogenate was used for calcium assay and calcium concentration was normalized by protein concentration of each sample after BCA assay.

While these samples do contain satellite cells, it is likely that within the short time frame of several hours, changes in calcium reflect modifications in post-injury calcium signalling in neurons. Typical signalling in satellite cells is activated at much later time points. As shown in Figure 3C, DRG do not show persistent elevated levels of calcium. In fact, calcium is unchanged 2 hours post-injury, it is elevated at 8 hours and it declines again at 24 hours. Therefore, this transient increase in calcium is likely important to trigger signalling pathways including the one shown in this paper (PP4/2) and as shown by others (Elziere et al. 2014; Cho et al. 2015), but it is unlikely to be toxic to the cells. Indeed, DRG cells do not show any sign of toxicity in these injury experiments.

Referee #2:

The paper describes a very extensive study that demonstrates a role for HDAC3 in the regenerative response of sensory neurons. The work uses a combination of inhibitors, mutants, genetic and promoter binding screens to make a comprehensive case for HDAC3 as a controller of regeneration. Because of its scope the study is a significant step forwards not only in control of regeneration by histone modification but also in methodology for regeneration studies.
The study is rather complete. Inevitably it is not possible to describe everything in detail in a paper of limited length, but the methods section is mostly good and comprehensive.

We are very grateful to this reviewer who has appreciated the extent of the effort we have put in our studies and the overall advance and quality of our work
1. There are instances where a short term treatment delivered shortly after axotomy has led to long-term changes. This implies that the key events happen at the time that the treatments are operative lead to fairly permanent effects. It would be useful to explain at some points why a particular treatment time was chosen, and to comment on its long-term effects. Examples are the calcium chelation experiment and the RGFP966 injection experiment. It this latter experiment it is not clear how the treatment was delivered- was it a single injection? The assay is 5 weeks later.

This is a very interesting point. The model as summarised in the summary cartoon includes early changes in calcium levels following peripheral but not central axotomy that lead to activation of PP4/2. This happens in the first several hours after injury, hence the post-injury early timing of calcium chelation experiments. These early signals lead to dephosphorylation of HDAC3 resulting in inhibition of HDAC3 activity that in turn increases histone acetylation in DRG neurons. The in vivo RGFP966 delivery experiments were not a single injection. They were conducted by intrathecal administration of RGFP966 through osmotic minipump for 14 days (Figure 8 and legend). This led to longer-lasting changes in histone acetylation.

2. In the in vivo experiments with genetic and pharmacological inhibition of HDAC3 with assessment of regeneration, if any tissue is available, it would be useful to check for upregulation of a couple of RAGs.
We have indeed measured the expression of several RAGs to find that their expression is enhanced after pharmacological inhibition of HDAC3 (Figure 6). Additionally, EV Fig 4 shows that these several RAGs display and increase in promoter acetylation following pharmacological HDAC3 inhibition.
3. In the various diagrams and bar graphs with gene expression and interaction data, it would help readers if known RAGs were identified by colour or backfill.
Following Reviewer#2 comment, we have modified the network diagram in Figure 5F as suggested.

2nd Editorial Decision 6th Mar 2019

Thank you for submitting your revised manuscript to the EMBO Journal. Your study has now been seen by referee #1 whose comments are provided below.

The referee appreciates that parts of the analysis has been improved, but there are still issues with the quality of the data and how it is presented that would have to be sorted out in order to consider publication here.

I also want to make a comment regarding Figure 4B. I don't think it is a good way to present the data by adding the image on a black background simply for stylistic reasons. We need the data presented as it was captured so that a better comparison can be made. Also, to me it looks like the images were cropped at different sizes as initially pointed out by the referee - please see attached screen shot. A similar issue goes for Fig S4. Would you also please make sure that the magnification boxes in 7 G-I matches with the image where they were taken from.

REFEREE REPORTS:

Referee #1:

The authors have addressed some of the concerns raised by the reviewers. For example, the quality and presentation of SCI images in Figure 7 and 8 have improved, the condensation of Figure 5 and 6 have also improved clarity of the presented results. But overall there still remains some quality concerns regarding how experiments were performed and quantified, which still dampens the enthusiasm for an otherwise interesting and potentially impactful manuscript.

Specific concerns:

This reviewer previously indicated that for the adult DRG in vitro growth assays, which were reported to be fixed 12 hours after plating, the growth in Fig 1B seemed too high and the time course for viral transduction in Fig. 1F was not feasible. The authors response is that the time course was simply not 12hr, but instead 24 and 36 hours for 1B and 1F, respectively.
The neurite length of the vehicle control on PDL-lam in 1B,C after 24 h in culture is around 300um, but in 1F,G the neurite length in AAV-GFP control on PDL-lam after 36 h in culture is 150um. Can the author comment on the smaller neurite length after longer time in culture?

This reviewer also previously suggested using images at the same scale throughout Fig 1B and 1F to make it easier for the reader to compare the effect of HDAC3 inhibition to HDCA3 dominant negative expression. The authors indicate in their response that they have corrected this, which they did, but by adjusting the scale bars rather than changing the images to present them at the same magnification. The scale bar in 1F used to be 50um, it is now twice the size and reported as 100um. The bottom panel of 1B (myelin) also now reports a scale bar of 100um (which was not present in the original submission), but the cells in 1B looks much larger than in 1F, despite identical scale bars.

This reviewer asked for clarification regarding the quantification of pHDAC3 (Fig 2A) and H3K9Ac (Fig 8E). Based on the authors explanations it remains unclear how the quantification can be done. Mainly, the authors do not indicate using a nuclear counterstain (e.g., DAPI) to identify Tuj1+ and Tuj1- nuclei. How can they identify all of the non-neuronal nuclei without a nuclear marker and quantify % of positive cells in Fig 2C, Fig 8G.

Further, Fig 8E-G provides images and quantification of TUJ1+ neurons. These images look more similar to a staining in Fig 6 that look at NF200+ neurons, as all positive neurons have very large cell diameters and there are considerable gaps between the labeled neurons. The staining in Fig 8 is more consistent with the NF200 staining as presented in Fig 6 than the TUJ1 staining presented in Fig2. Much of the H3Kac positive staining that does not overlap with the green marker have nuclear size more consistent with neuronal nuclei.
The cropping of images in Figure 4 has been addressed in the authors' response, who state that

"images have not been cropped at different sizes", but it remains that in 4B the green and red channels are not cropped similarly with respect to the position of the neuronal cell soma.

2nd Revision - authors' response 12th Mar 2019

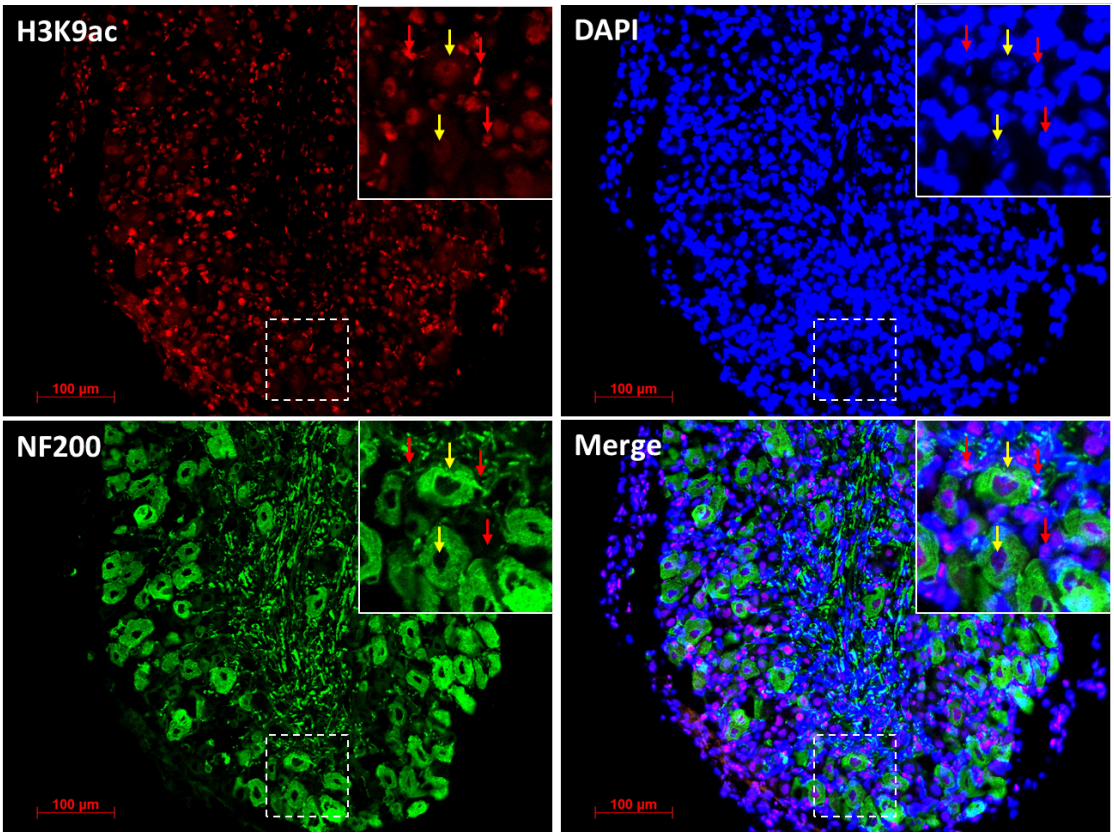
Referee #1
I would like to thank again this reviewer for the careful review (much appreciated), which have been very helpful to improve our manuscript and avoid inaccuracies.

Any change in the manuscript is now in blue.

The neurite length of the vehicle control on PDL-lam in 1B,C after 24 h in culture is around 300um, but in 1F,G the neurite length in AAV-GFP control on PDL-lam after 36 h in culture is 150um. Can the author comment on the smaller neurite length after longer time in culture?
We thank the reviewer for this question. Indeed, neurite length of the vehicle control on PDL-lam in 1B,C after 24 h in culture is around 300 vs 150µm after AAV transfection on the same substrates in 1F,G. This is likely due to the fact that cells in 1F,G were transfected with AAV at the time of plating. In our hands, viral transfection typically leads to slower growing neurites as opposed to naïve conditions such as in 1B,C. Additionally, although we strive for consistency when preparing cultures for neurite outgrowth assays, it is always difficult to directly compare neurite outgrowth between experiments done at different times. In fact, individual growth media batches and small differences in cell density for example, further contribute to variability in biological outcomes such as outgrowth.

*This reviewer also previously suggested using images at the same scale throughout Fig 1B and 1F to make it easier for the reader to compare the effect of HDAC3 inhibition to HDCA3 dominant negative expression. The authors indicate in their response that they have corrected this, which they did, but by adjusting the scale bars rather than changing the images to present them at the same magnification. The scale bar in 1F used to be 50um, it is now twice the size and reported as 100um. The bottom panel of 1B (myelin) also now reports a scale bar of 100um (which was not present in the original submission), but the cells in 1B looks much larger than in 1F, despite identical scale bars. Although scale bars are not identical, it would be wiser at this stage to provide the high mag images on 1F comparable to 1 A and B (for PDL and Myelin)***.***
We apologize for misunderstanding the reviewer's query in the first place. We have now changed the panels in order to show all of them at the same magnification for better comparison as suggested.

This reviewer asked for clarification regarding the quantification of pHDAC3 (Fig 2A) and H3K9Ac (Fig 8E). Based on the authors explanations it remains unclear how the quantification can be done. Mainly, the authors do not indicate using a nuclear counterstain (e.g., DAPI) to identify Tuj1+ and Tuj1- nuclei. How can they identify all of the non-neuronal nuclei without a nuclear marker and quantify % of positive cells in Fig 2C, Fig 8G.
We thank the reviewer for this comment. ***We did use DAPI to identify nuclei (please see an unedited example image here below, yellow arrows mark neuronal cells, red arrows non-neuronal cells). This has now been clearly stated in the methods and legends.***



Further, Fig 8E-G provides images and quantification of TUJ1+ neurons. These images look more similar to a staining in Fig 6 that look at NF200+ neurons, as all positive neurons have very large cell diameters and there are considerable gaps between the labeled neurons. The staining in Fig 8 is more consistent with the NF200 staining as presented in Fig 6 than the TUJ1 staining presented in Fig2. Much of the H3Kac positive staining that does not overlap with the green marker have nuclear size more consistent with neuronal nuclei.

We thank the reviewer for this observation, as indeed we mislabelled the panel. This tissue was immunostained with NF200, not Tuj1, this has now been amended it. We apologize for the mistake.

The cropping of images in Figure 4 has been addressed in the authors' response, who state that "images have not been cropped at different sizes," but it remains that in 4B the green and red channels are not cropped similarly with respect to the position of the neuronal cell soma.

We did address a similar issue in fig 4H in the previous revision, but missed to amend fig 4B, we apologize for this. Since only a few cells are transfected per sample and they are often surrounded by untransfected cells, making it hard at times to fully appreciate the cell of interest, cropping images is necessary- however we agree with the reviewer as we did now notice that cropping was not accurate across samples. In order to have a better comparison between fig 4A and B we have revised the representative panels that now contain comparable cropped images of the same size.

3rd Editorial Decision 11th Apr 2019

Thanks for submitting your revised manuscript to the EMBO Journal. Your study has now been re-reviewed by referee #1 and as you can see below the referee is happy with the introduced changes. I am therefore very pleased to let you know that we will accept your manuscript for publication here. Before I can send you the formal accept letter there are just a few editorial things to sort out.

REFEREE REPORT:

Referee #1:

The authors have adequately addressed this reviewers' latest comments

3rd Revision - authors' response 15th Apr 2019

The authors performed all requested editorial changes.

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: Simone Di Giovanni
Journal Submitted to: EMBO Journal
Manuscript Number: EMBOJ-2018-101032

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 χ2 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?	Sample size was chosen in order to ensure a power of at least 0.8, with a type I error threshold of 0.05, in view of the minimum effect size that was looked for.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	For all experiments involving animals, sample size was first estimated in order to ensure a power of at least 0.8, with a type I error threshold of 0.05, in view of the minimum effect size that was looked for.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	For all experiments, global outliers (data points that exceeded three standard deviations or more from the mean) were excluded from the data sets. This exclusion criteria was preestablished for all experiments.
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.	For all experiments, all samples were randomly assigned to the experimental group.
For animal studies, include a statement about randomization even if no randomization was used.	For all experiments involving animals, all animals were randomly assigned to the experimental group, with a 50-50% criteria to assign males and females
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.	For all experiments, analysis was performed blind to the experimental groups.
4.b. For animal studies, include a statement about blinding even if no blinding was done	For all animal studies, surgeries, treatments and behavioral assessments were performed blind to the experimental group whenever possible.
5. For every figure, are statistical tests justified as appropriate?	Normality of the distributions was checked via Shapiro-Wilk test, asterisks indicate a significant difference analyzed by ANOVA with Bonferroni post-hoc test or Student's t-test as indicated (* p<0.05; ** p<0.01; *** p<0.005; **** p<0.001). All tests performed were two-sided, and adjustments for multiple comparisons and/or significantly different variances (Fisher's F) were applied were indicated. All data analysis was performed blind to the experimental group.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	
Is there an estimate of variation within each group of data?	

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tun

http://datadryad.org

http://figshare.com

http://www.ncbi.nlm.nih.gov/gap

http://www.ebi.ac.uk/ega

http://biomodels.net/

http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

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

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).	HDAC3 (Abcam, ab7030, Ms, WB/IHC/ICC/IP), pHDAC3 (Cell Sginaling Technology (CST), #3815, Ms, WB/IHC/ICC), H3K9ac (CST, #9649, Ms, WB/IHC/ICC), Histone 3 (CST, #7915, Ms, WB), ERK (CST, #9102, Ms, WB), pERK (CST, #9101, Ms, WB/IHC), PP4c (C-6)(Santa Cruz, sc-374106, Ms, ICC), PP2A-Cα/β (1D6) (Santa Cruz, sc-80665, Ms, WB), GAPDH (14C10) (CST, #2118, Ms, WB), βIII Tubulin (5G8) (Promega, G7121, Ms, IHC/ICC), GFP (Abcam, ab13970, IHC/ICC), V5-tag (Millipore, AB3792, IHC/ICC), p-Stat3 (CST, #9145, Ms, IHC), c-Jun (CST, #9165, Ms, IHC), ATF3 (Santa Cruz, sc-188, Ms, IHC), IGF1R (CST, #3027, Ms, IHC), Myc (Sigma, M4439, Ms, IHC), NF200 (Sigma, N4142; Sigma, N0142, MS, IHC), GFAP (Millipore, AB5804, Ms, IHC), CD11b (Millipore, CBL1313, Ms, IHC), VGLUT1 (Synaptic Systems, 135 302, Ms, IHC), H3K9ac (AH3-120) (Abcam, ab12179, Ms, IHC)
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	No cell lines were used

* 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.	Wild-type C57Bl6/J (Harlan) mice ranging from 6 to 8 weeks of age were used for all experiments. Male and female were used in a 50-50% basis for each study.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	All animal procedures were approved by Imperial College London ethic committee, and were performed in accordance with the UK Animals Scientific Procedures Act (1986).
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.	All animal procedures were subjected to ARRIVE guidelines.

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	A data availability section has been added to the methods:RNAseq and H3K9ac ChIPseq data have been deposited at the Gene Expression Omnibus (GEO) with accession codes: GSE97090 for RNAseq, https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=anmdoqiodzgfvat&acc=GSE97090) and GSE108806 (exwheyyitzqlpoz) for ChIPseq.
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).	RNAseq and H3K9ac ChIPseq data have been deposited in the Gene Expression Omnibus (GEO) with accession codes: GSE97090 for RNAseq, https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=anmdoqiodzgfvat&acc=GSE97090) and GSE108806 (exwheyyitzqlpoz) for ChIPseq.
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