PBX1 acts as terminal selector for olfactory bulb dopaminergic neurons.

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MS TITLE: Isoform-specific functions of PBX1 in terminal differentiation of olfactory bulb dopaminergic neurons

AUTHORS: Laura Remesal, Isabel Roger-Baynat, Laura Chirivella, Miren Maicas, Rebeca Brocal-Ruiz, Ana Perez, Carme Cucarella, Marta Casado, and Nuria Flames

I have now received all the referees reports on the above manuscript, and have reached a decision. The referees' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

The overall evaluation is positive and we would like to publish a revised manuscript in Development, provided that the referees' comments can be satisfactorily addressed. Please attend to all of the reviewers' comments in your revised manuscript and detail them in your point-by-point response. If you do not agree with any of their criticisms or suggestions explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

In this manuscript Remesal addresses the function of PBX1 in the acquisition of dopaminergic phenotype during postnatal and adult olfactory bulb neurogenesis. Using a comprehensive set of well-designed experiments, the authors show that PBX1 has, in addition to an early function at the progenitor level that has been show before, a late function in dopaminergic OB neurons. Indeed, the authors show convincingly that PBX1 functions here as a terminal selector gene for neurotransmitter phenotype, a concept that has been put forward and extensively studied in C. elegans. The work represents a significant advance in our understanding of neuronal phenotype control.
The presented data is of very good quality. A wide spectrum of experimental approaches ranging from the use of transgenic mouse models over in vivo electroporation to behavior, is well used to

make the main points. Moreover, the paper is well written and easy to follow. I have only minor criticisms and comments.

Comments for the author

Fig. 2: For the quantification of Pax6 and DLX neurons the biological significance of the differences is not clear. Statistics are based on a n=3 in both cases with a "significant" 3% difference for DLX and a "non-significant" 5% difference for Pax6. Is this data (and the p=value) really useful?

Fig. 4: The authors show that the PBX deficient neurons of the TH lineage show morphological alterations with less dense and arborized dendritic trees. It would be interesting to know what happens to these strange neurons in the long range. Do they die?
However, this is not an essential experiment but just a suggestion.

Reviewer 2

Advance summary and potential significance to field

Remesal et al. examine the role of Pbx1 in terminal differentiation of olfactory bulb dopaminergic neurons.
Overall, this is a thorough and well executed study, and is an important contribution to our understanding of DA differentiation. The finding that Pbx1 is important both for neurogenesis and terminal differentiation is noteworthy, and also fits nicely with a model of terminal selection factors developed in C. elegans. Moreover I also appreciate the efforts to find a functional effect of loss of DA neurons by examining olfactory function.
This is a poorly studied area, and these studies help to provide some relevant behavioral impact as a result of loss of Pbx1 expression. The authors also begin to indicate the importance of isoform-specific Pbx1 function in DA differentiation, but this section is relatively poorly developed.

Comments for the author

Although the paper is titled "Isoform specific functions" of Pbx1, the majority of the paper has little to do with this. Further, the section on isoform-specific effects of Pbx1 is actually one of the least developed in the paper. I believe the main thrust of the paper is on characterizing the role of Pbx1 in terminal differentiation. As such, a change in title may be appropriate, unless the authors plan on doing much more to demonstrate the individual functions of the various isoforms.

Additional minor comments -
The two odorants tested (geraniol and carvone) were used at 1 and 13nM, respectively. Typically, odorant perception is dependent on concentration, and in order to control for this, odorants are presented at the same vapor pressure. The authors should explain how these concentrations were chosen for their study.

In Figure 8 - does expression of the long-form of Pbx1 rescue the morphological defects as well? It's not at all obvious from the image whether or not this is the case. Given the title, much more could have been included and tested on this isoform and the additional exon.

Fig.1 supplement 4 suggests that Pbx1 is not localized exclusively to TH expressing neurons. But CR and CB neurons are also unaffected in mutants (Fig. 1P,Q). What are these other Pbx1-positive cells, if not TH CR, or CB expressing neurons?

Fig. 1 supplement 3: although the figures appear clear, there is no quantification showing no change in SEZ or RMS upon deletion of Pbx1. This would be helpful to underscore the authors' contention that the deletion of Pbx1 by THCre only occurs during late stages of differentiation.

It's extremely difficult to visualize the putative glomeruli in the figures. For example, in figure 1A-D, some images appear to show two glomeruli (1A,B), while others show one (1D). This makes it quite difficult to easily compare the various results, as I had a difficult time determining what exactly I was looking at. It may be helpful to use dotted lines to circle one glomerulus somewhere

in this first figure to make it clearer what is being shown, as well as a typical example of an area that was quantified. I do not know how the authors decided on their given square area to be quantified, as most studies either analyze the number per mm, or the number per section.

Fig. 1 supplement 4: line 4 misspelled- should be lineage, not linage.

Reviewer 3

Advance summary and potential significance to field

Temporal control of transcription factor expression is often involved in regulating different cellular stages of neuronal development, e.g., specification vs. commitment vs. differentiation/maturation. However, some transcription factors are expressed at several or all stages of neuron development but have distinct roles during different times. How do cells use such continuously expressed transcription factors to control different cellular states at different developmental times? In this manuscript, the group addresses this question for a particular transcription factor, Pbx1, which is important for development of specific types of olfactory bulb neurons. Pbx1 was previously shown to be important for neurogenesis and survival of dopaminergic interneurons in the olfactory bulb, but its role in the terminal differentiation of DA interneurons has remained unknown.

Using genetic, molecular, cellular and behavioral approaches, the authors make the following major scientific findings/contributions: 1) Pbx1 acts as a terminal selector in DA neurons by controlling expression of DA-specific effector genes; 2) Pbx1 is required for general morphological maturation of DA neurons; 3) Pbx1 is additionally required to repress alternate interneuron subtype fates in DA neurons; 4) alternatively splicing of Pbx1 mRNA is temporally regulated, such that two functionally different isoforms of Pbx1 protein are expressed at different stages of DA neuron development. The authors conclude that Pbx1 has multiple roles in DA neuron specification and maturation, and these distinct functions are temporally regulated by differential alternative splicing.

This study is relevant to the readers of Development who are interested in neuronal development mechanisms of neuron differentiation, transcription factor regulation of subtype specification, terminal selection of cell fates and mRNA alternative splicing.

Comments for the author

Overall I found the experiments to be very well designed and executed, the presentation of the results to be complete and convincing, the figures to be nicely arranged and easy to understand, and the manuscript to be well written. I do have several comments and suggestions for improving the manuscript:

1. Page 2, Line 16 – This final sentence of the Abstract about alternative splicing possibly representing a “general mechanism” is not necessarily supported by the data presented in this manuscript and thus doesn’t seem to be appropriate here. I do think it’s an interesting discussion point (and even a likely scenario), but as written in the Abstract it leads the reader to believe that the current paper presents some evidence that this is a general mechanism used by cells. I recommend either removing the sentence from the abstract or replacing it with a statement supported by data in the current manuscript.

2. Similarly, Page 35 Lines 20-23 – “…repression of alternative fates might be a new general rule for terminal selectors…” is a bit strongly worded. There’s nothing presented here that indicates this is a general rule for terminal selectors, so wording it this way seems to be overstating the conclusion (even though it is only mentioned as a possibility). It seems more appropriate to say something like “…repression of alternative fates may be an important function of some terminal selectors…”.

3. Same idea on Page 38 Lines 10-13 – “This could be a widespread mechanism used to regulate…” could be changed to something like “It will be important in the future to investigate whether this is a widespread mechanism used to regulate…”.

4. There are a LOT of abbreviations in the main text of the manuscript; e.g., TF, DA, OB, AS, SEZ, HD, TH CR, CB, Ct, RMS, mESC, etc. Many of these are never used beyond the first time that they are spelled out and introduced. Others are non-standard and make it difficult to read the manuscript each time the abbreviation is used. I recommend using only standard abbreviations and getting rid of the others. For example, DA and OB seem to be standardly used in the field, but AS and HD should not be abbreviated.

5. The naming convention used for the Supplemental Figures is confusing. The first several instances I thought the reader was being referred to both Figure 1 AND Figure Supplement 1, before I realized the data were only in the supplement. Development's instructions indicate these figures should be listed in the main text as "Fig. S1", etc. Perhaps a good compromise would be to list as "Fig. S1, related to Fig. 1". Or simply state in the legends which main figure that particular supplemental figure relates to.

6. Statistical test and p values are generally well reported throughout the manuscript. There are a couple of times when actual p values are not reported in the legend: Fig. 1E, Fig. 8G. More importantly, in most of the instances there is a lot of redundancy in the reporting of the p values and quantifications. For example, the authors use *, **, *** to represent p value ranges on the graphs, but then state the actual p values in the legend. It's easier to read if they just replace the asterisks on the figure with the actual p values and remove values from the legend.

7. Many times the "n" unit is not defined. The value is always there, e.g., "n=3", but most of the time there is no definition of what unit "n" has. The methods state it's either cells or animals, but it should be stated each time. For example, "n=3 animals".

8. In graphs where cells are quantified as a percentage of marker+ cells, the labels on the X axes are kind of confusing. For example, in Fig. 1 N the authors quantify the percentage of BrdU+ cells that are also positive for marker TH. The graph is labeled "% TH cells/BrdU", which is not intuitive without reading the legends. A more conventional way to label this would be "TH+BrdU+/Brdu+", indicating the ratio of total BrdU+ cells that are also TH+. Alternatively, "% BrdU+ cells that are TH+". Same for Figs. 2, 4, 5, 8 and S1-4.

Minor Points:
9. Page 2, Line 6 – It's unclear who "they" refers to in "presently unclear how they adapt their regulatory programs…". Please clarify whether "they" refers to the cells being specified or the transcription factors that are expressed. If referring to the transcription factors, it does not seem accurate to say that they "adapt their regulatory programs," since the transcription factors themselves are not adapting their regulatory programs. I think a simple rephrasing of the point will suffice, but as written it is confusing.

10. Throughout the manuscript the authors state the quantification values in the main text AND in the graphs in the figures. Although I do appreciate seeing the true values, it seems redundant with the figures and makes the text somewhat hard to read at times. This is really up to the authors' style preference, I just wanted to point it out.

11. Page 35, Line 21 – "this data" should be "these data"

Signed,

Santos Franco, PhD
University of Colorado - Anschutz Medical Campus

First revision

Author response to reviewers' comments

Reviewer 1
Advance Summary and Potential Significance to Field

In this manuscript Remesal addresses the function of PBX1 in the acquisition of dopaminergic phenotype during postnatal and adult olfactory bulb neurogenesis. Using a comprehensive set of well-designed experiments, the authors show that PBX1 has, in addition to an early function at the progenitor level that has been show before, a late function in dopaminergic OB neurons. Indeed, the authors show convincingly that PBX1 functions here as a terminal selector gene for neurotransmitter phenotype, a concept that has been put forward and extensively studied in C. elegans. The work represents a significant advance in our understanding of neuronal phenotype control. The presented data is of very good quality. A wide spectrum of experimental approaches, ranging from the use of transgenic mouse models over in vivo electroporation to behavior, is well used to make the main points. Moreover, the paper is well written and easy to follow. I have only minor criticisms and comments.

Reviewer 1 Comments for the Author

Fig. 2: For the quantification of Pax6 and DLX neurons the biological significance of the differences is not clear. Statistics are based on a n=3 in both cases with a “significant” 3% difference for DLX and a “non-significant” 5% difference for Pax6. Is this data (and the p=value) really useful?

We thank the reviewer for this remark and we agree that the biological significance of this data is unclear. We present data for ETV1, DLX, PAX6, COUPTF1 and MEIS2 because these five factors are known regulators of OB DA fate and it was important to analyse if Pbx1 is transcriptionally upstream of any of them. Our quantification of DLX staining showed very little variability among animals and that is the reason why it is statistically (but not necessarily biologically) significant. In the case of PAX6 quantification even thought there is a slightly bigger difference with controls due to variability among animals this difference is not significant.
We think maintaining the data is important to show that Pbx1 is not required for DLX expression what supports a role for Pbx1 in terminal differentiation. However, as the reviewer points out, the potential repressive role of Pbx1 on DLX is very small and of uncertain biological relevance, accordingly we have added a sentence in the text to clarify what is the main conclusion of the experiment:
Page 8 line 26:
" $Pbx1^{Th}$ mutant mice showed a small increase in the number of DA lineage cells co-expressing DLX TFs (Fig. 2J-L, Table S1), demonstrating PBX1 is neither required for DLX expression. The potential biological relevance of the increase of DLX expressing cells is so far unclear."

Fig. 4: The authors show that the PBX deficient neurons of the TH lineage show morphological alterations with less dense and arborized dendritic trees. It would be interesting to know what happens to these strange neurons in the long range. Do they die? However, this is not an essential experiment but just a suggestion.

We thank the reviewer for this suggestion, a better understanding of the role of terminal selectors in aging could be an interesting follow up of the paper. Nevertheless, as this is not the main point of the paper and obtaining old-enough animals would delay its publication for at least 10 to 12 months, we appreciate that the reviewer considers these experiments not essential for the current manuscript.

Reviewer 2
Advance Summary and Potential Significance to Field

Remesal et al. examine the role of Pbx1 in terminal differentiation of olfactory bulb dopaminergic neurons. Overall, this is a thorough and well executed study, and is an important contribution to our understanding of DA differentiation. The finding that Pbx1 is important both for neurogenesis and terminal differentiation is noteworthy, and also fits nicely with a model of terminal selection factors developed in C. elegans. Moreover, I also appreciate the efforts to find a functional effect of loss of DA neurons by examining olfactory function. This is a poorly studied area, and these studies help to provide some relevant behavioral impact as a result of loss of Pbx1 expression. The authors also begin to indicate the importance of isoform-specific Pbx1 function in DA differentiation, but this section is relatively poorly developed.

Reviewer 2 Comments for the Author

Although the paper is titled "Isoform specific functions" of Pbx1, the majority of the paper has little to do with this. Further, the section on isoform-specific effects of Pbx1 is actually one of the least developed in the paper. I believe the main thrust of the paper is on characterizing the role of Pbx1 in terminal differentiation. As such, a change in title may be appropriate, unless the authors plan on doing much more to demonstrate the individual functions of the various isoforms.

We thank the reviewer for pointing this out. We agree the main message of the paper is the role of Pbx1 as terminal selector for OB DA fate. Following reviewer's suggestion we have now changed the title to better reflect our results:
"PBX1 acts as terminal selector for olfactory bulb dopaminergic neurons."

Additional minor comments

The two odorants tested (geraniol and carvone) were used at 1 and 13nM, respectively. Typically, odorant perception is dependent on concentration, and in order to control for this, odorants are presented at the same vapor pressure. The authors should explain how these concentrations were chosen for their study.

We thank the reviewer for this comment, we realise now this part of the text needs further clarification. As the reviewer explains, perception of odors dependends on its volatile concentration that in turns depends on vapor preassure. We first performed a threshold analysis with geraniol using a range of concentrations from the most diluted (1nM) increasing up to 341 nM (Data presented in Supplementary Figure 6). Control animals were able to detect geraniol at the lowest concentration (1nM), suggesting 1nM is above geraniol threshold value in control animals. Using the same behavioural paradigm and the same increasing concentrations, Pbx1 mutants failed to detect geraniol at the lowest 1nM concentration, demonstrating odor behavior imperments in Pbx1 mutants. Due to the high variability among mutant animals we failed to establish a clear threshold value for Pbx1 mutants.

In the next set of experiments, to expand our analysis of odor behavior deficits in Pbx1 mutant, we decided to use carvone, an additional neutral odor. Carvone vapor preassure is higher than Geraniol (carvone 0.115 mm Hg at 25 °C compared to geraniol 0.03 mmHg), what indicates that at the same soluble concentration more volatile carvone is available compared to geraniol. To make sure we were clearly above threshold detection in wildtype animals, we used a higher concentration than geraniol threshold (we chose one order of magnitude higher 13nM carvone instead of 1nM geraniol). As shown in Figure 6B, and as expected, control animals detect carvone at this high concentration, in contrast, Pbx1 mutants do not respond to carvone even at this high concentration.

The main point of carvone assay is not to compare responses between odors (carvone and geraniol) but to compare the response to this concentration of carvone between control and mutants animals. Thus we think data presented for both odors is relevant and we would like to maintain it in the manuscript. To clarify this section we have:

1) Changed Supplementary Figure 6 and added concentration values instead of dilutions, were it can be easily appreciated that control animals respond to 1nM geraniol

2) Modified the methods section to explain why we chose 13nM carvone in the next set of experiments: Page 22, line 2:

"Mice were then exposed for 1 min to successive cotton sticks with 3:1 increasing concentrations of geraniol ($C_{10}H_{18}O$; Vento s, S.A.) diluted in mineral oil: 1 nM, 3 nM, 11 nM, 36 nM, 111 nM, 341 nM. A different set of animals was exposed to carvone ($C_{10}H_{14}O$, Sigma) at 13 nM, after stick habituation. Carvone vapor preassure is slightly higher than geraniol (0.115 mm Hg and 0.03 mmHg respectively at 25°C), thus at the same concentration, more volatile carvone is available. Nevertheless, to ensure values above detection threshold we used 13 nM carvone instead of 1 nM used for geraniol."

In Figure 8 - does expression of the long-form of Pbx1 rescue the morphological defects as well? It's not at all obvious from the image whether or not this is the case. Given the title, much more could have been included and tested on this isoform and the additional exon.

We thank the reviewer for this comment. Presently, we do not know if morphological defects are rescued by Pbx1a, Pbx1b or both isoforms. In the morphological reconstruction experiments (Figure 4), the tissue is processed differently than in the experiments for quantification of TH expression (Figure 8), thus, to address reviewer's question we would need to perform new sets of electroporations. These experiments are easy to perform but will take three more months to get the data, delaying manuscript acceptance.
As the reviewer pointed out, the main message of the paper is the role of Pbx1 in DA terminal differentiation. We have modified the title and the abstract to make this more clear, following reviewer's suggestions. We now highlight Pbx1 role as terminal selector and we have removed from the abstract some statements on differential isoform functions.
We believe that, although isoform specific rescue of morphological defects is a very interesting experiment, it does not significantly reinforce the main message of the paper.
As the reviewer points out, further experiments both on progenitors and on differentiating neurons should be designed and performed to be able to make stronger claims on isoform specific functions of Pbx1, which could be a logical follow up of the paper but at this moment, is out of the scope of this manuscript.
All other comments or questions raised by reviewers have been now addressed. Thus, we would like to kindly ask the reviewer if the manuscript could be considered suitable for publication without the isoform- specific morphological rescue. Nevertheless, if reviewer considers these experiments will significantly increase the quality of the paper we will be happy to perform them.

Fig.1 supplement 4 suggests that Pbx1 is not localized exclusively to TH expressing neurons. But CR and CB neurons are also unaffected in mutants (Fig. 1P,Q). What are these other Pbx1-positive cells, if not TH, CR, or CB expressing neurons?

As the reviewer points out, Pbx1 is not exclusively expressed in TH neurons. We have now quantified both Pbx1a and Pbx1b expression in CR and CB populations. Pbx1b is not expressed in neither of these interneuron types, while 16% of CR cells express Pbx1a (corresponding to 15% of the total of Pbx1a cells) and 42% of CB cells express Pbx1a (corresponding to 14% of the total of Pbx1a cells).
We have now included this data in Supplementary Figure S4.
Regarding reviewer's comment: "But CR and CB neurons are also unaffected in mutants (Fig. 1P,Q)"

It is important to consider that our conditional approach (Th^{CRE}) only removes Pbx1 from the dopaminergic lineage and not from CR or CB lineage. Thus, we cannot assess if, in addition to its role on DA fate, Pbx1 has a role in the percentage of CR or CB Pbx1a positive populations. In *C. elegans* terminal selectors often regulate different neuronal fates, acting with different combinations of transcription factors, thus Pbx1 could be acting similarly in the OB, this is an interesting hypothesis that could be explored in the future with specific CRE lines for CR and CB lineages.
To clarify this question we have added double CR/Pbx1 and CB/Pbx1 expression data on Supplementary Figure S4. And modified the text:
Page 7 line 30:
" As expected, deletion of Pbx1 exclusively from the DA lineage (Pbx1Th) did not affect the other two types of adult-generated periglomerular interneurons labeled with calretinin (CR) and calbindin (CB) (Fig. 1P, Q, Table S1). Nevertheless, we found PBX1 is expressed in a 16% of CR and 42% of CB interneurons (Fig. S4). Pbx1 expression in non-DA lineages is unaffected in Pbx1Th animals, thus the role of Pbx1 on CR and CB differentiation remains to be explored."

Fig. 1 supplement 3: although the figures appear clear, there is no quantification showing no change in SEZ or RMS upon deletion of Pbx1. This would be helpful to underscore the authors' contention that the deletion of Pbx1 by THCre only occurs during late stages of differentiation.

We thank the reviewer for this suggestion, we have quantified ki67 and DCX expressing cells in three controls and three mutant animals and found no differences. Data is now added in Supplementary Figure 3.

It's extremely difficult to visualize the putative glomeruli in the figures. For example, in figure 1A-D, some images appear to show two glomeruli (1A,B), while others show one (1D). This makes it quite difficult to easily compare the various results, as I had a difficult time determining what exactly I was looking at. It may be helpful to use dotted lines to circle one glomerulus somewhere in this first figure to make it clearer what is being shown, as well as a typical example of an area that was quantified. I do not know how the authors decided on their given square area to be quantified, as most studies either analyze the number per mm, or the number per section.

We apologise for the inconvenience. As suggested by the reviewer we have used dotted lines to label glomeruli in the first figure (See Figure 1A-D). Regarding quantification, for experiments in which total numbers are shown (in contrast to percentages) we randomly sampled PGL in the dorsal, ventral, medial and lateral regions and along the rostro-caudal level (6 sections analysed). Random fields are selected, in which all the area photographed (184 × 184 µm corresponding to the field at 63X magnification) is part of the PGL. As there is no difference in the mean glomerulus area between controls and mutants (Figure 4) we likely sample the same amount of total cells in both genotypes. Similar quantification strategies for TH cells in the PGL has been followed before (Bovetti et al. Figure 3, Development 2013, DOI: 10.1242/dev.089961). We have modified the corresponding section of methods to make it more clear: Page 21 line 14:
" For OB quantification dorsal, ventral, medial and lateral regions of the glomerular cell layer were randomly sampled with a predetermined area (184 × 184 µm corresponding to the field at 63X magnification) and all immunopositive cells in these selected areas were counted. In all cases, cells were sampled from six sections along the rostro-caudal axis of the olfactory bulb."

Fig. 1 supplement 4: line 4 misspelled- should be lineage, not linage.

Thanks, we apologise for the typo. It has now been corrected (Supplementary Figure S4)

Reviewer 3
Advance Summary and Potential Significance to Field:

Temporal control of transcription factor expression is often involved in regulating different cellular stages of neuronal development, e.g., specification vs. commitment vs. differentiation/maturation. However, some transcription factors are expressed at several or all stages of neuron development but have distinct roles during different times. How do cells use such continuously expressed transcription factors to control different cellular states at different developmental times? In this manuscript, the group addresses this question for a particular transcription factor, Pbx1, which is important for development of specific types of olfactory bulb neurons. Pbx1 was previously shown to be important for neurogenesis and survival of dopaminergic interneurons in the olfactory bulb, but its role in the terminal differentiation of DA interneurons has remained unknown. Using genetic, molecular, cellular and behavioral approaches, the authors make the following major scientific findings/contributions: 1) Pbx1 acts as a terminal selector in DA neurons by controlling expression of DA- specific effector genes; 2) Pbx1 is required for general morphological maturation of DA neurons; 3) Pbx1 is additionally required to repress alternate interneuron subtype fates in DA neurons; 4) alternatively splicing of Pbx1 mRNA is temporally regulated, such that two functionally different isoforms of Pbx1 protein are expressed at different stages of DA neuron development. The authors conclude that Pbx1 has multiple roles in DA neuron specification and maturation, and these distinct functions are temporally regulated by differential alternative splicing.

This study is relevant to the readers of Development who are interested in neuronal development, mechanisms of neuron differentiation, transcription factor regulation of subtype specification, terminal selection of cell fates and mRNA alternative splicing.

Reviewer 3
Comments for the Author:

Overall I found the experiments to be very well designed and executed, the presentation of the results to be complete and convincing, the figures to be nicely arranged and easy to understand, and the manuscript to be well written. I do have several comments and suggestions for improving the manuscript:

1. Page 2, Line 16 – This final sentence of the Abstract about alternative splicing possibly representing a "general mechanism" is not necessarily supported by the data presented in this manuscript and thus doesn't seem to be appropriate here. I do think it's an interesting discussion point (and even a likely scenario), but as written in the Abstract it leads the reader to believe that the current paper presents some evidence that this is a general mechanism used by cells. I recommend either removing the sentence from the abstract or replacing it with a statement supported by data in the current manuscript.

We thank the reviewer for this remark, we have now removed the sentence from the abstract.

2. Similarly, Page 35 Lines 20-23 – "…repression of alternative fates might be a new general rule for terminal selectors…" is a bit strongly worded. There's nothing presented here that indicates this is a general rule for terminal selectors, so wording it this way seems to be overstating the conclusion (even though it is only mentioned as a possibility). It seems more appropriate to say something like "…repression of alternative fates may be an important function of some terminal selectors…".

We apologise for the overstatement, we have modified the text following reviewer's suggestion. In addition, we have added a reference to a recently paper published (Feng 2020, eLife). This paper describes that *unc-3*, a terminal selector for *C. elegans* motorneuron fate, has an additional role repressing alternative fates, similar to what we found for Pbx1 in mice, we think this reference adds support to our conclusion: Page 17 line 14:
"Repression of alternative fates might be an important function of some terminal selectors, in line with this hypothesis, a recent report shows that unc-3, a terminal for C. elegans motorneuron specification, also acts as repressor of alternative neuronal fates (Feng et al., 2020)."

3. Same idea on Page 38 Lines 10-13 – "This could be a widespread mechanism used to regulate…" could be changed to something like "It will be important in the future to investigate whether this is a widespread mechanism used to regulate…".

We apologise again for the overstatement and thank the reviewer for the remark. We have modified the text according to the suggestion:
Page 19 line 17
" It will be important in the future to investigate whether this is a widespread mechanism used to regulate the rapid rewiring of transcriptional regulatory programs that is required in the cell-state transitions taking place in the development of many tissues and organs."

4. There are a LOT of abbreviations in the main text of the manuscript; e.g., TF, DA, OB, AS, SEZ, HD, TH, CR, CB, Ct, RMS, mESC, etc. Many of these are never used beyond the first time that they are spelled out and introduced. Others are non-standard and make it difficult to read the manuscript each time the abbreviation is used. I recommend using only standard

abbreviations and getting rid of the others. For example, DA and OB seem to be standardly used in the field, but AS and HD should not be abbreviated.

We apologise for the over-abundance of abbreviations, we have now reduced them as suggested.

5. The naming convention used for the Supplemental Figures is confusing. The first several instances I thought the reader was being referred to both Figure 1 AND Figure Supplement 1, before I realized the data were only in the supplement. Development's instructions indicate these figures should be listed in the main text as "Fig. S1", etc. Perhaps a good compromise would be to list as "Fig. S1, related to Fig. 1". Or simply state in the legends which main figure that particular supplemental figure relates to.

We have now named figures according to Development's instructions.

6. Statistical test and p values are generally well reported throughout the manuscript. There are a couple of times when actual p values are not reported in the legend: Fig. 1E, Fig. 8G. More importantly, in most of the instances there is a lot of redundancy in the reporting of the p values and quantifications. For example, the authors use *, **, *** to represent p value ranges on the graphs, but then state the actual p values in the legend. It's easier to read if they just replace the asterisks on the figure with the actual p values and remove values from the legend.

We thank the reviewer for the suggestion, we have now removed asterisks and added p values to the figures. In addition, we added an * to any p value below 0.05 as we think it is easier for the reader to identify statistically significant values.

7. Many times the "n" unit is not defined. The value is always there, e.g., "n=3", but most of the time there is no definition of what unit "n" has. The methods state it's either cells or animals, but it should be stated each time. For example, "n=3 animals".

We apologise for the ambiguity and thank reviewer for pointing it out. We have now clarified what n refers to each time the term is used in the text.

8. In graphs where cells are quantified as a percentage of marker+ cells, the labels on the X axes are kind of confusing. For example, in Fig. 1 N the authors quantify the percentage of BrdU+ cells that are also positive for marker TH. The graph is labeled "% TH cells/BrdU", which is not intuitive without reading the legends. A more conventional way to label this would be "TH+BrdU+/Brdu+", indicating the ratio of total BrdU+ cells that are also TH+. Alternatively, "% BrdU+ cells that are TH+". Same for Figs. 2, 4, 5, 8 and S1-4.

We thank the reviewer for the suggestion and have modified figures it accordingly.

Minor Points:

9. Page 2, Line 6 – It's unclear who "they" refers to in "presently unclear how they adapt their regulatory programs…". Please clarify whether "they" refers to the cells being specified or the transcription factors that are expressed. If referring to the transcription factors, it does not seem accurate to say that they "adapt their regulatory programs," since the transcription factors themselves are not adapting their regulatory programs. I think a simple

We thank the reviewer for this comment, we have modified the sentence to make it more clear: Page 2, line 9:
" but it is presently unclear how these factors modify their targets as cells transition through different stages of specification."

rephrasing of the point will suffice, but as written it is confusing.

10. Throughout the manuscript the authors state the quantification values in the main text AND in the graphs in the figures. Although I do appreciate seeing the true values, it seems redundant with the figures and makes the text somewhat hard to read at times. This is really up to the authors' style preference, I just wanted to point it out.

We thank the reviewer for the comment, we realise including primary data in the text might make it a bit harder to read. Nevertheless, we think it is important to show these data, thus we have removed it from the text but included it in a Supplementary Table (Table S1).

11. Page 35, Line 21 – "this data" should be "these data"

We apologise for the typo. Thanks for pointing that out. This paragraph has been now rephrased: Page 17 line 14:
" Repression of alternative fates might be an important function of some terminal selectors, in line with this hypothesis, a recent report shows that *unc-3*, a terminal for *C. elegans* motorneuron specification, acts as repressor of alternative neuronal fates (Feng, 2020)"

Signed,
Santos Franco, PhD
University of Colorado - Anschutz Medical Campus

Second decision letter

MS ID#: DEVELOP/2019/186841

MS TITLE: PBX1 acts as terminal selector for olfactory bulb dopaminergic neurons.

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ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Development, pending our standard ethics checks.