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A PBX1 transcriptional network controls dopaminergic neuron development and it is impaired in Parkinson's disease

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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 05 February 2016

Thank you for submitting your manuscript to The EMBO Journal. Sorry for the delay in getting back to you, but due to the Xmas holidays things got a bit delayed. I have now received the comments from the two referees that you will find enclosed.

As you can see below, the referees find the analysis interesting and suitable for consideration here. However they also raise a number of issues that should be sorted out before publication here. The regulation of Pbx1 target genes need to be better supported by additional data and the same goes for a protective role of Nfe2l1 during oxidative stress. Should you be able to extend the analysis along the lines as indicated by the referees then I would like to invite you to submit a suitable revised manuscript. I should add that it is EMBO Journal policy to allow only a single major round of revision and that it is therefore important to resolve the concerns raised at this stage.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: http://emboj.embopress.org/about#Transparent_Process

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

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REFEREE REPORTS

Referee #1:

The manuscript by Villaescusa et al addresses the function of Pbx1 in developing midbrain dopaminergic (mDA) neurons. It starts by using mouse genetics to show that Pbx1 is required for proper differentiation and survival of this neuronal population. These findings are complemented with the characterization of transcriptional targets of Pbx1 by combining ChIP-seq from mouse embryonic chromatin, with expression profiling by RNA-Seq of distinct midbrain cell populations. The authors conclude that Pbx1 functions both as an activator and repressor, and generate a list of Pbx1 targets that include genes expected to regulate the differentiation of mDA neurons (Pitx3), the repression of alternative cell fates (Onecut2) or protect against oxidative stress (Nfe2l1, aka Nrf1). They develop this last point by showing that knocking down Nfe2l1 in mDA cells in culture decreases survival upon oxidative stress. In addition, the analysis of brain samples from PD patients show reduced expression of Pbx1 and Nfe2l1 in neuromelanin neurons of the Substantia Nigra (SN), and suggest that downregulation of this pathway could be of importance in PD.

The identification of novel regulators of development and survival of mDA neurons is of great interest, given its potential use in the development of cell replacement strategies, but also as it may contribute to a better understanding of the etiology of the disease. In that sense the work here provided if of potential relevance to the field. The strong point is that it is the first report that uses extensively mouse genetics to dissect out the function of Pbx1 quite convincingly, circumventing the problem of redundancy amongst Pbx family members. Also, it is to the best of my knowledge the first time that Nrf1 deregulation is shown in the midbrain of PD patients, although oxidative stress in PD has been extensively studied. The manuscript has however several weaknesses, mostly concerning the regulation and function of Pbx1 target genes (see below).

Previous studies (properly cited by the authors) have already characterized the expression of Pbx1 in developing mDA neurons. In addition, a very mild phenotype of Pbx1 null embryos in mDA neurons has previously been reported by Sgado et al (Neural Dev. 2012 Jul 2; 7:24). The authors cite this article ("expression of Pbx genes has been detected in the mammalian midbrain and mDA neurons") while not mentioning the initial description of the Pbx1 null embryos that it describes. Moreover, the expression of Pbx1 in the adult mouse Substantia Nigra and the conserved expression in ventral midbrain of human embryos has already been reported by Ganat et al (J Clin Invest. 2012 Aug 1; 122(8): 2928-2939), and are thus not novel.

An important part of the work consists of the identification of Pbx1 direct target genes. The ChIP-seq data could be better presented, as it is difficult to evaluate its quality on its present form. ChIP-seq enrichment profiles at representative genomic regions (namely at the targets discussed) should be presented. In addition, a larger scale validation of Pbx1 bound regions by ChIP-PCR should be performed.

The identification of a Pbx1 binding motif is described, and used to support the ChIP-seq data. However no numbers or statistics associated with this observation are shown.
The authors suggest a dual role for Pbx1 in activation and repression of gene expression. Although the molecular basis for Pbx1 function is not a major focus of the manuscript, the authors could further explore their data. For example, are there additional motifs present in Pbx1 bound regions associated with activated versus repressed genes? This should be looked at, and discussed.

The case for the identification of Nfe2l1 (aka Nrf1) as a target of Pbx1 is poor. The regulation data shown derived from overexpression in a cell line (Figure 6B) is not convincing, and further evidence based on gain or loss-of-function of Pbx1 is required. As it was done for two other targets, Nf2l1 expression should be analyzed in Pbx1/3 mutants. This is particularly important, if one wants to establish the link with the decreased survival observed in the compound mutants.

The experiment described in Figure 6D is aimed at investigating "whether Nfe2l1 plays a role in preventing oxidative stress in human mDA cells". From the data presented, it is not possible to conclude that the increase in Casp3 expression occurs in fully differentiated mDA neurons, as

opposed to any other cell type present in the culture. In addition, the quantification of the shRNA mediated knock-down shown in Figure EV11 is very mild. The results must therefore be corroborated with a second shRNA virus, in order to minimize the possibility of occurrence of off-target effects.

The reduction of Pbx1/Nfe2l1 in NM+ neurons from PD patients presented in Figure 6 is quite relevant. I understand the difficulty of obtaining material for these experiments, however results shown in Figure 6E should be properly quantified. In addition, the data showing the specificity of these observations to NM+ neurons are as far as I can see based on one single sample (Figure EV11) and require further work.

Referee #2:

In this study, Villaescusa et al. identifies Pbx1 transcription factor as a crucial intrinsic determinant for midbrain dopaminergic neuronal specification during development. Inactivation of Pbx1 and its close homolog Pbx3, leads to severe loss of mDA neurons. In addition, they carried out a ChIP-Seq analysis using fetal midbrain tissue to identify the Pbx1 genomic binding occupancy and its direct target genes, among which Pitx3 is a well-known determinant of mDA neuronal identity. A second Pbx1 direct target results Nfe2l1 (Nrf1), one of the activators of the cell antioxidant response, whose silencing in human NES-derived mDA neurons is detrimental for their survival in oxidative stress conditions. Interestingly, both Pbx1 and Nfe2l1 are expressed in adult human substantia nigra and are downregulated in tissues from Parkinson's disease patients. This is a relevant set of findings that identify for the first time the function and downstream effectors of a new key factor in mDAn specification and survival. Thus, these results place Pbx1 within the transcription factor regulatory network which controls mDAn genesis upstream to Pitx3. Few but important points need to be addressed as below:

- Loss of mDA neurons in Pbx1/Pbx3 double mutants is analyzed exclusively with TH staining Pbx1/Pbx3 double mutants. It remains unclear how the neurons in SN and VTA are differentially affected. The use of mDA neuronal subtype markers such as Otx2, Calbindin, Sox6 or GIRK2 would help to clarify this aspect.

- To better define the magnitude of mDA neuronal fiber loss in the Pbx1/Pbx3 double mutants, it would be very informative the immunohistochemistry (DAB staining) for TH in the striatum which represent the main target of the DA projections.

- The increase in aCASP-3 staining at E18.5 is limited in the mutants questioning whether TH expression lost is exclusively caused by cell death. On this regard, it would be helpful to repeat this staining at E14.5, a stage of high neurogenesis, to verify whether cell death is enriched at earlier time points. Alternatively, it should be considered that cell identity misspecification could occur as well. Thus, a staining for alternative neuronal cell types like 5-HT could address this eventuality.

- In order to better appreciate the protective role of Nfe2l1 during oxidative stress in NES-derived mDA neurons, it would be relevant to evaluate if the overexpression of this gene can rescue cell death in oxidative stress conditions.

1st Revision - authors' response 21 April 2016

Referee #1:

1) <u>Previous studies</u> (properly cited by the authors) have already characterized the expression of Pbx1 in developing mDA neurons. In addition, a very mild phenotype of Pbx1 null embryos in mDA neurons has previously been reported by Sgado et al (Neural Dev. 2012 Jul 2; 7:24). The authors cite this article ("expression of Pbx genes has been detected in the mammalian midbrain and mDA neurons") while not mentioning the initial description of the Pbx1 null embryos that it describes. Moreover, the expression of Pbx1 in the adult mouse Substantia Nigra and the conserved expression in ventral midbrain of human embryos has already been reported by Ganat et al (J Clin Invest. 2012 Aug 1; 122(8): 2928-2939), and are thus not novel.

The first paragraph of the introduction has now been modified in order to incorporate these suggestions. We mention the five articles that to our knowledge describe *Pbx* expression in midbrain dopaminergic neurons in mouse and humans.
We agree that PBX-immunoreactivity has been previously detected in the mouse and human midbrain, although our study is the first to validate PBX1, PB1A, PBX1B and PBX3 antibodies in ventral midbrain tissue in KO sections.
Ganat et al., described a diffuse nuclear and perinuclear PBX1 staining throughout the three layers of the ventral midbrain (from ventricular to marginal zone), which does not match the cell-type specific expression in the intermediate and marginal zones that we describe, which we find to be very similar between mouse and human development. Differences in the concentration of primary and secondary antibodies, blocking reagents and antigen retrieval methods, among other things, could explain these differences.
Another difference with previous studies (Sgado et al. 2012; Thompson et al. 2006) is that they used a pan-PBX antibody that identifies all PBX members (PBX1-4) and their different isoforms. These studies could not resolve which member of this family of transcription factors is actually present in the dopaminergic lineage.
Finally, our study shows co-expression of PBX1 with other markers (LMX1A, NURR1, PITX3 and TH) and carefully analyses the temporal pattern of expression. Indeed, our immunofluorescence analysis identifies that the first PBX1+ cells appear at E10, and that PBX1 is present in a subpopulation of NURR1+ neuroblasts, which has not been previously described. Moreover, we identify PBX1A as the isoform present in the ventral midbrain, which was not previously known. We thus think that our analysis of PBX family members by *in situ* hybridization and immunohistochemistry reveal important new information about these transcription factors.

2) An important part of the work consists of the identification of Pbx1 direct target genes. The ChIP-seq data could be better presented, as it is difficult to evaluate its quality on its present form. ChIP-seq enrichment profiles at representative genomic regions (namely at the targets discussed) should be presented.
Following the suggestion from this reviewer, we now show the enrichment profiles in Fig 5B and EV4E. Former Fig 5B is now shown in EV4F.

In addition, a larger scale validation of Pbx1 bound regions by ChIP-PCR should be performed.
We have also performed a large-scale validation of PBX1 bound regions by ChIP-PCR. Our analysis includes all identified regions with p-values equal to, or greater than, 10^{-3}. We have thus performed ChIP-PCR on a total of 18 putative target genes, which are now shown in Fig 5E and EV6B.

3) The identification of a Pbx1 binding motif is described, and used to support the ChIP-seq data. However no numbers or statistics associated with this observation are shown.
Statistical E-value is now shown in Fig 5A (E-value = $1.1E^{-55}$).

The authors suggest a dual role for Pbx1 in activation and repression of gene expression. Although the molecular basis for Pbx1 function is not a major focus of the manuscript, the authors could further explore their data. For example, are there additional motifs present in Pbx1 bound regions associated with activated versus repressed genes? This should be looked at, and discussed.
We thank reviewer #1 for these suggestions. We have now included in Fig EV5 de novo motif analysis that has focused on the 200bp regions which are centered on PBX1 peaks to identify additional motifs. Moreover, we also now show the analysis of the promoter regions of PBX target genes for transcription factor binding site enrichment. All these results are described in pages 5-6 and shown in a new figure, Fig EV5.

4) The case for the identification of Nfe2l1 (aka Nrf1) as a target of Pbx1 is poor. The regulation data shown derived from overexpression in a cell line (Figure 6B) is not convincing, and further evidence based on gain or loss-of-function of Pbx1 is required. As it was done for two other targets, Nf2l1 expression should be analyzed in Pbx1/3 mutants. This is particularly important, if one wants to establish the link with the decreased survival observed in the compound mutants.

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Following the advice of this reviewer, we analyzed the expression of *Nfe2l1* in loss of function experiments. Our results show a near complete loss of NFE2L1 in TH+ cells of *Pbx1$^{-/-}$; Pbx3$^{+/-}$* embryos at E12.5 (new Fig 6B) but not in PBX1-free structures such as the branchial arches (Appendix Fig S6B), indicating that PBX1 is required for the expression of *Nfe2l1* in TH+ cells. This result is now described in page 6.
Former Fig 6A-B is now part of Fig 5E and Appendix Fig S6A.

5) The experiment described in Figure 6D is aimed at investigating "whether Nfe2l1 plays a role in preventing oxidative stress in human mDA cells". From the data presented, it is not possible to conclude that the increase in Casp3 expression occurs in fully differentiated mDA neurons, as opposed to any other cell type present in the culture. In addition, the quantification of the shRNA mediated knock-down shown in Figure EV11 is very mild. The results must therefore be corroborated with a second shRNA virus, in order to minimize the possibility of occurrence of off-target effects.
Following the suggestion from reviewer #1, we have done new experiments using a pool of concentrated, transduction-ready viral particles (sc-43575-V, Santa Cruz Inc) containing 3 target-specific constructs that encode 19-25 nt (plus hairpin) shRNAs designed to knock down gene expression of human *NFE2L1*, which results in a much clearer knockdown in the absence of target effects (Appendix Fig S6C). Using this approach, we examined whether shNFE2L1 increased the number of dying human mDAn (double TH+aCASP3+ cells) in lt-NES cell cultures treated with H_2O_2, compared to shControl. Our results show that shNFE2L1 increases the number of double-positive TH+aCASP3+ cells by two fold (Fig 6C) in response to both 10 and 100 microM H_2O_2, (p-values 0.0066 and 0.0016, respectively), indicating that NFE2L1 protects human mDAn from oxidative stress. We also show in Appendix Fig S6E that shNFE2L1 impairs the survival of other cells (increase of aCASP3+ cells over Dapi), so showing that NFE2L1 has a broader role in survival, which is not limited to mDA neurons or its regulation by PBX1. These results are described in pages 6-7.

6) The reduction of Pbx1/Nfe2l1 in NM+ neurons from PD patients presented in Figure 6 is quite relevant. I understand the difficulty of obtaining material for these experiments, however results shown in Figure 6E should be properly quantified.
We now show quantification of PBX1 intensity levels in the same patients in which NFE2L1 stainings were performed. The new results are shown in Fig 6F. Analysis of the intensity of PBX1 staining in the nuclei of multiple NM+ cells in the same patients (3 control and 5 PD) revealed a remarkable decrease in the levels of PBX1 in NM+ cells of PD patients compared to controls. These results are presented in a new Fig 6F and described on page 7.

In addition, the data showing the specificity of these observations to NM+ neurons are as far as I can see based on one single sample (Figure EV11) and require further work.
Fig EV12E showed one example of the 5 PD brains analyzed. In the revised version of the manuscript we now show 3 representative examples of the PD patients analyzed (70, 81 and 85 years old). This data is shown now in Appendix Fig S6F. We have also added a sentence, on pages 7 and 9, to explain that we had very strict inclusion criteria for PD patient sections. We only considered sections from patients and controls in which nuclear immunoreactivity for NFE2L1 antibody was clearly detected, and used consecutive sections for PBX1 analyses. We excluded material in which no NFE2L1 immunoreactivity was detected in NM+ or NM- cells.

Referee #2:

1- Loss of mDA neurons in Pbx1/Pbx3 double mutants is analyzed exclusively with TH staining Pbx1/Pbx3 double mutants. It remains unclear how the neurons in SN and VTA are differentially affected. The use of mDA neuronal subtype markers such as Otx2, Calbindin, Sox6 or GIRK2 would help to clarify this aspect.
Following the suggestion from reviewer #2, we have added additional markers to our analysis. However, we would like to point out that our analysis of *Pbx1$^{-/-}$;Pbx3$^{+/-}$* mutants and cKO mice is not exclusively based on TH staining. In our original version of the manuscript we had already examined PITX3+ and NURR1+ cells in cKO and *Pbx1$^{-/-}$;Pbx3$^{+/-}$* mutants (see new versions of Fig 3C, 3D, 5F and Appendix Fig S4). We have now added NURR1 stainings in *Pbx1$^{-/-}$;Pbx3$^{+/-}$* at E12.5 in Fig EV2A. Our results show a reduction of TH+ cells but not of

NURR1+ cells, indicating that the differentiation of NURR1+TH- neuroblasts into NURR1+TH+ mDA neurons is impaired. In the revised version of the paper we also report that the levels or number of neurogenin2+ (NGN2) cells do not change (Fig EV2B), indicating no effect on neurogenesis.
In response to the suggestion of this reviewer, we examined whether there is a differential impairment in SN vs VTA mDA cells in *Pbx1$^{-/-}$;Pbx3$^{+/-}$* mutant mice. As the reviewer suggested, we performed immunofluorescence for SOX6 and OTX2, the two early transcription factors that control SN vs VTA mDA subtype specification. We did not use markers appearing latter in development (Calbindin and GIRK2), because *Pbx1$^{-/-}$;Pbx3$^{+/-}$* embryos die before E16.5.
Our results show no alteration in the number or position of SOX6+ or OTX2+ cells in *Pbx1$^{-/-}$;Pbx3$^{+/-}$* mutant mice at E12.5 (Fig EV2C), suggesting that *Pbx1* is not involved in this process. These results are now described on page 4. This new data is in line with our observation that both medial and lateral TH+PITX3+ cells are lost in *Pbx1$^{-/-}$;Pbx3$^{+/-}$* mice at E12.5 (Fig 5F) and that TH+NURR1+ cells and TH+PITX3+ cells are also lost in the cKO mice at E18.5 (Fig 3D).

2- To better define the magnitude of mDA neuronal fiber loss in the Pbx1/Pbx3 double mutants, it would be very informative the immunohistochemistry (DAB staining) for TH in the striatum which represent the main target of the DA projections.
In order to address this question, we have performed TH immunohistochemistry (DAB) in the striatum of cKO mice at E18.5, compared to cHET. Our results show a decrease in the TH immunoreactivity in both the dorsal and the ventral striatum of cKO mutant mice, which reflects the loss of mDAn in both SN and VTA. This information is described on page 5 and shown in new Fig EV3D.

3- The increase in aCASP-3 staining at E18.5 is limited in the mutants questioning whether TH expression lost is exclusively caused by cell death. On this regard, it would be helpful to repeat this staining at E14.5, a stage of high neurogenesis, to verify whether cell death is enriched at earlier time points. Alternatively, it should be considered that cell identity misspecification could occur as well. Thus, a staining for alternative neuronal cell types like 5-HT could address this eventuality.
Following the suggestion from this reviewer, we have quantified aCASP3 in cHet and cKO at E14.5. We found that the number of aCASP3+ cells was increased in cKO compared to cHet (cHet = 43 ± 8.7 vs cKO = 71 ± 4.4, mean ±SD, p-value 0.016). This result is now presented on page 5.
In addition, we have examined the possibility of a misspecification and stained for 5HT in cKO mutant mice at E18.5. Our results show that in the area where cell loss takes place there is no increase in 5HT immunoreactivity, which is limited to fibers (Fig EV2D). Combined, these data indicate that there is no midbrain to hindbrain misspecification in cKO mutant mice, but rather a loss of mDA neurons. This result is discussed on pages 4-5.

4- In order to better appreciate the protective role of Nfe2l1 during oxidative stress in NES-derived mDA neurons, it would be relevant to evaluate if the overexpression of this gene can rescue cell death in oxidative stress conditions.
We thank reviewer #2 for the suggestion. In the revised version of the manuscript we now show that shNFE2L1 increases the number of double-positive TH+aCASP3+ cells by two fold (Fig 6C) in response to both 10 and 100 microM of H_2O_2, (p-values 0.0066 and 0.0016, respectively), indicating that NFE2L1 protects human mDAn from oxidative stress. However, the effect of NFE2L1 is not limited to TH+ cells. Indeed, shNFE2L1 impaired the survival of other cells in the cultures (increase of aCASP3+ cells over Dapi; Appendix Fig S6E). These results indicate that NFE2L1 has a broader role in survival than that elicited by PBX1 on mDAs. This is in agreement with the broad expression pattern of *NFE2L1* compared to that of *PBX1*.
In response to the suggestion by this reviewer, we performed a gain of function experiment in which we examined whether the antioxidant effect of NFE2L1 can be elicited by PBX1 in mDA neurons. Lentiviral overexpression of *PBX1* in human Lt-NES cells differentiated into mDA neurons decreased by half the loss of mDA neurons (TH+aCASP3+ cells) after exposure to 100 microM H_2O_2 (p-value 0.0301), compared to control vector (new Fig 6D). These results fit well with our observation of a PBX1 binding site in the proximity of the TSS of *Nfe2l1* (new Fig 5B and E), the increase in the levels of NFE2L1 protein by *PBX1* overexpression (Appendix Fig S6A) and the requirement of *Pbx1/3* for the expression of NFE2L1 in mDA neurons (new Fig

6B). Moreover, it complements the data presented in Fig 6C, showing that shNFE2L1 increases the number of double-positive TH+aCASP3+ cells two fold (Fig 6C), indicating that NFE2L1 protects human mDAn from oxidative stress. Combined, our results show that PBX1 controls the levels of the antioxidant protein NFE2L1 and that PBX1 is sufficient to reduce oxidative stress. We thus conclude that the PBX1-NFE2L1 axis promotes resistance to oxidative stress in mDA neurons.

2nd Editorial Decision 23 May 2016

Thanks for submitting your revised version to The EMBO Journal. Your revision has now been seen by the two referees and their comments are below. As you can see both referees appreciate the introduced changes and support publication here. There are just a few minor changes needed for acceptance here.

Referee #1 remaining point concerns the conclusion that NFE2L1 has a "broader role in survival" that goes beyond mDAs. I think the referee has a point, but if you have good arguments for this conclusion also OK to leave. Please take a look at the comment and respond (point-by-point response and/or manuscript)

The ChIP and microarray data should be deposited in a public database and the accession numbers should be added to the manuscript.

REFEREE REPORTS

Referee #1:

After reading carefully the manuscript, I can confirm the authors have addressed all issues that I have previously raised. I have at this stage only one comment that concerns the conclusion that knocking down NFE2L1 results in increased cell death of TH+ and "other cells in the cultures", demonstrating NFE2L1 has a "broader role in survival" that goes beyond mDAs. I just cannot understand how the authors can claim that the Casp3+/DAPI+ cells in Figure S6E are not TH+ cells. Maybe I am missing something, but given the present data I would conclude for an effect on TH positive cells, without making any strong statement about other cell types. I leave it to the editor the last word on this detail.

Also, I have not found any evidence showing that ChIP-seq and transcriptomic data sets were deposited in any publicly available data base, according to the journal´s policy. Accession numbers must be available in materials and methods section.

Referee #2:

The authors provided new results which adequately answer to my previous remarks. Thus, this revised version is fully acceptable for publication for this reviewer. I am confident that this study will be of great interest presenting valuable insights in the fields of neurodevelopment, neurodegeneration and Parkinson's disease.

2nd Revision - authors' response 01 June 2016

Referee #1:

After reading carefully the manuscript, I can confirm the authors have addressed all issues that I have previously raised. I have at this stage only one comment that concerns the conclusion that knocking down NFE2L1 results in increased cell death of TH+ and "other cells in the cultures", demonstrating NFE2L1 has a "broader role in survival" that goes beyond mDAs. I just cannot understand how the authors can claim that the Casp3+/DAPI+ cells in Figure S6E are not TH+

cells. Maybe I am missing something, but given the present data I would conclude for an effect on TH positive cells, without making any strong statement about other cell types. I leave it to the editor the last word on this detail.

We thank reviewer #1 for these suggestions. We have now changed the text concluding than role of NFE2L1 is in preventing oxidation in TH+ cells.

Also, I have not found any evidence showing that ChIP-seq and transcriptomic data sets were deposited in any publicly available data base, according to the journal´s policy. Accession numbers must be available in materials and methods section.

ChIP-seq and RNA-seq data are now deposited in a public NCBI database. Accession numbers are now indicated in the manuscript with numbers GSE82098, GSE82099 and GSE82100.

Referee #2:

The authors provided new results which adequately answer to my previous remarks. Thus, this revised version is fully acceptable for publication for this reviewer. I am confident that this study will be of great interest presenting valuable insights in the fields of neurodevelopment, neurodegeneration and Parkinson's disease.

We thank reviewer #2 for all suggestions to improve our study.

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Corresponding Author Name:	Ernest Arenas
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Reporting Checklist For Life Sciences Articles (Rev. July 2015)

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?
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  - exact statistical test results, e.g., P values = x but not P values < x;
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Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).

B- Statistics and general methods

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

Question	Answer
1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	page 14-15
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	page 14-15
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	page 14-15
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.	page 14-15
For animal studies, include a statement about randomization even if no randomization was used.	page 14-15
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.	page 14-15
4.b. For animal studies, include a statement about blinding even if no blinding was done	page 14-15
5. For every figure, are statistical tests justified as appropriate?	page 14-15
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	page 14-15
Is there an estimate of variation within each group of data?	page 14-15
Is the variance similar between the groups that are being statistically compared?	page 14-15

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).	page 10 and 12
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	page 12

* 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.	page 9
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	page 9
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.	ok

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	page 9
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.	page 9
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	n/a
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	page 9
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	n/a
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.	n/a
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.	n/a

F- Data Accessibility

18. Provide accession codes for deposited data. See author guidelines, under '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	n/a
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).	n/a
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).	n/a
21. As far as possible, primary and referenced data should be formally cited in a Data Availability section. Please state whether you have included this section. Examples: **Primary Data** Wetmore KM, Deutschbauer AM, Price MN, Arkin AP (2012). Comparison of gene expression and mutant fitness in Shewanella oneidensis MR-1. Gene Expression Omnibus GSE39462 **Referenced Data** Huang J, Brown AF, Lei M (2012). Crystal structure of the TRBD domain of TERT and the CR4/5 of TR. Protein Data Bank 4O26 AP-MS analysis of human histone deacetylase interactions in CEM-T cells (2013). PRIDE PXD000208	n/a
22. 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.	n/a

G- Dual use research of concern

23. 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.	n/a