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Tox: A Multifunctional Transcription Factor and Novel Regulator of Mammalian Corticogenesis

Benedetta Artegiani, Antonio de Jesus Domingues, Sara Bragado-Alonso, Elisabeth Brandl, Simone Massalini, Andreas Dahl and Federico Calegari

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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 21 October 2014

Thank you for submitting your manuscript to The EMBO Journal. Two referees have now seen your study and their comments are provided below.

Both referees find the analysis interesting, but also find that some further analysis is needed to strengthen the findings reported. The concerns are clearly outline in the referee reports and should be straightforward to address. Should you be able to address the concerns raised in full then I would like to invite you to submit a suitably revised manuscript for our consideration.

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 considering us and I look forward to seeing the revision.

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REFEREE REPORTS:

Referee #1:

In their manuscript Artegiani et al. describe the function of the transcription factor Tox, which has previously only been described in hematopoiesis, during development of the mouse neocortex.

Being expressed in the ventricular zone and in mature neurons they attribute a role of a "switch-gene", which is downregulated in differentiative divisions and fulfills likely a different role during neuronal maturation. The study is very well conducted and thoroughly carried out, giving some fundamental insight into the molecular nature of this TF and its function during telencephalic develoment. However, several concerns remain to be clarified that will strengthen the findings of the data presented here.

1) The authors chose three timepoints to characterize the expression of Tox during development. However E9.5 and E15.5 are six days apart and E15.5 represents already late stages of neurogenesis. It would be helpful to include an intermediate timepoint, when APs switch from symmetric to asymmetric divisions (e.g. E12.5 or E13.5), especially as this stage is used for in-utero electroporations studies later on.

2) When analyzing the expression of Tox in Sox2+ APs and Btg2+ BPs the authors claim that it is "expressed higher in cells undergoing proliferative divisions", but no co-staining together with a mitosis marker (e.g. pHH3) is shown or analyzed. This should be included.

3) Nfat4 overexpression increases Tox levels to 30x fold and rescues the application of CsA. The authors should discuss the possible other targets by which Nfat4 overexpression influences progenitor fate and which the extracellular triggers for calcium influx in cortical progenitors might be. In addition, a staining confirming the elevated levels of Tox in the different GFP positive cells should be shown.

4) The overexpression of Tox by in utero electroporation (IUE) leads to an increase in GFP positive cells in the SVZ. This is in part due to a larger number of Tbr2+ BPs in the SVZ. However it remains unclear, which identity cells have in the SVZ (especially the mislocalized Sox2+ cells). Are these aRG with an extended interkinetic nuclear migration or delaminated bRG? Careful analysis of cellular morphology, e.g. inheritance of apical and basal processes, or staining with phospho-vimentin might be helpful in this context. As Tox levels are high in PP, does Tox overexpression lead to proliferative divisions also in BPs (that may be addressed by Btg2 co-stainings) possibly changing their cell cycle length?

5) The impact of Tox on neuronal migration and maturation remains still unclear with the data presented in this manuscript. How is the difference in neuronal migration 48h (Fig.5A) and 24h after IUE (Fig.6B) explained? From the graph in Fig.6B` one can not see a reduction in the number of neurons by 30%, as also a neuronal costain is missing. Further, these experiments are not able to address wether neurons going through the stage of multiple processes in the IZ after Tox overexpression are finally able to complete their migration to the CP. I suggest live imaging of GFP+ cells in embryonic brainslices after IUE to address these points.

6) Are there effects (distribution, identity) on progenitor cells upon CsA administration/Tox downregulation?
In summary, we believe that this is an interesting study that will be of interest to the field.

Referee #2:

The article by Artigiani reports a tour de force of techniques to imply Tox as an important regulator of corticogenesis. They first report the expression of Tox during cortical development. Then they use a novel method DamID-seq to identify targets of Tox. They use in utero electroporation to show the effect of Tox overexpression on neural stem cells and neurons and to identify calcineurin/NFAT4 signaling as a regulator of Tox expression. This are very interesting data, but in some important details still preliminary.

Major points:
1. Besides western blot it would be important to show immuno-fluorescence of Tox after constitutively active NFAT4.

2. I am not an expert to judge the validity of the DamID-Seq method, but I raise two points which the authors should address at least by discussion (In fact the discussion should contain a paragraph dedicated to the methodology): 1) does the adenine methylation sequence motif GATC cause any bias in the sequences which can be recovered by DamID-Seq? 2) Why did the authors not use neural stem cells for DamID-Seq? They make an argument that using Hela cells avoids bias, but then in this context we are interested in the targets in neural cells rather than lymphocytes. Do Hela cells have a particularly open chromatin so to render all relevant sites accessible to the modified Tox protein?

3. Show the validity of using Dam-Tox by functionally comparing its phenotype following in utero electroporation.

4. It did not become entirely clear why Tbr2 is positively regulated by Tox given that Tox is not expressed in the SVZ where many Tbr2 cells reside. Why is Tbr2 specifically upregulated in SVZ cells overexpressing Tox? While this data may make sense, the authors should provide their explanation.

Minor points:
1. Most readers are not familiar with DamID so this abbreviation should not be used in the abstract.
2. Mislabeling of figure 1D in the text on page 6 (which should be 1C')
3. Show co-expression of Tox and Pax6 or Tbr2 during development and following Tox overexpression.
4. On page 7, Tox is stated to be co-expressed in Ctip2+ cells in the adult. But it also holds true for Satb2. So state Ctip2+/Satb2+ cells
5. Given that the authors mention Tbr1 and Satb2 in the text, they should provide the PCR data in figure 4C.

1st Revision - authors' response 08 November 2014

Referee #1:
In their manuscript Artegiani et al. describe the function of the transcription factor Tox, which has previously only been described in hematopoiesis, during development of the mouse neocortex. Being expressed in the ventricular zone and in mature neurons they attribute a role of a "switch-gene", which is downregulated in differentiative divisions and fulfills likely a different role during neuronal maturation. The study is very well conducted and thoroughly carried out, giving some fundamental insight into the molecular nature of this TF and its function during telencephalic development. However, several concerns remain to be clarified that will strengthen the findings of the data presented here.

Authors' reply
We thank the reviewer for the positive comments and interest in our work.

Referee #1; point #1:
The authors chose three timepoints to characterize the expression of Tox during development. However E9.5 and E15.5 are six days apart and E15.5 represents already late stages of neurogenesis. It would be helpful to include an intermediate timepoint, when APs switch from symmetric to asymmetric divisions (e.g. E12.5 or E13.5), especially as this stage is used for in-utero electroporations studies later on.

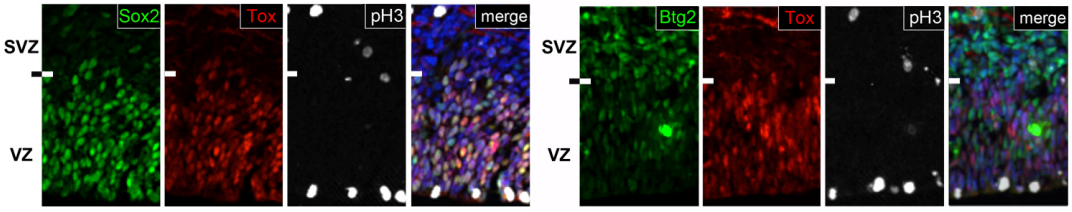
Authors' reply
We performed the stainings at E12.5 as suggested by the reviewer (see revised Fig. 1A; right). These data confirm the gradient of Tox between E9.5 and E15.5.

Referee #1; point #2:
When analyzing the expression of Tox in Sox2+ APs and Btg2+ BPs the authors claim that it is

"expressed higher in cells undergoing proliferative divisions", but no co-staining together with a mitosis marker (e.g. pHH3) is shown or analyzed. This should be included.

Authors' reply

We stained pHH3 as requested. Concerning the term "proliferative division" we would like to point out that this is not meant to indicate that Sox2+ APs divide while Btg2+ BPs do not. Both APs and BPs divide and both can be labeled by pHH3. The difference is in regard to the daughters generated after such divisions: additional progenitors or neurons, respectively. Accordingly, we refer to these divisions as "proliferative" or "differentiative", with progenitors undergoing the former expressing high levels of Tox. To avoid misunderstandings, we explain this terminology in page 2-3 and the use of Sox2 and Btg2 to identify each progenitor type is well documented in the literature (citations within the manuscript). Below the reviewer can find the stainings requested showing pHH3+ cells in the VZ and SVZ that are either Sox2+ or Btg2+, respectively. Only the former express high levels of Tox, according to its gradients (Fig. 1). Since all progenitors undergo mitosis and Tox down-regulation in Btg2+ BPs was shown in our previous (Aprea et al., 2013) and current study we thought that it would not be very informative to also include these pHH3 stainings in the manuscript.



Immunohistochemistry of the E14.5 mouse cortex as indicated in each panel. Due to the lack of antibodies, Btg2 was detected using the Btg2^{RFP} reporter line (Aprea et al., 2013) and pseudocolored in green.

Referee #1; point #3:

Nfat4 overexpression increases Tox levels to 30x fold and rescues the application of CsA. The authors should discuss the possible other targets by which Nfat4 overexpression influences progenitor fate and which the extracellular triggers for calcium influx in cortical progenitors might be. In addition, a staining confirming the elevated levels of Tox in the different GFP positive cells should be shown.

Authors' reply

As suggested, we confirmed the upregulation of Tox by immunohistochemistry upon CA-Nfat4 overexpression (new Fig 2G). We quantified this by measuring Tox in nuclei of randomly chosen GFP+ cells and using GFP– cells as an internal negative control. Here an important distinction exists between VZ and SVZ. Cells in the VZ physiologically express high levels of Tox and after CA-Nfat4 overexpression this increases even further by 35%. In contrast, cells in the SVZ physiologically do not express any detectable level of Tox with CA-Nfat4 inducing its ectopic expression in a subset of cells to levels comparable to the VZ. Clearly, this latter effect in the SVZ cannot be quantified because the ratio between "high levels" and "zero levels" is infinite. Hence, we show the 35% increase in the VZ (Fig. 2G) but only describe the SVZ effect in the text. Notwithstanding the differences in affinity, sensitivity and saturation of the two methods, the 30-fold effect shown by WB (Fig. 2E) is clearly a combination of both VZ and SVZ effects seen by IF.

With regard to the other targets of Nfat4, and calcium activation, we expanded the discussion in page 19 including 6 citations to the main relevant studies in these areas.

Referee #1; point #4:

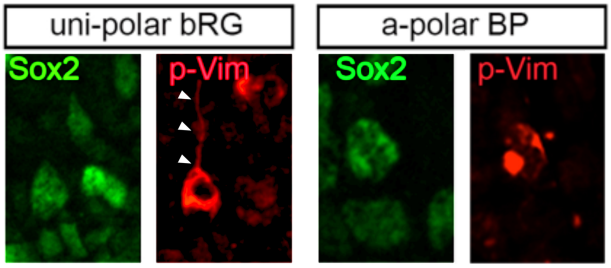
The overexpression of Tox by in utero electroporation (IUE) leads to an increase in GFP positive cells in the SVZ. This is in part due to a larger number of Tbr2+ BPs in the SVZ. However it remains unclear, which identity cells have in the SVZ (especially the mislocalized Sox2+ cells). Are these aRG with an extended interkinetic nuclear migration or delaminated bRG? Careful analysis of cellular morphology, e.g. inheritance of apical and basal processes, or staining with phospho-

vimentin might be helpful in this context. As Tox levels are high in PP, does Tox overexpression lead to proliferative divisions also in BPs (that may be addressed by Btg2 co-stainings) possibly changing their cell cycle length?

Authors' reply

We thank the reviewer for raising these important points. As suggested, we assessed the bi-polar, a-polar or uni-polar morphology of Tox overexpressing cells by i) p-Vim in mitosis and ii) cell morphology of GFP+ cells in interphase to infer their identity as aRG, BP or bRG, respectively. p-Vim showed that Tox overexpressing cells in the SVZ are neither aRG with extended nuclear migration nor newly generated bRG because the almost totality (34/36; i.e. 94%) maintained their a-polar morphology as it was the case in both unmanipulated brains or electroporation with control plasmids (together 132/138; i.e. 96%). This was also confirmed by the a-polar morphology of interphase, GFP+ cells in the SVZ, which also confirmed our previous results (Fig. 5D) showing that the proportion of a-polar, GFP+, SVZ cells that were Sox2+ increased after Tox overexpression by almost 2-fold. At the end of this reply, we have included examples of our analyses for the reviewer that are also mentioned in our manuscript (page 16 and new 5D).

As also suggested, and similarly to Btg2 quantifications in the VZ (Fig. 5B), we assessed Btg2 expression in the SVZ after Tox electroporation. This was done using the Btg2^{RFP} reporter line because antibodies for Btg2 do not exist. Contrary to the VZ, however, differences in the SVZ were not significant, which we think is due to the fact that in this line the RFP protein is known to last for very long after silencing of its transcription (Aprea et al., 2013; Fig. 1). Specifically, Btg2 in the VZ is induced in the transition from Btg2– to Btg2+ cells making it simple to measure a change in the proportion of the two cell populations. In contrast, virtually all cells in the SVZ are Btg2+ making it necessary to assess a decrease in the relative levels of the Btg2^{RFP} signal in individual cells. This, however, cannot be done reliably because of the long half-life of the RFP protein persisting irrespective of its expression. To overcome this, we even considered ISH but, as the reviewer probably knows, this provides very poor cellular resolution making it hard to reliably identify single electroporated GFP+ cells. The main evidence to conclude that BPs expand more when Tox is overexpressed comes from the i) overall decrease in neurogenesis and ii) substantial increase in Sox2+ cells in the basal compartment of the SVZ, which in normal conditions is virtually absent. This may not necessarily require a change in cell cycle length because DamID and qRT-PCRs identified several transcription factors that inhibit differentiation without having any reported role in cell cycle progression (e.g. Fig. 4 and File S1).



Examples of p-Vim+/Sox2+ cells in the SVZ/IZ that have been identified as uni-polar bRG (left) or a-polar BP (right) by the presence, or lack, of a basal process (arrowheads; left), respectively. The almost totality (ca. 95%) of p-Vim+ cells belonged to the latter group in both controls and Tox overexpressing brains

Referee #1; point #5:

The impact of Tox on neuronal migration and maturation remains still unclear with the data presented in this manuscript. How is the difference in neuronal migration 48h (Fig.5A) and 24h after IUE (Fig.6B) explained? From the graph in Fig.6B` one can not see a reduction in the number of neurons by 30%, as also a neuronal costain is missing. Further, these experiments are not able to address wether neurons going through the stage of multiple processes in the IZ after Tox overexpression are finally able to complete their migration to the CP. I suggest live imaging of GFP+ cells in embryonic brainslices after IUE to address these points.

Authors' reply
We are sorry for the misunderstanding and apology for mislabeling panel 6B'. The reduction in GFP+ cells in the CP (Fig. 5A) could potentially be due to i) a decrease in progenitor differentiation and/or ii) impaired neuronal migration. The experiments in Fig. 5B-D confirm the former while Fig. 6A-B' excludes the latter since we did not find any effect on neuronal migration. First, the 30% decrease in neurogenesis is shown in Fig. 5 and Fig. 6B'-right (before wrongly labeled Fig. 6B). As suggested, this effect was now also validated by co-staining with the neuronal marker Tubb3 (all cells in the IZ/CP being Tubb3; data not shown). Second, irrespective of neuron numbers, we addressed their migration by BrdU birthdating 1 day after electroporation at E13.5 and collecting brains at E15.5. We found that migration was unchanged because after normalizing the number of neurons to 100% in each condition their distribution was similar in 5 equidistant bins within the IZ (Fig. 6B'-left; before wrongly labeled Fig. 6). Finally, to address the reviewer's comment and verify if neurons are finally able to undergo maturation and specification, independently from migration and numbers, we repeated electroporations at E13.5 collecting brains at E18.5 and assessed the proportion of cells positive for markers of deeper/upper layers (Ctip2 and Satb2). This showed that neurons can complete their migration to the CP, however their identity was changed, such that a reduced proportion of Ctip2+ neurons was found upon Tox electroporation (page 18 and Fig. 6E). This is an important addition to our study and we thank the reviewer for this suggestion.

Referee #1; point #6:
Are there effects (distribution, identity) on progenitor cells upon CsA administration/Tox downregulation?

Authors' reply
Unfortunately not much, CsA did not induce any evident change in the distribution of progenitors (not shown) but perhaps this is because CsA induced only a minor decrease in Tox by barely 30% (Fig. 2B'); probably not enough to induce obvious phenotypes.

With regard to downregulation, we invested a substantial amount of time to RNAi Tox even before our first submission. In short, we tested 4 shRNAs by electroporation at E13.5 and immunolabeled Tox at E15.5 to test the efficacy of RNAi. We found that one shRNA induced a knock-down by ca. 70% in neurons of the IZ/CP but this was not the case in the VZ were Tox was unchanged. This is easily explained by the fact that Tox is an "off-switch" gene whose locus is silenced, and protein degraded, in neurogenic progenitors resulting in newborn neurons that start "their life" with minimal, if any, Tox inherited transcript and protein. In contrast, both transcript and protein are physiologically high in VZ progenitors with our transcriptome sequencing (Aprea et al., 2013) classifying Tox among the top 25% most highly expressed genes. In this context it is clear that RNAi can be maximally effective in neurons but not progenitors. Inducible mutant mice would need to be done to address this satisfactorily but we cannot provide this for the revision of this study.

Referee #1:
In summary, we believe that this is an interesting study that will be of interest to the field.

Authors' reply
We again thank the reviewer and hope that our revised manuscript can satisfactorily address his/her comments and concerns.

Referee #2:
The article by Artigiani reports a tour de force of techniques to imply Tox as an important regulator of corticogenesis. They first report the expression of Tox during cortical development. Then they use a novel method DamID-seq to identify targets of Tox. They use in utero electroporation to show the effect of Tox overexpression on neural stem cells and neurons and to identify calcineurin/NFAT4 signaling as a regulator of Tox expression. This are very interesting data, but in some important details still preliminary.

Authors' reply

We also thank this reviewer for the positive comments and interest in our work.

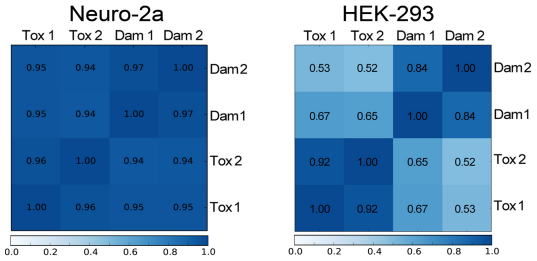
Major points
Referee #2; Major point #1:
1. Besides western blot it would be important to show immuno-fluorescence of Tox after constitutively active NFAT4.

Authors' reply
This is a valid point also raised by reviewer 1. As requested, we measured Tox immunoreactivity in nuclei of cells electroporated with GFP or CA-Nfat4 finding a significant increase in both the VZ and SVZ. Details about this can be found in our response to reviewer 1 (#3; page 2 above).

Referee #2; Major point #2:
I am not an expert to judge the validity of the DamID-Seq method, but I raise two points which the authors should address at least by discussion (In fact the discussion should contain a paragraph dedicated to the methodology): 1) does the adenine methylation sequence motif GATC cause any bias in the sequences which can be recovered by DamID-Seq? 2) Why did the authors not use neural stem cells for DamID-Seq? They make an argument that using Hela cells avoids bias, but then in this context we are interested in the targets in neural cells rather than lymphocytes. Do Hela cells have a particularly open chromatin so to render all relevant sites accessible to the modified Tox protein?

Authors' reply
These are good suggestions, we expanded the description of the method including a scheme on DamID and additional controls (new Fig. S2). Next, a bias in GATC-containing loci is theoretically possible because regions with less than 2 motifs cannot generate methylated fragments for sequencing. However, four evidences suggest that this is not the case. (1) Methylation can occur at a considerable distance from the TF binding site, up to about 2 kb, meaning that only GATC sites located further away would be unavailable. (2) GATC motifs are 10-fold more frequent with a median occurrence every 180 nucleotides providing a high and homogenous density of Dam-methylated (control) sites and excluding the presence of particular genomic clusters (own bioinformatics analysis). (3) DamID and ChIP are known to be in good agreement (e.g. Filion et al, 2010 Fig S1b and references within) suggesting that possible biases do not affect the overall results. Finally, (4) differences in chromatin accessibility and untargeted binding and methylation are controlled for by the use of unfused Dam (*details in Greil et al., 2006).

The second point about using "neural" cells is also excellent. Indeed, we originally performed all experiments in HEK-293T (not HeLa) and Neuro-2a in parallel. Surprisingly, however, no difference was observed between Dam and Tox-Dam peaks in Neuro-2a. We are still puzzled by this discrepancy between cell lines but this can be explained by considering that the endogenous Tox competes with Tox-Dam for DNA binding. HEK-293T do not express Tox (Fig. S2B) allowing the ectopic Tox-Dam to bind its loci without competition. We originally excluded the data on Neuro-2a since we thought that little can be inferred from a "lack of difference" (see Paerson's correlation below). Yet, after the reviewer's comment we envision that other groups may profit from our experience and have now included Neuro-2a (page 11). Finally, HEK-293T gave us a high enrichment in GO terms related to neurogenesis despite their non-neural background (Fig. 4A) and no matter how close a cell line is to the real tissue validation in vivo is always essential, which we attempted at the molecular, cellular and functional level (Fig.4-6).



Paerson's correlation of Dam and Tox-Dam (Tox) peaks in biological replicates of Neuro-2a (left) and HEK-293T (right) cells. Note the virtually identical values in the former and robust and reproducible differences in the latter.

Referee #2; Major point #3:
Show the validity of using Dam-Tox by functionally comparing its phenotype following in utero electroporation.

Authors' reply
As suggested by the inventors of DamID, we show that the Tox-fusion construct retains its nuclear localization. Additionally, as requested by the reviewer, we validated the functionality of Tox-Dam by in utero electroporation. No significant difference was observed between Tox and Tox-Dam constructs in neither of the two set of experiments (new Fig. S2B and S2C).

Referee #2; Major point #4:
It did not become entirely clear why Tbr2 is positively regulated by Tox given that Tox is not expressed in the SVZ where many Tbr2 cells reside. Why is Tbr2 specifically upregulated in SVZ cells overexpressing Tox? While this data may make sense, the authors should provide their explanation.

Authors' reply
It is correct, an artificial overexpression of Tox induced a modest upregulation of Tbr2 by 30% but this does not imply that the opposite must also be true and that in a physiological context cells expressing low Tox should express low Tbr2. Beside, the reviewer is correct that an increase in Tbr2 upon Tox electroporation is somehow counterintuitive because Tox overexpression decreases neurogenesis. Hence, Tbr2 would be expected to go down. However, it is unlikely that Tox, Tbr2, or any other TF acts alone as a "master regulator of neurogenesis". The effect of Tox manipulation is clearly the result of the concerted action of all of its targets with DamID indicating that these targets are many. As one example, Tox was found to bind the loci of Gli1, Yap1 and Sox2 (two potent oncogenes and one reprogramming factor!) increasing their expression by ca. 50%, 100% and 80%, respectively. Under these conditions, proliferation is expected to increase even despite the relatively minor effect on Tbr2 by 30%. We realize that this is an important aspects of our study that we now discuss in our revised manuscript (page 20).

Minor points
Referee #2; Minor point #1:
Most readers are not familiar with DamID so this abbreviation should not be used in the abstract.

Authors' reply
DamID is now spelled out as requested.

Referee #2; Minor point #2:
Mislabeling of figure 1D in the text on page 6 (which should be 1C')

Authors' reply
We have corrected this.

Referee #2; Minor point #3:
Show co-expression of Tox and Pax6 or Tbr2 during development and following Tox overexpression.

Authors' reply
Co-expression of Tox with Pax6 or Tbr2 was unfeasible since the three antibodies (Atlas Antibodies, Covance and Abcam, respectively) are all rabbit polyclonal. This is the reason why we chose to show co-expression of Tox with Sox2 and Btg2 (Fig. 1C) since Pax6 and Sox2 are essentially equivalent markers identifying the same population of neural progenitors (e.g. Gomez-Lopez et al., 2011 and references within) and Btg2 and Tbr2 show an 80% overlap (Arai et al. 2011). Therefore, we find it extremely unlikely that labeling with Tbr2 and Pax6 may yield substantially different results. Coexpression of Tox-GFP/Btg2, Tox-GFP/Tbr2 and Tox-GFP/Sox2 following

electroporation are shown in Fig. 5.

Referee #2; Minor point #4:
On page 7, Tox is stated to be co-expressed in Ctip2+ cells in the adult. But it also holds true for Satb2. So state Ctip2+/Satb2+ cells

Authors' reply
We have included this information for Ctip2 in page 7 (i.e. 72%), which we found to be essentially equivalent to the proportion of Ctip2+/Satb2+. In fact, as the reviewer can see (Fig. S1D''), most Ctip2+ are also Satb2+ while, conversely, only a small proportion of Satb2 are Ctip2. In essence, Ctip2 and Tox appear as very similar markers, which is the reason why we emphasize the values for Ctip2 rather than Satb2.

Referee #2; Minor point #5:
Given that the authors mention Tbr1 and Satb2 in the text, they should provide the PCR data in figure 4C.

Authors' reply
We attempted this for Tbr1, Satb2 and many other neuronal markers even before the submission of our first manuscript. However, establishment of qRT-PCRs for several such markers of this cell population was particularly troublesome. In many cases, and even testing different primer sets per gene, this resulted in technical problems, e.g. dissociation curves with more than one peak or amplification of genomic DNA. The fact that this was rarely the case for the really same markers in control-electroporated brains as well as for progenitor markers makes us to conclude that this may be due to the very low number of neurons that we can retrieve after Tox overexpression, particularly considering the poor yields associated with the FAC-sorting of electroporated cells even after pooling together several electroporated embryos added to the decrease in neurogenesis induced by Tox. In essence, all qRT-PCRs that we performed for any marker can be divided in two groups i) the ones that were technically successful showing reproducible results and ii) the ones that technically failed giving us no result. Of the former, essentially all validated the effect of Tox (9/10). About the latter (9), nothing can be concluded. Hence, it seems inappropriate to completely remove from the text the reference to important genes that we could not validate by qRT-PCR.

2nd Editorial Decision 02 December 2014

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

As you can see below, both referees appreciate the introduced revisions and support publication here. There are just a few minor things to resolve before formal acceptance here. The remaining issues can be addressed with text changes and a better presentation of the data.

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REFEREE REPORTS:

Referee #1:

The authors have revised their manuscript and substantially improved it by adding novel data and modifying their discussion. In its current form the manuscript by Artegiani et al. will be of interest to the field.
However, two minor points remain to be addressed by the authors:

Point 3: The examples of GFP/CA-Nfat4 and Tox upregulation are not convincing. Two of the cells indicated by arrowheads appear to be actually GFP negative. Better example images should be included.

Point 4: How the increase in BPs upon Tox OE can be explained by an increased number of Sox2 positive cells remains unclear, as BPs in the developing cortex are typically not Sox2 positive. How can this be explained?

Referee #2:

The authors addressed most of my concerns/questions satisfactorily or pointed out where this was technically not possible. The only amendment I would still request is that the four evidences for the validity of DamID-seq will not remain "ad reviewer" but included in the discussion. It is not about convincing the reviewer about the methodology employed here, but the reader.

2nd Revision - authors' response 02 December 2014

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The authors have revised their manuscript and substantially improved it by adding novel data and modifying their discussion. In its current form the manuscript by Artegiani et al. will be of interest to the field. However, two minor points remain to be addressed by the authors:

Authors' reply
We thank once again the reviewer for the positive comments.

Referee #1; point #3:
The examples of GFP/CA-Nfat4 and Tox upregulation are not convincing. Two of the cells indicated by arrowheads appear to be actually GFP negative. Better example images should be included.

Authors' reply
Assessing the colocalization of GFP and Tox was difficult in the small space available in Fig. 2 because GFP is cytosolic and Tox nuclear. We now have taken a much higher magnification of a detail within the SVZ and, in addition, have shown the GFP and Tox individual channels separately to better judge each. The figure remains representative and virtually no Tox+ cell in the SVZ could be found in control electroporations.

Referee #1; point #2:
How the increase in BPs upon Tox OE can be explained by an increased number of Sox2 positive cells remains unclear, as BPs in the developing cortex are typically not Sox2 positive. How can this be explained?

Authors' reply
Correctly so, Sox2 is not expressed in BP in physiological conditions. It is expressed upon Tox overexpression. Considering that Tox binds the Sox2 promoter (DamID-Seq data) we conclude that Tox induces Sox2 expression in BP as explained in the discussion.

Referee #2:
The authors addressed most of my concerns/questions satisfactorily or pointed out where this was technically not possible. The only amendment I would still request is that the four evidences for the validity of DamID-seq will not remain "ad reviewer" but included in the discussion. It is not about convincing the reviewer about the methodology employed here, but the reader.

Authors' reply
We thank once again the reviewer for the positive comments and have now included the points about DamID-Seq in page 11 when first introducing the technique (text in red).

