Rnf220/Zc4h2-mediated monoubiquitylation of Phox2 is required for noradrenergic neuronal development

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MS TITLE: RNF220/ZC4H2-mediated monoubiquitination of Phox2 is required for noradrenergic neuronal development

AUTHORS: Ning-Ning Song, Pengcheng Ma, Qiong Zhang, Lei Zhang, Longlong Zhang, Liang Zhu, Chun-Hui He, Bingyu Mao, and Yu-Qiang Ding

I have now received the reports of three referees on your manuscript and I have reached a decision. The reports are appended below and you can access them online: for this please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

As you will see, all the referees express great interest in your work, but they also have significant criticisms and recommend a substantial revision of your manuscript before we can consider publication. In particular, they request a more direct demonstration of ubiquitination of Phox2 proteins, better quantification of the data and better controlled experiments. If you are able to revise the manuscript along the lines suggested by the referees, which may involve further experiments, I will be happy to receive a revised version of the manuscript. Your revised paper will be re-reviewed by the original referees, and its acceptance will depend on your addressing satisfactorily all their major concerns. Please also note that Development will normally permit only one round of major revision.

Please attend to all of the reviewers' comments and ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion. I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

The manuscript by Song et al points to a novel mechanism of function of Phox2a and Phox2b transcription factors, previously shown to be critical for the development/specification of noradrenergic (NA) neurons. Given the importance of these neurons for normal nervous system function, their development is of considerable interest and despite the work of JF Brunet et al, it remains only partly understood. Another novelty factor is the potential regulation of Phox2 transcription factors by ubiquitination, which is also the mechanistically novel work in this manuscript. This is particularly significant, given the involvement in a neurodevelopmental disorder of one of the proteins that apparently regulate Phox2 function.

Comments for the author

The authors first use genetics to show that the ubiquitin E3 ligase RNF220 and its cofactor ZC4H2 are required for the development of NA neurons. Next, they use biochemical experiments to show interactions between these two proteins and Phox2 transcription factors, in vitro and in vivo, and that the ubiquitination function of RNF220 is required for Phox2 modification, in addition to providing structure-function evidence regarding the modification site and genetic evidence of these effects. Further biochemical experiments are used to argue that these interaction are important for transcriptional activity of Phox2 proteins on promoters related to NA specification. These conclusions are supported by in ovo manipulations in chick.

General comments and major issues:
The authors argue for ubiquitination of Phox2 proteins, and this is one of the main conclusions of the study, but the authors do not directly show that Phox2 modification (band size shift) is a result of direct ubiquitination by RNF220. Some sort of a direct experiment is required to draw this conclusion. For example, using tagged Ubiquitin and showing that it ends up on Phox2 proteins.

The in ovo electroporations, which are basically in vivo demonstration that RNF220/ ZC4H2 work together with Phox2's should have RNF220/ZC4H2-alone and Phox-alone controls. Without these, the experiment is difficult to interpret.

There are essentially no instances of the authors reporting the number of replicates for any of the experiments. This is not acceptable and without these values, the manuscript should not be published. For the biochemical analyses, quantification of the blots must be done for experiments in which there are quantitative effects (Fig. 4KLM). Number of replicates must be reported for all the blots. To be more explicit: all experiments must describe number of replicates.

Overall, the quality of the data is good, but the authors' arguments would be more compelling with some specific improvements to the manuscript (see below).

Detailed comments:
Introduction:
-Line 71 – Please elaborate on the nature of ZC4H2. Is it DNA binding? How does it exert its function?
Compare this to RNF220 (an E3 ligase) which is better described.
-Line 76 – What is Sin3B and what is its relevance here? Is it related to Wnt signalling?
-Line 79-83 – Please elaborate on these mechanisms, briefly. The nature of the interaction between ZC4H2 and RNF220 should be proposed clearly here.
-Numerous spelling and grammatical errors throughout the manuscript. Consider revising.

Results:
Generally the quality of images is low and unconvincing. The authors are suggested to provide high resolution images to replace lower resolution images and to provide high magnification insets to bolster their claims. These (and other suggestions) are listed in the order of chapters:

ZC4H2 is required for LC NA neuron development Fig. 1b) Consider adding high magnification/resolution insets to e12.5 experiments, as the color contrast between DBH and the other genes are difficult to distinguish. Fig. 1d) Resolution is very low. Consider providing increased resolution images as well as high resolution insets.

Line 104 & Supplemental – Consider demonstrating proliferation with hindbrain progenitor zone markers or PCNA/Ki67. Nevertheless, the location of cells seems to be well outside the ventricular zone. Fig. 1g) The reduction in Phox2b signal here is very slight and not convincing. Consider an inset to illustrate this.
Supplemental Figure 2 – It's not necessary to include these data, as the point was already well made, but there should be cell counts for at least an n=3 for each group and a statistical comparison.

RNF220 is required for LC NA neuron development
Supplemental 1d) RNF220 and ZC4H2 are expressed similarly, but it is not convincing that these are expressed in the same cells. I recommend high resolution microscopy with insets as well as counts of RNF220, ZC4H2 and their overlap. At this resolution it is not possible to identify individual cells, so it is not possible to quantify overlap.
Figure 3 – General comments
•Restructure this figure for clarity. The first two rows have ZC4H2 KO and then beneath RNF220 KO, however the last row is a mix. Add two extra rows structured the same as the first two rows, and demonstrate Peri/TH staining in the ZC4H2 LC as well (as it is indicated in the text but not shown).
Line 151 – Elaborate the meaning of "residual mRNA". What is the nature of the KO? Does a truncated transcript persist in KO mice?
Supplemental Fig 3 – This is not convincing evidence for the loss of neurons, as a multitude of reasons could result in a loss of mRNA. It may be beyond the scope of this paper to prove this claim, but potential future experiments could use a Cre-marker to label these neurons prior to the effects of the knockout (e.g Phox2b:Cre + Cre-reporter + KO) and demonstrating that Cre-reporter signal is lost in KO mice. Again, this is involved and unnecessary, but you cannot make the claim that neurons do not survive with your current data.
Line 163 – Clarify that TH staining in Sympathetic ganglia was not affected. Staining at a later stage (e.g e16.5 would be more convincing to demonstrate that no disruption has occurred).
Line 165-167 – There is no suggestion that these proteins coordinate their functions based on the data presented so far. Please revise this conclusion.

The RNF220/ZC4H2 complex interacts with and monoubiquitinates Phox2a/Phox2b

Line 194-196 – The ability of a dual knock-down approach to demonstrate a decrease in TH transcript is a good argument for interaction between these genes. This should be emphasized when discussing their coordination, not the individual KO studies in prior figures.
- Figure 4K) The decrease in Phox2a here is slight. Consider performing a densitometry analysis on multiple samples.

RNF220/ZC4H2-mediated monoubiquitination of Phox2a/Phox2b favors Phox2a/Phox2b transactivity

-A missing control here should be done: Phox2a/b + ZC4H2 together, without other factors.
-Appropriate controls should be done for in-ovo electroporation experiments to demonstrate that the constructs used express their corresponding proteins (see above).

Reviewer 2

Advance summary and potential significance to field

In their manuscript, Song et al investigate the role of the ubiquitin E3 ligase RNF220 and its co-factor ZC4H2 in the development of noradrenergic (NA) neurons in the locus coeruleus (LC). They show that both genes are expressed in these neurons and that mutations in RNF220 or in ZC4H2 interfere with their development. They continue to demonstrate that RNF220 and ZC4H2 form a ternary complex with the Phox2a/b transcription factors and mono-ubiquitinates these factors in

cell culture and in vivo. This post-translational modification increases the transcriptional activity of Phox2a/b. Taken together, these are interesting findings which shed new light on how the development of LC NA neurons is controlled at a molecular level through modification of crucial transcription factors but there are a number of caveats the author need to address:

Comments for the author

Major points

1) The finding that RNF220 and ZC4H2 interact to modify a critical transcription factor during neural development is not entirely novel and was described in a several papers by this and another group (see Kim et al., 2018; Ma et al., 2019a/b).

2) The authors postulate that mono-ubiquitination of Phox2a/b increases the transcriptional activity of these transcription factors but their measurements of DBH and TH transcription levels leaves open other interpretations. Moreover, a mechanism was not investigated. It is conceivable that Phox2a/b regulate DBH and TH transcription indirectly by controlling the expression of another transcription factor. The authors should confirm that Phox2a/b directly bind to the DBH and TH promoters by ChIP experiments. They should also investigate in bandshift analyses whether mono-ubiquitinylation of Phox2a/b affects their DNA binding. Moreover, they should use luciferase assays using the DBH and TH promoter regions to show that Phox2a/b can transactivate these promoters and how ubiquitinylation affects this transactivation. Finally, RFN220 and ZC4H2 might control nuclear localisation of the Phox2a/b transcription factors.

3) The authors focus their analysis on the effect of Rnf220/ZC4H2 on the Phox2a/b transcription factors but they previously reported that ZC4H2 also controls Bmp signalling. As Bmp signalling is required for the specification of NA progenitors this raises the possibility that NA progenitor development is affected but this is not investigated by the authors.

4) In general, the manuscript contains very little quantification. The authors should at least aim to quantify the levels of mono-ubiquitinated Phox2a/b in CATH1a cells and in the mid/hindbrain (Figure 4k-m). Moreover, the authors have to ensure that the correct statistical tests are employed. Multiple t-test were used to evaluate the transcriptional activities of Phox2a/b but this increases the risk of type 1 errors. The authors should use an ANOVA followed by a post-hoc test.

Minor points

1) On page 8, the authors should explain the strategy used to inactivate Rnf220 and why an exon 1-3 probe can be used to detect the fate of mutant cells. In this respect, it remains unclear what the fate of the mutant cells is. Do they die or do they switch fate?

2) In the discussion, the authors state that a lack of Phox2a/b modification may account for the down-regulated expression of Phox2a (line 245) but it remains unclear how this could work. Does Phox2a auto-regulate its own expression? The authors should cite evidence for such a mechanism or modify their statement.

3) There are a number of sloppy formulations:
Line 40: … and some other groups
Line 73: … and other issues …
Line 81: “reactors” should read transcription factor
Line 112: it should read: … is expressed …
Line 120: it should read: …nor Phox2a/Phox2b ….
Line 173: “but not other factors”. What are these factors and why were they investigated?
Line 253: is it share or exhibit?

Reviewer 3

Advance summary and potential significance to field

In the manuscript, the authors build on their recent characterisation of RNF220 as an important factor for non-proteolytic ubiquitinylation of factors for neural development. The authors provide evidence to indicate that the expression of markers of NA neurons within the locus coeruleus are dependent upon RNF220 and ZC4H2 expression, and that transcription factors Phox2a and Phox2b are influenced by the functions of RNF220 and ZC4H2 which, in turn, mediate downstream expression of genes for NA neuron development.
A series of immunostaining and in situ hybridisation studies are conducted to show temporal expression patterns for RNF220, ZC4H2, Phox2a and Phox2b in concert with DBH. Investigations with RNF220 KO embryos and ZC4H2 embryos demonstrate that detection of a panel of markers of NA neurons is significantly reduced in these. These are very interesting findings and highlight a putative causal relationship between RNF220 and ZC4H2, and LC NA development.

Next, a series of biochemical studies is conducted to provide evidence that RNF220 and ZC4H2 appear to covalently modify Phox2a and Phox2b protein on their respective lysine residues, such that a higher-molecular weight species of immunoblotted Phox2a and Phox2b (interpreted to be ubiquitinylated species of Phox2a and Phox2b) is evidence in co-transfection assays. The presence of the higher molecular weight species is related to the ligase activity of RNF220 in concert with ZC4H2, since experiments with a W539R ligase-dead variant of RNF220 does not lead to the presence of such signals for Phox2a and Phox2b. Similar findings for the absence of the higher molecular weight immunoblotted species for Phox2a/ and Phox2b are illustrated in lysates of RNF220 KO and ZC4H2 KO embryonic hindbrain tissues. The high molecular weight species is alluded to as a "monoubiquitinated Phox2a/Phox2b", but direct evidence of ubiquitination would need to be provided. Nevertheless, the evidence is interesting and constitutes an intriguing hypothesis that non-proteolytic ubiquitinylation underlies LC NA development in mice. However, there are several major concerns for the immunoblotting data which makes it difficult to evaluate what some of the panels really do represent (see below), and whether data or lysates, or both, might need to be checked for lanes represented across several figures.

Finally, the authors investigate the impact of RNF220 and ZC4H2 on TH and DBH mRNA expression in a series of transient transfection assays in which Phox2a and Phox2b is also co-transfected. The data is illustrated in Figure 5, and is interpreted by the authors to suggest that RNF220 and ZC4H2 influence Phox2a and Phox2b to drive TH and DBH expression. However, the data has several observations which are difficult to reconcile, considering that several experiments (5e, g, h) lack conditions which are necessary to evaluate the stimulating effects of RNF220 and ZC4H2 on Phox2a/b. My specific concerns for these are described below in detail.
Overall, the authors present a compelling hypothesis to substantiate the observations that reductions in LC NA markers in RNF220 and ZC4H2 deficient mouse embryos arises from a consequence of non-proteolytic ubiquitinylation actions on Phox2a and Phox2b. However, given the lack of direct evidence for ubiquitnylated Phox2a/2b species, questionable immunoblot data, and transfection experiments which are difficult to interpret; the authors will have to extensively revise the current manuscript in order for it to be reconsidered for publication.

Comments for the author

Major issues:
1. The paper describes "RNF220/ZC4H2 complex monoubiquitinates Phox2a/Phox2b", yet monoubiquitinylation is not directly demonstrated for Phox2a and Phox2b. Rather, data from Figure 4 interprets the presence of a larger molecular weight species on Western blots for Phox2a and Phox2b across multiple panels in Figure 4 to represent monoubiquitnylated Phox2a or -2b.
2. The premise to the mechanism for NA development by RNF220/ZC4H2/Phox2a/b is predicated on (mono)ubiquitinylated forms. As it stands, the data on ubiquitinylation is inferential, relying on studies with the ligase-dead W539R variant of RNF220 to suggest this. As a start, the higher molecular species for Phox2a and Phox2b have to be identified as ubiquitnylated forms of the protein.

3. There are several concerns regarding the data in Figure 4. Firstly, the series of immunoblotting signals for Figure 4e appear to be identical to 4f. Have the authors encountered some kind of problem with the data presentation in this figure? Secondly, the data from experiments in Figure 4i and 4j appear near-identical. Can the authors please check their data and primary lab recordsto confirm that the correct samples which are meant to be illustrated in this figure are in fact accurately portrayed? The same question is asked of data from Figures 4l and 4m– could the authors demonstrate that the lysates were in fact assayed from tissues of RNF220 KO embryos in Figure 4l, and that samples were collected from ZC4H2 KO embryos in Figure 4m?
4. This reviewer would like to see the original blots for all of the signals presented in data across experiments illustrated in Figure 4. As a minimum, please show this raw data(labelled for representative lanes) in any revised version of this manuscript.
5. The trends for data in Figure 5a-h seem near identical across the board. Yet, for Fig. 5e, why is the relative expression of TH markedly different between lane 1 and lane 5, which is clearly an experiment with identical conditions. This is difficult to understand. Furthermore, data in Fig. 5e is lacking a lane in which Phox2a(K178R) variant alone is transfected. Does Phox2a(K178R) alone stimulate TH expression?
6. For Fig 5g, why is the relative expression of TH markedly different between lane 1 and lane 5 (labelled from left to right of the graph), which is clearly an experiment with identical conditions. This is difficult to understand. Furthermore, data in Fig. 5g is lacking a lane in which Phox2b(K185R) variant alone is transfected, and relative TH levels are detected.
7. For Fig 5h, why is the relative expression of TH markedly different between lane 1 and lane 5 (labelled from left to right of the graph), which is clearly an experiment with identical conditions. This is difficult to understand. Furthermore, data in Fig. 5h is lacking a lane in which Phox2b(K185R) variant alone is transfected, and relative TH levels are detected.
8. Also, are equal quantities of plasmid constructs transfected between conditions, or are Lanes 1 and 5 mock transfections and, in fact, do not have plasmid DNA transfected in these samples?
9. Given that RNF220 is on chromosome X, have embryos been sexed and only male embryos used for experiments in this paper? Can the authors please account for this issue in their Methods section, such that readers are clear as to what lysates and tissues were collected for experiments with RNF220 KO animals in this study.
10. Paired and unpaired t-tests are underpowered and wholly inappropriate for use across the manuscript, particularly in the interpretation of data in Figure 5. An analysis of variance should be applied, together with an appropriate posthoc t-test corrected for multiple testing.

Minor issues:
1. In Lines 114-120, the authors discuss significant differences to the levels of ISH signal from genes investigated. This would suggest that the authors have quantified these signals in multiple independent E10.5 embryos to draw this statistical conclusion. Can the authors please provide this data to support their claim? If they didn't conduct statistical analysis, the authors should revise their statement to indicate that "there is no difference in ISH signal intensity and signal distribution for DBH, Phox2a and Phox2b in ZC4H2 KO embryos at E10.5".
2. The statement in Lines 117-118 “…dramatic reductions in DBH and Phox2a expression were found in ZC4H2 embryos….” Is incorrect and misleading. As shown in their data, DBH signal appears to be reduced, but Phox2a ISH signal is reduced to a lesser extent than DBH. Dramatic reductions were not found in both BDH and Phox2a.
3. In Lines 131-132, it is indicated that RNF220 mRNA distribution in E10.5 overlapped DBH transcripts, but it is difficult to determine the degree of overlap as the sections illustrated in Fig. 2a are not of the same size.
Can the authors please improve this, or repeat the experiments on thinner (7-8 micrometer thickness), serial sections to demonstrate this point better?
4. The authors have to provide an improved explanation as to how their findings lead to a better understanding of how NA deficiency may be involved in the development of intellectual disability in humans with ZC4H2 mutations? Merely citing a paper (Vogel-Hopker and Rohrer, 2002) which investigates BMP signalling in the development of locus coeruleus neurons is inadequate.
5. The representation of the data can be improved. ZC4H2 immunostaining in Figure 1a is difficult to interpret, given that there is no corresponding negative control to appreciate what constitutes as background signal.

6. In Figure 1a, fields for visualisation of DBH double-staining with Phox2a and Phox2b are drawn from different territories. Boxed inserts within the low-magnification field should be noted as to where these are located within each field of tissue imaged.

First revision

Author response to reviewers' comments

Reviewer 1
Advance Summary and Potential Significance to Field:
The manuscript by Song et al points to a novel mechanism of function of Phox2a and Phox2b transcription factors, previously shown to be critical for the development/specification of noradrenergic (NA) neurons. Given the importance of these neurons for normal nervous system function, their development is of considerable interest and despite the work of JF Brunet et al, it remains only partly understood. Another novelty factor is the potential regulation of Phox2 transcription factors by ubiquitination, which is also the mechanistically novel work in this manuscript. This is particularly significant, given the involvement in a neurodevelopmental disorder of one of the proteins that apparently regulate Phox2 function.
Thank you very much for your comments.

Reviewer 1
Comments for the Author:
The authors first use genetics to show that the ubiquitin E3 ligase RNF220 and its cofactor ZC4H2 are required for the development of NA neurons. Next, they use biochemical experiments to show interactions between these two proteins and Phox2 transcription factors, in vitro and in vivo, and that the ubiquitination function of RNF220 is required for Phox2 modification, in addition to providing structure-function evidence regarding the modification site and genetic evidence of these effects. Further biochemical experiments are used to argue that these interaction are important for transcriptional activity of Phox2 proteins on promoters related to NA specification. These conclusions are supported by in ovo manipulations in chick.

General comments and major issues:
The authors argue for ubiquitination of Phox2 proteins, and this is one of the main conclusions of the study, but the authors do not directly show that Phox2 modification (band size shift) is a result of direct ubiquitination by RNF220. Some sort of a direct experiment is required to draw this conclusion. For example, using tagged Ubiquitin and showing that it ends up on Phox2 proteins.
Thanks for your valuable suggestion.
We carried out the *in vivo* ubiquitination assays using HA-tagged ubiquitin in HEK293 cells and found that the modifications of both Phox2a and Phox2b were indeed a result of mono-ubiquitination by RNF220. The results are shown in the revised Figure 5D and Supplementary Figure 4D.

The in ovo electroporations, which are basically in vivo demonstration that RNF220/ZC4H2 work together with Phox2's should have RNF220/ZC4H2-alone and Phox-alone controls. Without these, the experiment is difficult to interpret.
Thanks again for your suggestion.
As you suggested, three additional groups of *in ovo* electroporations were performed: wild type Phox2a/Phox2b, Phox2a/Phox2b KR mutants and RNF220/ZC4H2.
Compared with data of in ovo electroporation of both RNF220/ZC4H2 and Phox2, ectopic expression of DBH was weakly induced in wide-type Phox2a/2b or their KR mutants overexpressed chick neural tube while hardly detected in RNF220/ZC4H2 complex overexpressed one. The results are shown in the revised Supplementary Figure 5M.

There are essentially no instances of the authors reporting the number of replicates for any of the experiments. This is not acceptable and without these values, the manuscript should not be published.

All of our IF and ISH assays were carried out on at least 3 indicated embryos. All of our WB, EMSA, luciferase reporter and real-time qPCR assays were conducted at least 3 times. This information is included in the revised Methods and Results.

For the biochemical analyses, quantification of the blots must be done for experiments in which there are quantitative effects (Fig. 4KLM). Number of replicates must be reported for all the blots. To be more explicit: all experiments must describe number of replicates.
All the mentioned experiments were carried out 3 times, and we added the information and statistic data in the revised Figure 5G-I and its legends.

Overall, the quality of the data is good, but the authors' arguments would be more compelling with some specific improvements to the manuscript (see below).

Detailed comments:
Introduction:
-Line 71 – Please elaborate on the nature of ZC4H2. Is it DNA binding? How does it exert its function? Compare this to RNF220 (an E3 ligase) which is better described.
ZC4H2 encodes a C4H2 type zinc-finger nuclear factor and is required to prevent RNF220 ubiquitination and degradation (Ma *et al.*, 2019b; and our unpublished observations). Whether ZC4H2 is DNA binding remains to be tested. Related information is included in the revised manuscript.

-Line 76 – What is Sin3B and what is its relevance here? Is it related to Wnt signalling?
Sin3B is a global regulator of gene transcription, which serves as an essential scaffold protein of the Sin3/HDAC corepressor complex. Our previous study indicated that RNF220 binds with Sin3B for the polyubiquitination modification and proteosomal degradation, with the physiological role so far unknown (Kong *et al.*, 2010). RNF220 enhances Wnt signaling through USP7-mediated deubiquitination of β-catenin (Ma *et al.*, 2014), likely independent of Sin3B. We revised the related expressions in the manuscript.

-Line 79-83 – Please elaborate on these mechanisms, briefly. The nature of the interaction between ZC4H2 and RNF220 should be proposed clearly here.
Our and Kim's recent studies have reported that ZC4H2 and RNF220 show similar expression patterns in the spinal tubes and both are required for normal vertebrate spinal cord patterning through modulation of Shh/Gli signaling (Ma *et al.*, 2019a and Ma *et al.*, 2019b). In addition, we also demonstrated that ZC4H2 interacts with RNF220 and enhance RNF220 protein stability *in vitro* and *in vivo* (Ma *et al.*, 2019a; Kim *et al.*, 2018). We revised the related expressions in the manuscript.

-Numerous spelling and grammatical errors throughout the manuscript. Consider revising.
Thank you. We carefully revised our manuscript to avoid such errors.

Results:
Generally the quality of images is low and unconvincing. The authors are suggested to provide high resolution images to replace lower resolution images and to provide high magnification insets to bolster their claims. These (and other suggestions) are listed in the order of chapters:

ZC4H2 is required for LC NA neuron development
Fig. 1b) Consider adding high magnification/resolution insets to e12.5 experiments, as the color contrast between DBH and the other genes are difficult to distinguish.
Fig. 1d) Resolution is very low. Consider providing increased resolution images as well as high resolution insets.
Line 104 & Supplemental – Consider demonstrating proliferation with hindbrain progenitor zone markers or PCNA/Ki67. Nevertheless, the location of cells seems to be well outside the ventricular zone.
Fig. 1g) The reduction in Phox2b signal here is very slight and not convincing. Consider an inset to illustrate this.
Thank you for your suggestion.

We added insets with high magnification in Figures 1, 2 and Supplementary Figure 1. For all of ZC4H2^{+} neurons in presumptive LC is non-BrdU^{+} (Supplementary Figure 1C) suggesting that ZC4H2 is expressed in post-mitotic NA neurons.

Supplemental Figure 2 – It's not necessary to include these data, as the point was already well made, but there should be cell counts for at least an n=3 for each group and a statistical comparison.
Thank you for your suggestion. With this figure we hope to show the reduction of NA neurons in ZC4H2$^{+/-}$ mice, which is likely due to random inactivation of the wild type ZC4H2 allele on the X-chromosome. We counted the DBH^{+} neurons in ZC4H2 and RNF220 KO mice and included the statistics in the revised Figure 1 and Figure 3.

RNF220 is required for LC NA neuron development
Supplemental 1d) RNF220 and ZC4H2 are expressed similarly, but it is not convincing that these are expressed in the same cells. I recommend high resolution microscopy with insets as well as counts of RNF220, ZC4H2 and their overlap. At this resolution it is not possible to identify individual cells, so it is not possible to quantify overlap.
Thank you for your suggestion. We performed the double labeling of RNF220 mRNA and ZC4H2 protein again and added high magnification insets in the revised Supplementary Figure 1D.

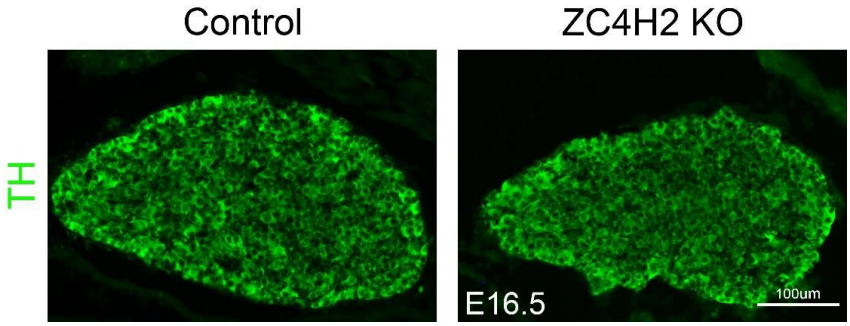
Figure 3 – General comments
Restructure this figure for clarity. The first two rows have ZC4H2 KO and then beneath RNF220 KO, however the last row is a mix. Add two extra rows structured the same as the first two rows, and demonstrate Peri/TH staining in the ZC4H2 LC as well (as it is indicated in the text but not shown).
Thank you for your suggestion. We performed the Peri/TH staining in control and ZC4H2 KO embryos, rearranged the panels and added the results in the revised Figure 4.

Line 151 – Elaborate the meaning of "residual mRNA". What is the nature of the KO? Does a truncated transcript persist in KO mice?
Thank you for your comments. In the KO mice, the first coding exon (which encodes roughly the first half of the RNF220 protein) was deleted. The "residual mRNA" means the truncated transcript, which should not be functioning even if a truncated protein is produced. To avoid confusion to the readers and for the point below, these sentences were deleted in the revised manuscript.

Supplemental Fig 3 – This is not convincing evidence for the loss of neurons, as a multitude of reasons could result in a loss of mRNA. It may be beyond the scope of this paper to prove this claim, but potential future experiments could use a Cre-marker to label these neurons prior to the effects of the knockout (e.g Phox2b:Cre + Cre-reporter + KO) and demonstrating that Cre-reporter signal is lost in KO mice. Again, this is involved and unnecessary, but you cannot make the claim that neurons do not survive with your current data.
Thank you for your comment. We agree with you that our present data cannot reach the conclusion that NA neurons were dead at E16.5 and we deleted the related description in the revised manuscript.

Line 163 – Clarify that TH staining in Sympathetic ganglia was not affected. Staining at a later stage (e.g e16.5 would be more convincing to demonstrate that no disruption has occurred).
Line 165-167 – There is no suggestion that these proteins coordinate their functions based on the data presented so far. Please revise this conclusion.

Thank you for your suggestion. We performed TH immunofluorescence in E16.5 sympathetic ganglia and found no difference between control and ZC4H2 KO mice (please refer to Figure R1 below).
Figure R1. TH immunostaining in sympathetic ganglion in control and ZC4H2 KO mice at E16.5.



The RNF220/ZC4H2 complex interacts with and monoubiquitinates Phox2a/Phox2b
Line 194-196 – The ability of a dual knock-down approach to demonstrate a decrease in TH transcript is a good argument for interaction between these genes. This should be emphasized when discussing their coordination, not the individual KO studies in prior figures.
Thank you for your suggestion. The TH/DBH expression was unchanged in single knockdown of ZC4H2/RNF220, which may be due to possible presence of residual ZC4H2/RNF220 complex still working in the process of monoubiquitination of Phox2a/Phox2b. We discussed it in the revised manuscript.

- Figure 4K) The decrease in Phox2a here is slight. Consider performing a densitometry analysis on multiple samples.
Thank you for your suggestion. We added the statistical information in the revised Figure 5.

RNF220/ZC4H2-mediated monoubiquitination of Phox2a/Phox2b favors Phox2a/Phox2b transactivity
-A missing control here should be done: Phox2a/b + ZC4H2 together, without other factors. **Thank you for your suggestion. We repeated the experiments with the indicated groups and new data are included in the revised Figure 5.**

-Appropriate controls should be done for in-ovo electroporation experiments to demonstrate that the constructs used express their corresponding proteins (see above).
As you suggested, three additional groups of *in ovo* electroporations were performed: wild type Phox2a/Phox2b, Phox2a/Phox2b KR mutant and RNF220/ZC4H2. We have checked the overexpressing efficiency of the constructs *in vitro* and *in vivo* by Western blot or *in situ* hybridization (data not shown). The results are shown in the revised Supplementary Figure 5M.

References

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Kong, Q., Zeng, W., Wu, J., Hu, W., Li, C. and Mao, B. (2010) 'RNF220, an E3 ubiquitin ligase that targets Sin3B for ubiquitination', Biochem Biophys Res Commun 393(4): 708-13.
Ma, P., Yang, X., Kong, Q., Li, C., Yang, S., Li, Y. and Mao, B. (2014) 'The ubiquitin ligase RNF220 enhances canonical Wnt signaling through USP7-mediated deubiquitination of beta-catenin', Mol Cell Biol 34(23): 4355-66.
Ma, P., Song, N. N., Cheng, X., Zhu, L., Zhang, Q., Zhang, L., Yang, X., Wang, H., Kong, Q., Shi, D. et al. (2019a) 'ZC4H2 stabilizes RNF220 to pattern ventral spinal cord through modulating Shh/Gli signaling', J MOL CELL BIOL.
Ma, P., Song, N. N., Li, Y., Zhang, Q., Zhang, L., Zhang, L., Kong, Q., Ma, L., Yang, X., Ren, B. et al. (2019b) 'Fine-Tuning of Shh/Gli Signaling Gradient by Non-proteolytic Ubiquitination during Neural Patterning', Cell Rep 28(2): 541-553 e4.

Reviewer 2

Advance Summary and Potential Significance to Field:
In their manuscript, Song et al investigate the role of the ubiquitin E3 ligase RNF220 and its co-factor ZC4H2 in the development of noradrenergic (NA) neurons in the locus coeruleus (LC). They show that both genes are expressed in these neurons and that mutations in RNF220 or in ZC4H2 interfere with their development. They continue to demonstrate that RNF220 and ZC4H2 form a ternary complex with the Phox2a/b transcription factors and mono-ubiquitinates these factors in cell culture and in vivo. This post-translational modification increases the transcriptional activity of Phox2a/b. Taken together, these are interesting findings which shed new light on how the development of LC NA neurons is controlled at a molecular level through modification of crucial transcription factors but there are a number of caveats the author need to address:

Reviewer 2 Comments for the Author:
Major points

1) The finding that RNF220 and ZC4H2 interact to modify a critical transcription factor during neural development is not entirely novel and was described in a several papers by this and another group (see Kim et al., 2018; Ma et al., 2019a/b).
It is not surprising to identify more targets for RNF220. We feel that the specific roles and mechanism of RNF220/ZC4H2 in LC NA neurons established in this study should be of general interest.

2) The authors postulate that mono-ubiquitination of Phox2a/b increases the transcriptional activity of these transcription factors but their measurements of DBH and TH transcription levels leaves open other interpretations. Moreover, a mechanism was not investigated. It is conceivable that Phox2a/b regulate DBH and TH transcription indirectly by controlling the expression of another transcription factor. The authors should confirm that Phox2a/b directly bind to the DBH and TH promoters by ChIP experiments.

They should also investigate in band shift analyses whether mono-ubiquitinylation of Phox2a/b affects their DNA binding.

Moreover, they should use luciferase assays using the DBH and TH promoter regions to show that Phox2a/b can transactivate these promoters and how ubiquitinylation affects this transactivation.
Thank you for your suggestions.
There is no available commercial Phox2a/2b antibodies for ChIP experiments. The binding motif in human DBH promoter was previously identified (Seo *et al*, 2002). We thus carried out a series of EMSA experiments to demonstrate whether the monoubiquitination of Phox2a/2b by RNF220/ZC4H2 complex is able to enhance the DNA binding activities of Phox2a/2b. In addition, we construct a human DBH promoter reporter and carried out luciferase reporter assays. The results showed that the mono-ubiquitination of Phox2a/2b by RNF220/ZC4H2 complex indeed enhanced the DNA binding ability and transactivities of Phox2a/2b. These data are included in the revised Figure 6 and Supplementary Figure 5.

Finally, RFN220 and ZC4H2 might control nuclear localisation of the Phox2a/b transcription factors.
Thank you for your suggestion.
We conducted the IF assays in HEK293 cells to examine whether the subcellular localization of Phox2a and Phox2b is affected by co-expression RNF220/ZC4H2. The results showed that that both Phox2a and Phox2b showed nuclear location irrelevant with the presence of RNF220/ZC4H2 (please refer to Figure R2 below).

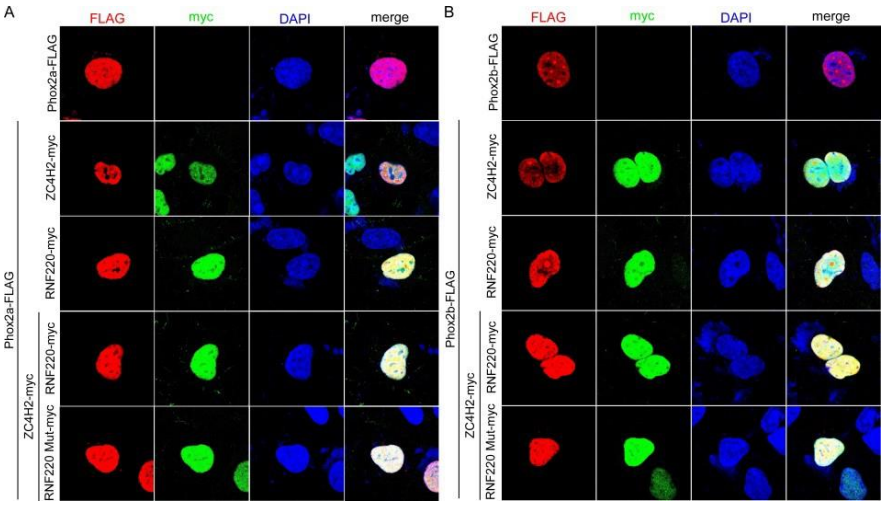

Figure R2. IF assays show that Phox2a (A) and Phox2b (B) cellular location when RNF220/ZC4H2 complex were overexpressed in HEK293 cells.

3) The authors focus their analysis on the effect of Rnf220/ZC4H2 on the Phox2a/b transcription factors but they previously reported that ZC4H2 also controls Bmp signalling. As Bmp signalling is required for the specification of NA progenitors this raises the possibility that NA progenitor development is affected but this is not investigated by the authors.

As ZC4H2 is expressed in non-BrdU^{+} post-mitotic NA neurons (outside the ventricular zone) in E10.5 and older embryos (Figure 1 and Supplementary Figure 1), we expected ZC4H2/RNF220-involved regulation of NA neuron development is more likely acts on post- mitotic NA cells rather than directly regulating BMPs. Besides, we checked the expression of Mash1, a downstream direct target of BMPs, in E10.5 embryos, and its expression was comparable in both control and RNF220/ZC4H2 KO mice. Based on the observations, we think that the BMP signaling is less likely involved in RNF220/ZC4H2-mediated NA neuron differentiation.

4) In general, the manuscript contains very little quantification. The authors should at least aim to quantify the levels of mono-ubiquitinated Phox2a/b in CATH1a cells and in the mid/hindbrain (Figure 4k-m). Moreover, the authors have to ensure that the correct statistical tests are employed. Multiple t-test were used to evaluate the transcriptional activities of Phox2a/b but this increases the risk of type 1 errors. The authors should use an ANOVA followed by a post-hoc test.

Thank you for your comments.

We included the statistics and related description in the revised Figure 5 and its legend. Also, we replaced all the *t*-test evaluation with the one-way ANOVA evaluation in the indicated figures.

Minor points

1) On page 8, the authors should explain the strategy used to inactivate Rnf220 and why an exon 1-3 probe can be used to detect the fate of mutant cells. In this respect, it remains unclear what the fate of the mutant cells is. Do they die or do they switch fate?

Thank you for your suggestion.

We agree that we cannot reach a conclusion on the fate of the NA neurons based on the current data, as pointed out also by reviewer 1. We revised the description and discussed this in the revised manuscript.

2) In the discussion, the authors state that a lack of Phox2a/b modification may account for the down-regulated expression of Phox2a (line 245) but it remains unclear how this could work. Does Phox2a auto-regulate its own expression? The authors should cite evidence for such a mechanism or

modify their statement.
Thank you for your suggestion.
Phox2a autoregulation was indeed reported previously (Rychlik *et al*., 2005). We assume that Phox2a downregulation is likely due to deficient autoregulation. We discussed it in the revised manuscript.

3) There are a number of sloppy formulations:
Line 40: … and some other groups …
Line 73: … and other issues …
Line 81: "reactors" should read transcription factor
Line 112: it should read: … is expressed …
Line 120: it should read: …nor Phox2a/Phox2b ….
Line 173: "but not other factors". What are these factors and why were they investigated? Line 253: is it share or exhibit?
Thank you for your careful reading. We revised all the sloppy formulations throughout the manuscript carefully.

References
Rychlik, J. L., Hsieh, M., Eiden, L. E. and Lewis, E. J. (2005) 'Phox2 and dHAND transcription factors select shared and unique target genes in the noradrenergic cell type', J Mol Neurosci 27(3): 281-92.
Seo, H., Hong, S. J., Guo, S., Kim, H. S., Kim, C. H., Hwang, D. Y., Isacson, O., Rosenthal, A. and Kim, K. S. (2002) 'A direct role of the homeodomain proteins Phox2a/2b in noradrenaline neurotransmitter identity determination', J Neurochem 80(5): 905-16.

Reviewer 3
Advance Summary and Potential Significance to Field
In the manuscript, the authors build on their recent characterisation of RNF220 as an important factor for non-proteolytic ubiquitinylation of factors for neural development. The authors provide evidence to indicate that the expression of markers of NA neurons within the locus coeruleus are dependent upon RNF220 and ZC4H2 expression, and that transcription factors Phox2a and Phox2b are influenced by the functions of RNF220 and ZC4H2 which, in turn, mediate downstream expression of genes for NA neuron development. A series of immunostaining and in situ hybridisation studies are conducted to show temporal expression patterns for RNF220, ZC4H2, Phox2a and Phox2b in concert with DBH. Investigations with RNF220 KO embryos and ZC4H2 embryos demonstrate that detection of a panel of markers of NA neurons is significantly reduced in these. These are very interesting findings and highlight a putative causal relationship between RNF220 and ZC4H2, and LC NA development.
Next, a series of biochemical studies is conducted to provide evidence that RNF220 and ZC4H2 appear to covalently modify Phox2a and Phox2b protein on their respective lysine residues, such that a higher-molecular weight species of immunoblotted Phox2a and Phox2b (interpreted to be ubiquitinylated species of Phox2a and Phox2b) is evidence in co-transfection assays. The presence of the higher molecular weight species is related to the ligase activity of RNF220 in concert with ZC4H2, since experiments with a W539R ligase-dead variant of RNF220 does not lead to the presence of such signals for Phox2a and Phox2b. Similar findings for the absence of the higher molecular weight immunoblotted species for Phox2a/ and Phox2b are illustrated in lysates of RNF220 KO and ZC4H2 KO embryonic hindbrain tissues. The high molecular weight species is alluded to as a "monoubiquitinated Phox2a/Phox2b", but direct evidence of ubiquitination would need to be provided. Nevertheless, the evidence is interesting and constitutes an intriguing hypothesis that non-proteolytic ubiquitinylation underlies LC NA development in mice. However, there are several major concerns for the immunoblotting data which makes it difficult to evaluate what some of the panels really do represent (see below), and whether data or lysates, or both, might need to be checked for lanes represented across several figures.
Finally, the authors investigate the impact of RNF220 and ZC4H2 on TH and DBH mRNA expression in a series of transient transfection assays in which Phox2a and Phox2b is also co- transfected. The data is illustrated in Figure 5, and is interpreted by the authors to suggest that RNF220 and ZC4H2 influence Phox2a and Phox2b to drive TH and DBH expression. However, the data has several observations which are difficult to reconcile, considering that several experiments (5e, g, h) lack conditions which are necessary to evaluate the stimulating effects of RNF220 and ZC4H2 on

Phox2a/b. My specific concerns for these are described below in detail. Overall, the authors present a compelling hypothesis to substantiate the observations that reductions in LC NA markers in RNF220 and ZC4H2 deficient mouse embryos arises from a consequence of non-proteolytic ubiquitinylation actions on Phox2a and Phox2b. However, given the lack of direct evidence for ubiquitnylated Phox2a/2b species, questionable immunoblot data, and transfection experiments which are difficult to interpret; the authors will have to extensively revise the current manuscriptin order for it to be reconsidered for publication.

Thank you for comments. We carefully revised the manuscript and figures according to your suggestions/comments, and hope our revised manuscript meet the criteria of publication in Development.

Comments for the Author:
Reviewer 3 Comments for the Author:

Major issues:

1. The paper describes "RNF220/ZC4H2 complex monoubiquitinates Phox2a/Phox2b", yet monoubiquitinylation is not directly demonstrated for Phox2a and Phox2b. Rather, data from Figure 4 interprets the presence of a larger molecular weight species on Western blots for Phox2a and Phox2b across multiple panels in Figure 4 to represent monoubiquitnylated Phox2a or -2b.

Thank you for your suggestion.

We carried out *in vivo* ubiquitination assays using HA-tagged ubiquitin in HEK293 cells and found that the modifications of both Phox2a and Phox2b were indeed a result of mono- ubiquitination by RNF220/ZC4H2 complex. These results are included in the revised Figure 5 and Supplementary Figure 4.

2. The premise to the mechanism for NA development by RNF220/ZC4H2/Phox2a/b is predicated on (mono)ubiquitinylated forms. As it stands, the data on ubiquitinylation is inferential, relying on studies with the ligase-dead W539R variant of RNF220 to suggest this. As a start, the higher molecular species for Phox2a and Phox2b have to be identified as ubiquitnylated forms of the protein.

Thank you for your suggestion. Due to the fact that the higher molecular species for Phox2a and Phox2b are indeed ubiquitnylated forms (revised Figure 5 and Supplementary Figure 4) and the changes of the molecular weight, we assume that the modification is most likely mono-ubiquitination.

3. There are several concerns regarding the data in Figure 4. Firstly, the series of immunoblotting signals for Figure 4e appear to be identical to 4f. Have the authors encountered some kind of problem with the data presentation in this figure? Secondly, the data from experiments in Figure 4i and 4j appear near-identical. Can the authors please check their data and primary lab records to confirm that the correct samples which are meant to be illustrated in this figure are in fact accurately portrayed? The same question is asked of data from Figures 4l and 4m– could the authors demonstrate that the lysates were in fact assayed from tissues of RNF220 KO embryos in Figure 4l, and that samples were collected from ZC4H2 KO embryos in Figure 4m?

Thanks for pointing out this critical point. The original Figure 4e and 4f were indeed identical, which is accidently duplicated when we prepared the figure, as the images for Phox2a and Phox2b are very similar. The other panels are all correct. To make it more clearly for readers to follow, we included the data on only Phox2a in the revised Figure 5 and put that on Phox2b in the Supplementary Figure 4. The revised panel for Phox2b is now include in the revised Supplementary Figure 4. We provided all the original blot for each panel in revised Figure 5 and Supplementary Figure 4 (all from the original figure 4) for your reference. We do appreciate your careful reading and pointing this mistake.

4. This reviewer would like to see the original blots for all of the signals presented in data across experiments illustrated in Figure 4. As a minimum, please show this raw data (labelled for representative lanes) in any revised version of this manuscript.

Thanks again. We included all the original blot for every panel in the revised Figure 5 and Supplementary Figure 4 (all from the original Figure 4) for your reference.

5. The trends for data in Figure 5a-h seem near identical across the board. Yet, for Fig. 5e, why is

the relative expression of TH markedly different between lane 1 and lane 5, which is clearly an experiment with identical conditions. This is difficult to understand. Furthermore, data in Fig. 5e is lacking a lane in which Phox2a (K178R) variant alone is transfected. Does Phox2a(K178R) alone stimulate TH expression?

6. For Fig 5g, why is the relative expression of TH markedly different between lane 1 and lane 5 (labelled from left to right of the graph), which is clearly an experiment with identical conditions. This is difficult to understand. Furthermore, data in Fig. 5g is lacking a lane in which Phox2b(K185R) variant alone is transfected, and relative TH levels are detected.

7. For Fig 5h, why is the relative expression of TH markedly different between lane 1 and lane 5 (labelled from left to right of the graph), which is clearly an experiment with identical conditions. This is difficult to understand. Furthermore, data in Fig. 5h is lacking a lane in which Phox2b(K185R) variant alone is transfected, and relative TH levels are detected.

8. Also, are equal quantities of plasmid constructs transfected between conditions, or are Lanes 1 and 5 mock transfections and, in fact, do not have plasmid DNA transfected in these samples?

For your issues 5-8:

Thank you for your careful reading.

For the original Figure 5e, g, h, we indeed mislabeled a "-" as "+", which results in the misunderstanding. We corrected this in the revised Figure 6E and Supplementary Figure 5I 5J.

9. Given that RNF220 is on chromosome X, have embryos been sexed and only male embryos used for experiments in this paper? Can the authors please account for this issue in their Methods section, such that readers are clear as to what lysates and tissues were collected for experiments with RNF220 KO animals in this study.

It is ZC4H2, not RNF220, on chromosome X.

We have found similar phenotypes in NA neurons of female and male ZC4H2 KO mice, the results shown in ZC4H2 KO mice were from both female and male KO embryos. The results related to ZC4H2 heterozygotes are all from female embryos. We add this statement in the Methods and Material part of the revised manuscript.

10. Paired and unpaired t-tests are underpowered and wholly inappropriate for use across the manuscript, particularly in the interpretation of data in Figure 5. An analysis of variance should be applied, together with an appropriate posthoc t-test corrected for multiple testing.

Thank you for your comments. We replaced all the *t*-test evaluation with one way ANOVA evaluation for the statistics in the revised Figure 6 and Supplementary Figure 5.

Minor issues:

1. In Lines 114-120, the authors discuss significant differences to the levels of ISH signal from genes investigated. This would suggest that the authors have quantified these signals in multiple independent E10.5 embryos to draw this statistical conclusion. Can the authors please provide this data to support their claim? If they didn't conduct statistical analysis, the authors should revise their statement to indicate that "there is no difference in ISH signal intensity and signal distribution for DBH, Phox2a and Phox2b in ZC4H2 KO embryos at E10.5".

Thanks for your question.

All of our IF and ISH assays were carried out on least 3 indicated embryos. Because ZC4H2 is not expressed in the primodium (dorsal r1) of LC NA neurons at the stage of E10.5, we expect the expression of DBH was not changed in E10.5 KO mice. However, as we did not qualify the ISH signal at E10.5, we revised the description to "The ISH signal intensity and signal distribution for DBH, Phox2a and Phox2b in ZC4H2 KO embryos appeared not to be changed significantly at E10.5".

2. The statement in Lines 117-118 "…dramatic reductions in DBH and Phox2a expression were found in ZC4H2 embryos…." Is incorrect and misleading. As shown in their data, DBH signal appears to be reduced, but Phox2a ISH signal is reduced to a lesser extent than DBH. Dramatic reductions were not found in both BDH and Phox2a.

Thanks for your suggestion. The reduction of Phox2a mRNA is not as significant as DBH mRNA and we revised the description in the manuscript

3. In Lines 131-132, it is indicated that RNF220 mRNA distribution in E10.5 overlapped DBH transcripts, but it is difficult to determine the degree of overlap as the sections illustrated in Fig.

2a are not of the same size. Can the authors please improve this, or repeat the experiments on thinner (7-8 micrometer thickness), serial sections to demonstrate this point better?
Thanks for your suggestion. We performed the ISH of DBH and RNF220 in the same wild type embryos again and the new images with high quality are included in the revised Figure 3A.

4. The authors have to provide an improved explanation as to how their findings lead to a better understanding of how NA deficiency may be involved in the development of intellectual disability in humans with ZC4H2 mutations? Merely citing a paper (Vogel-Hopker and Rohrer, 2002) which investigates BMP signalling in the development of locus coeruleus neurons is inadequate.
Mutations in ZC4H2 have been reported to be associated with various disorders, including arthrogryposis multiplex congenita (AMC), X-linked intellectual disability (XLID), Wieacker- Wolff syndrome and Miles-Carpenter syndrome (Hirata *et al*., 2013; Kondo *et al*., 2018; May *et al*., 2015). Dysfunction of the NA system has been implicated in both psychiatric and neurodegenerative disorders, such as cognition decline, anxiety, depression, and Parkinson's and Alzheimer's diseases (Itoi and Sugimoto, 2010; Sara, 2009; Szot, 2012). Although we lack the direct evidence to show NA differentiation defect is linked to ZC4H2 related diseases, here we provided a possible clue for future studies.

5. The representation of the data can be improved. ZC4H2 immunostaining in Figure 1a is difficult to interpret, given that there is no corresponding negative control to appreciate what constitutes as background signal.
Thanks for your comment. We performed ZC4H2 immunostaining at E10.5, E11.5 and E12.5, no specific signals at E10.5 was found as compared with that at E11.5 and E12.5 in presumptive NA neurons, so we think that ZC4H2 is not expressed in NA neurons at E10.5.

6. In Figure 1a, fields for visualisation of DBH double-staining with Phox2a and Phox2b are drawn from different territories. Boxed inserts within the low-magnification field should be noted as to where these are located within each field of tissue imaged.
Thanks for your suggestion. We added inserts with high magnification in revised Figure 1 and Figure 2.

References

Hirata, H., Nanda, I., van Riesen, A., McMichael, G., Hu, H., Hambrock, M., Papon, M. A., Fischer, U., Marouillat, S., Ding, C. et al. (2013) 'ZC4H2 mutations are associated with arthrogryposis multiplex congenita and intellectual disability through impairment of central and peripheral synaptic plasticity', Am J Hum Genet 92(5): 681-95.
Itoi, K. and Sugimoto, N. (2010) 'The brainstem noradrenergic systems in stress, anxiety and depression', J Neuroendocrinol 22(5): 355-61.
Kondo, D., Noguchi, A., Takahashi, I., Kubota, H., Yano, T., Sato, Y., Toyono, M., Sawaishi, Y. and Takahashi, T. (2018) 'A novel ZC4H2 gene mutation, K209N, in Japanese siblings with arthrogryposis multiplex congenita and intellectual disability: characterization of the K209N mutation and clinical findings', Brain Dev 40(9): 760-767.
May, M., Hwang, K. S., Miles, J., Williams, C., Niranjan, T., Kahler, S. G., Chiurazzi, P., Steindl, K., Van Der Spek, P. J., Swagemakers, S. et al. (2015) 'ZC4H2, an XLID gene, is required for the generation of a specific subset of CNS interneurons', Hum Mol Genet 24(17): 4848-61.
Sara, S. J. (2009) 'The locus coeruleus and noradrenergic modulation of cognition', Nat Rev Neurosci 10(3): 211-23.
Szot, P. (2012) 'Common factors among Alzheimer's disease, Parkinson's disease, and epilepsy: possible role of the noradrenergic nervous system', Epilepsia 53 Suppl 1: 61-6.

OriginalblotofFigure5 andFigureS5

For reviewer 3reference

Figure 5a

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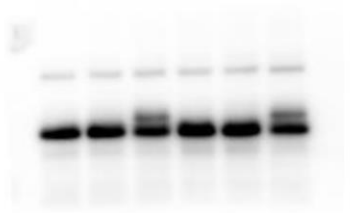

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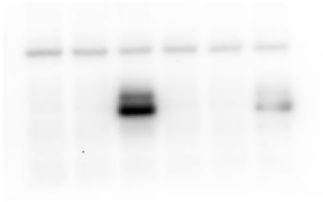

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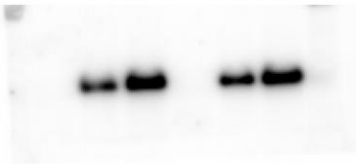

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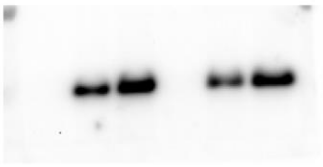

Figure 5b

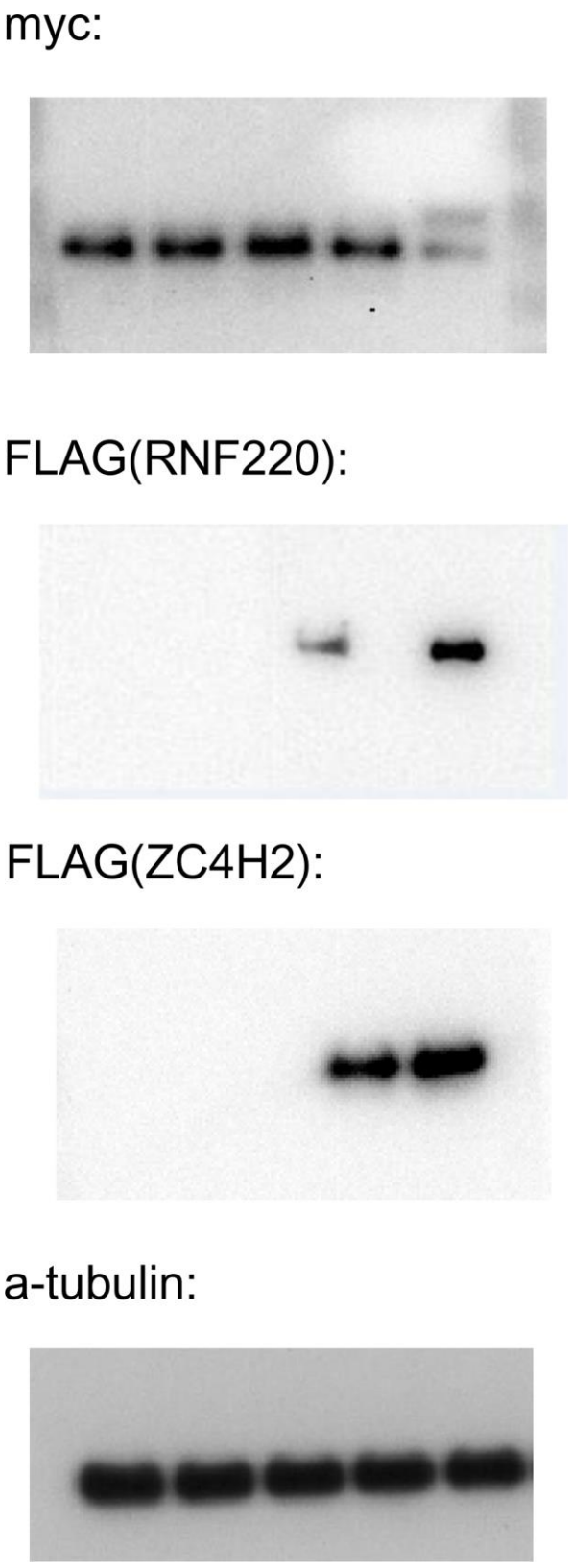

Figure 5c

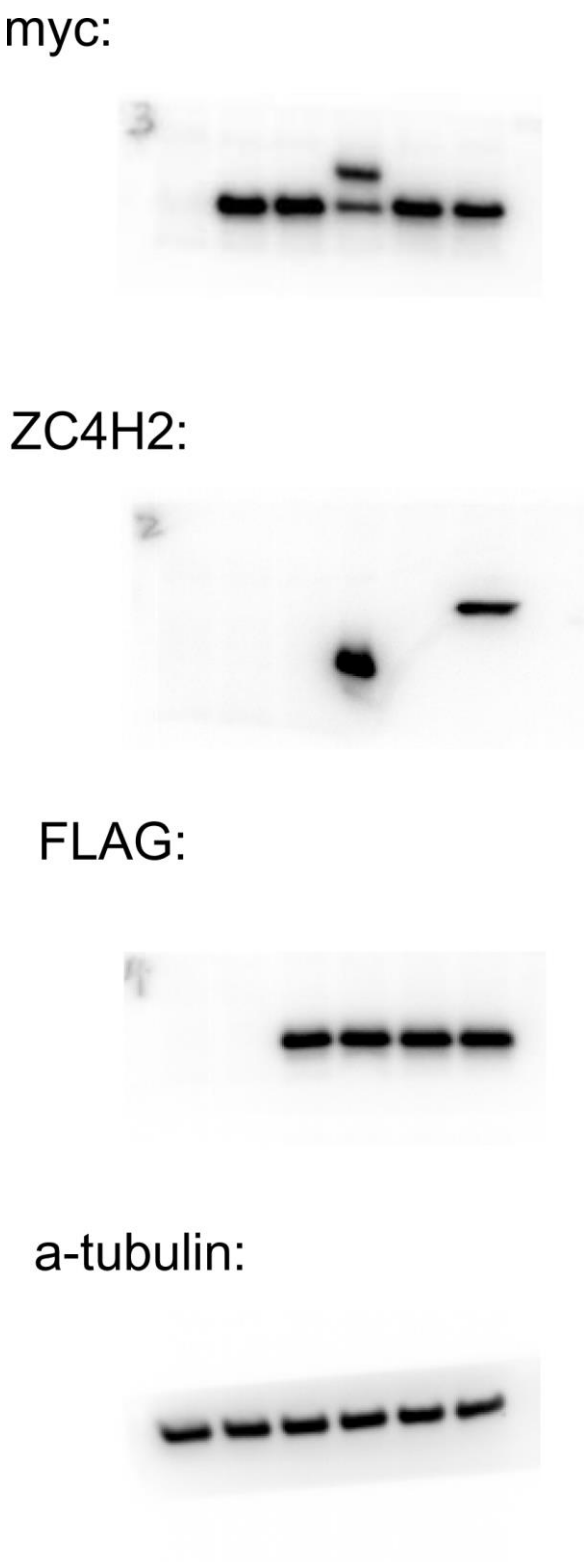

Figure 5d

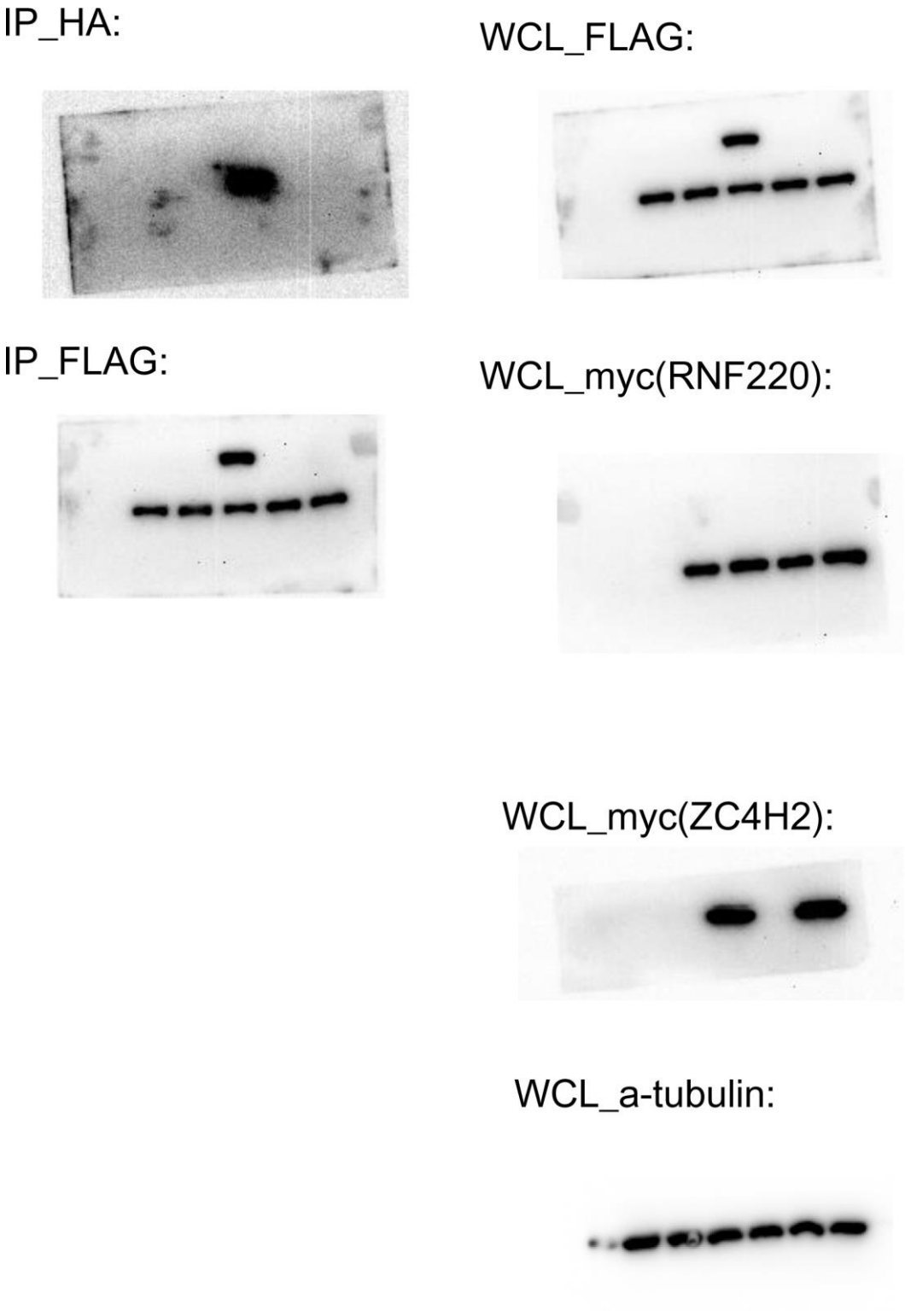

Figure 5e

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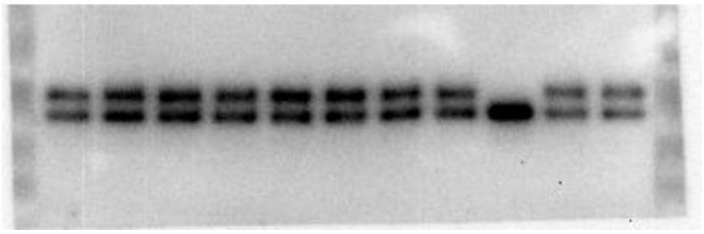

Figure 5f

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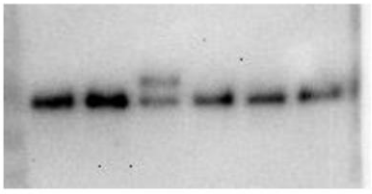

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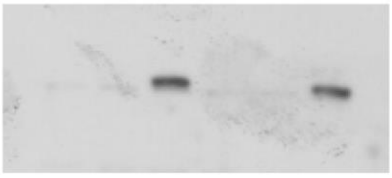

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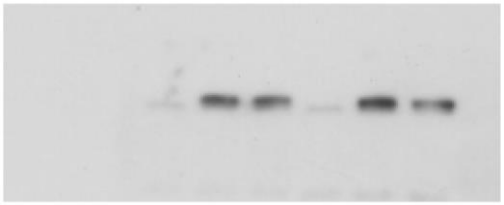

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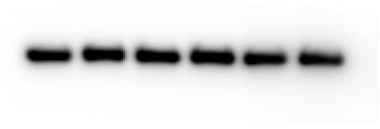


Figure 5g

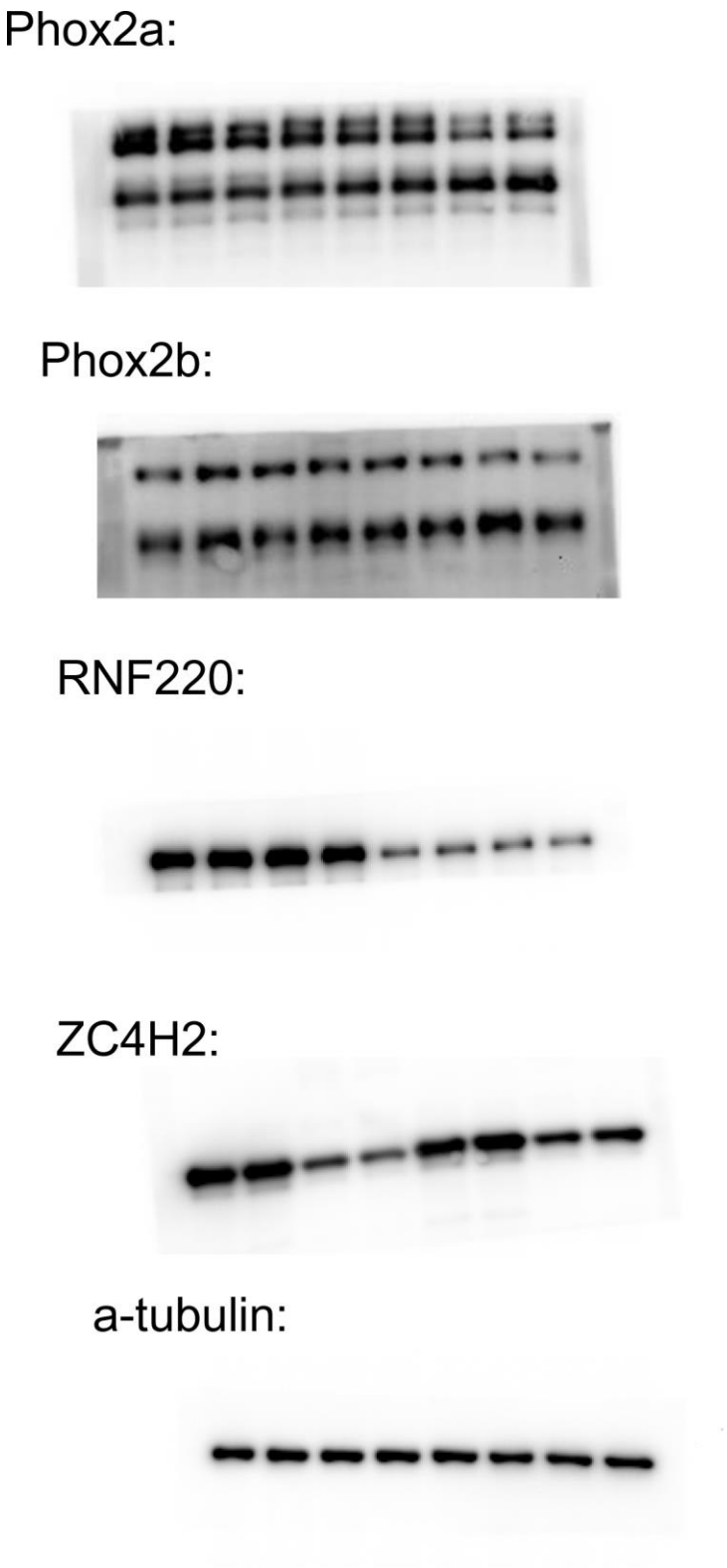


Figure 5h

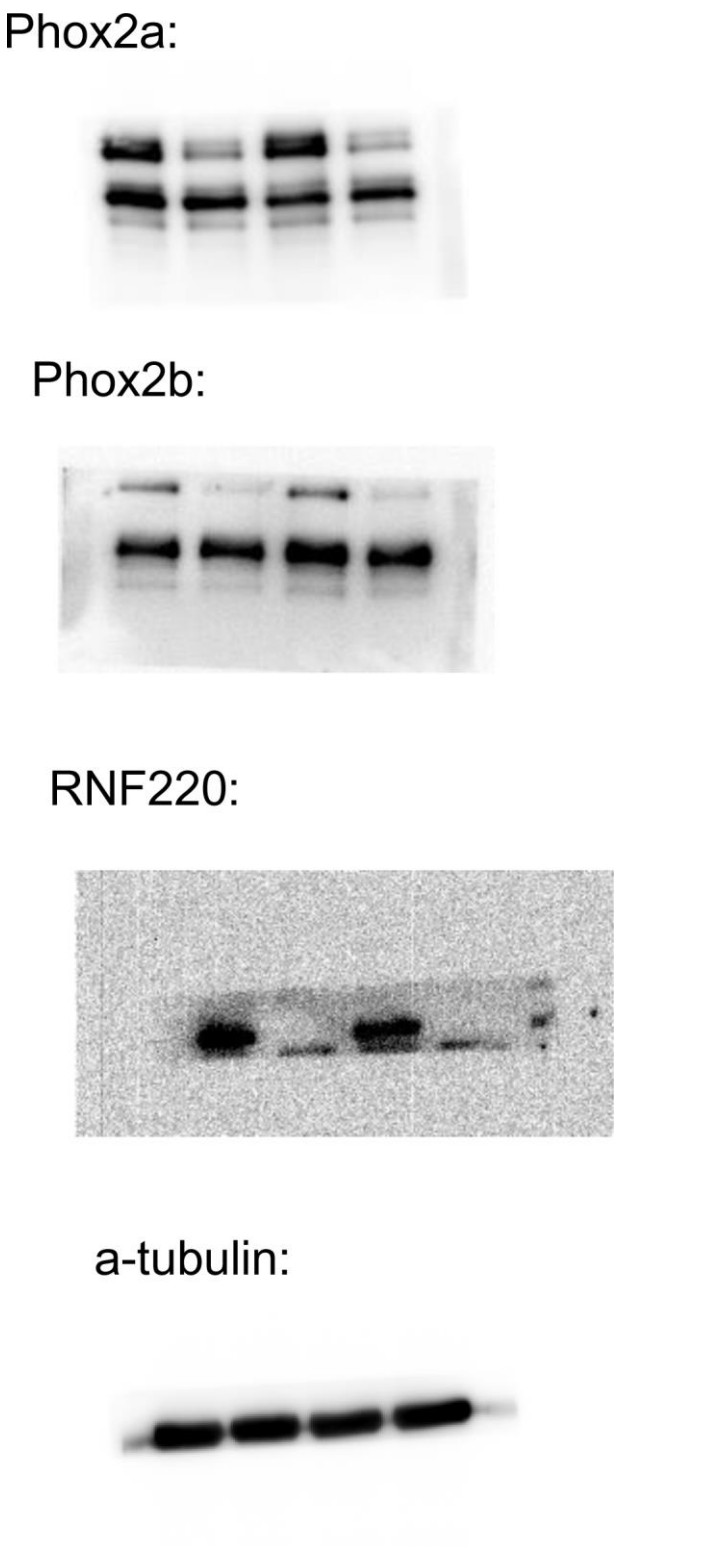
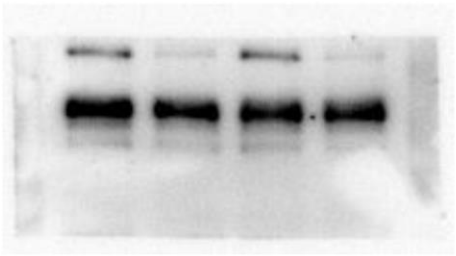


Figure 5i

Phox2a:

Phox2b:



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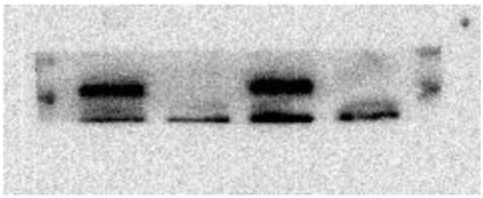

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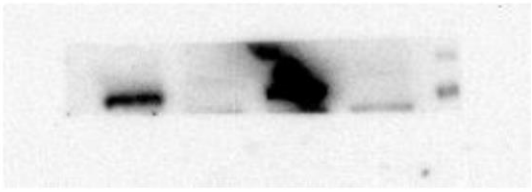


a-tubulin:

Figure S5a

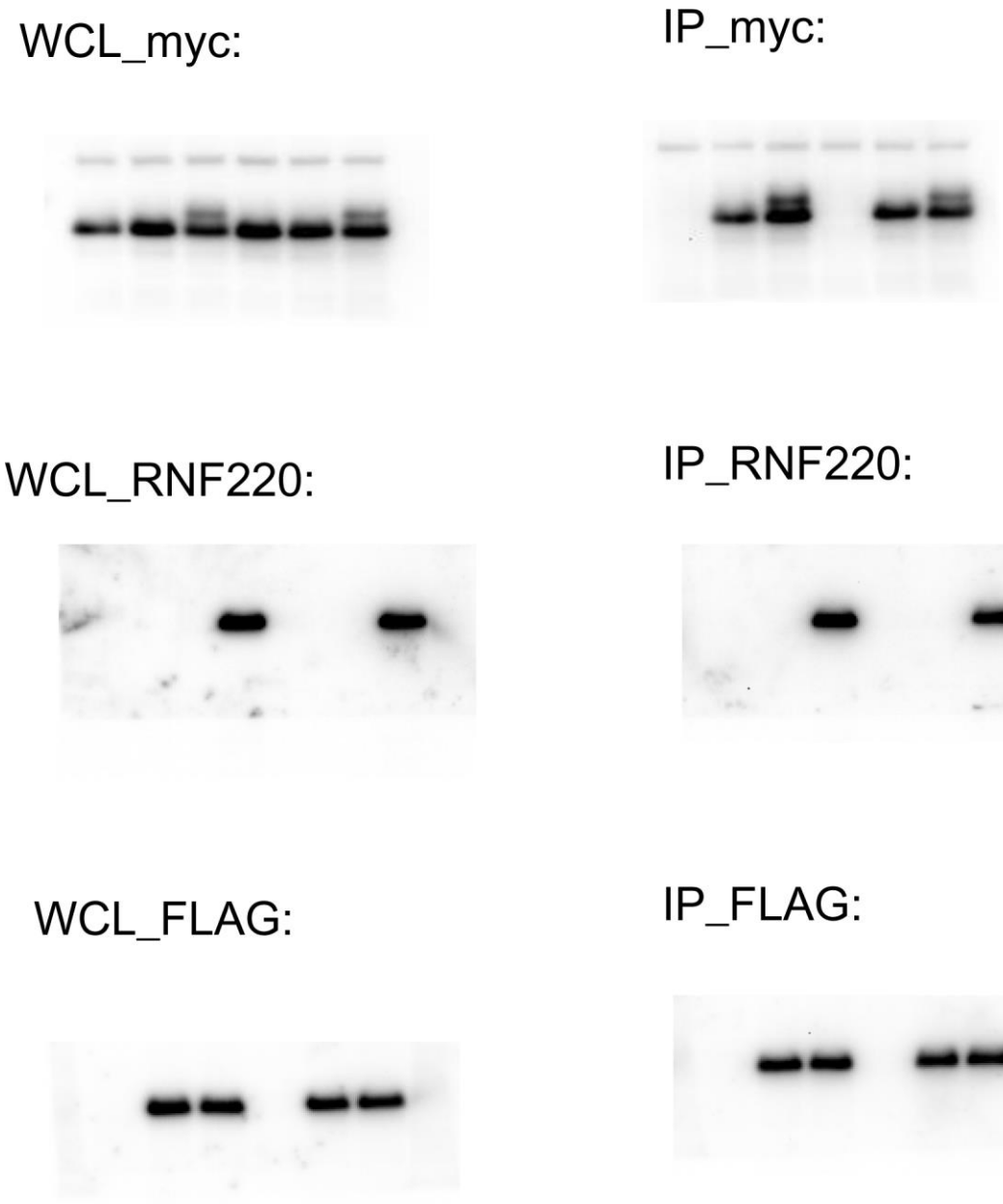
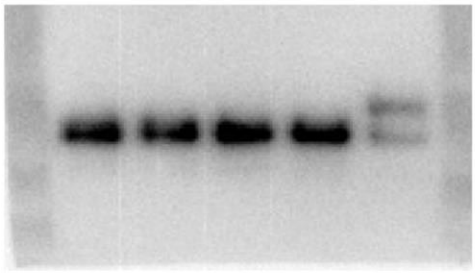


Figure S5b

myc:



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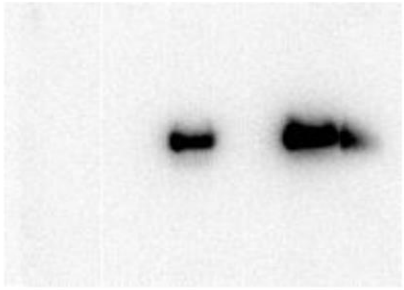

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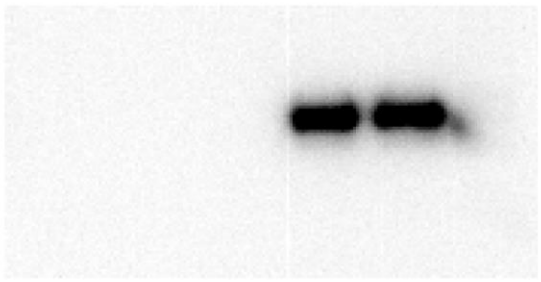

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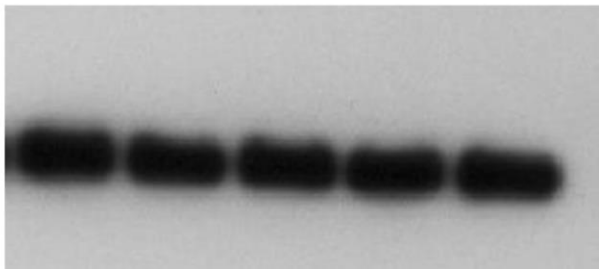

Figure S5c

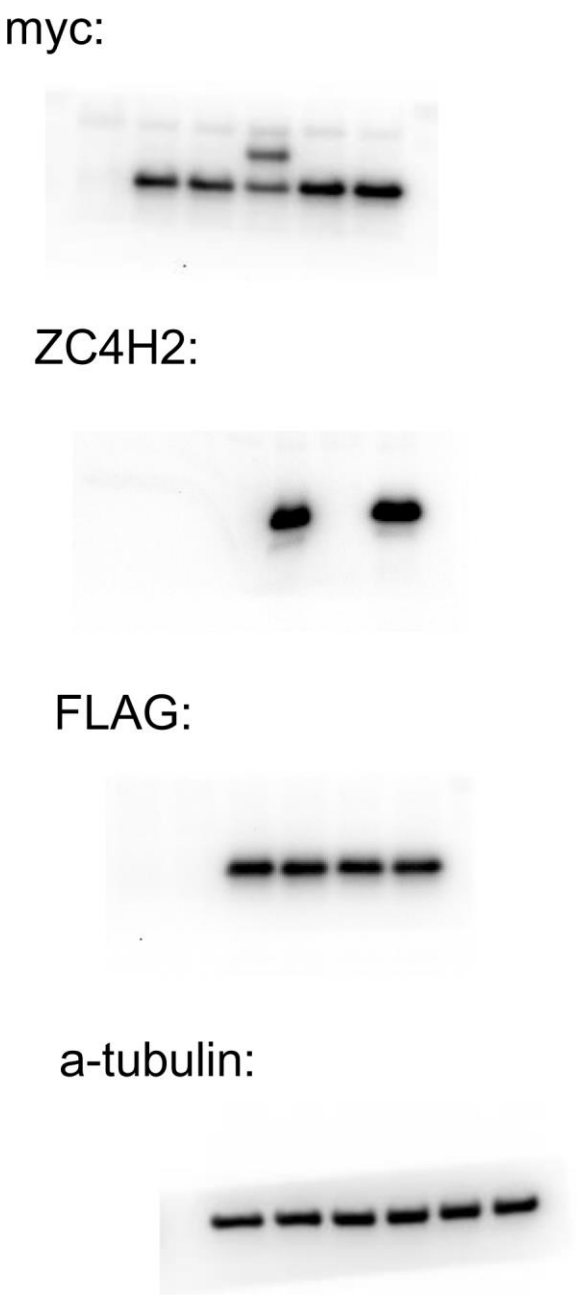

Figure S5d

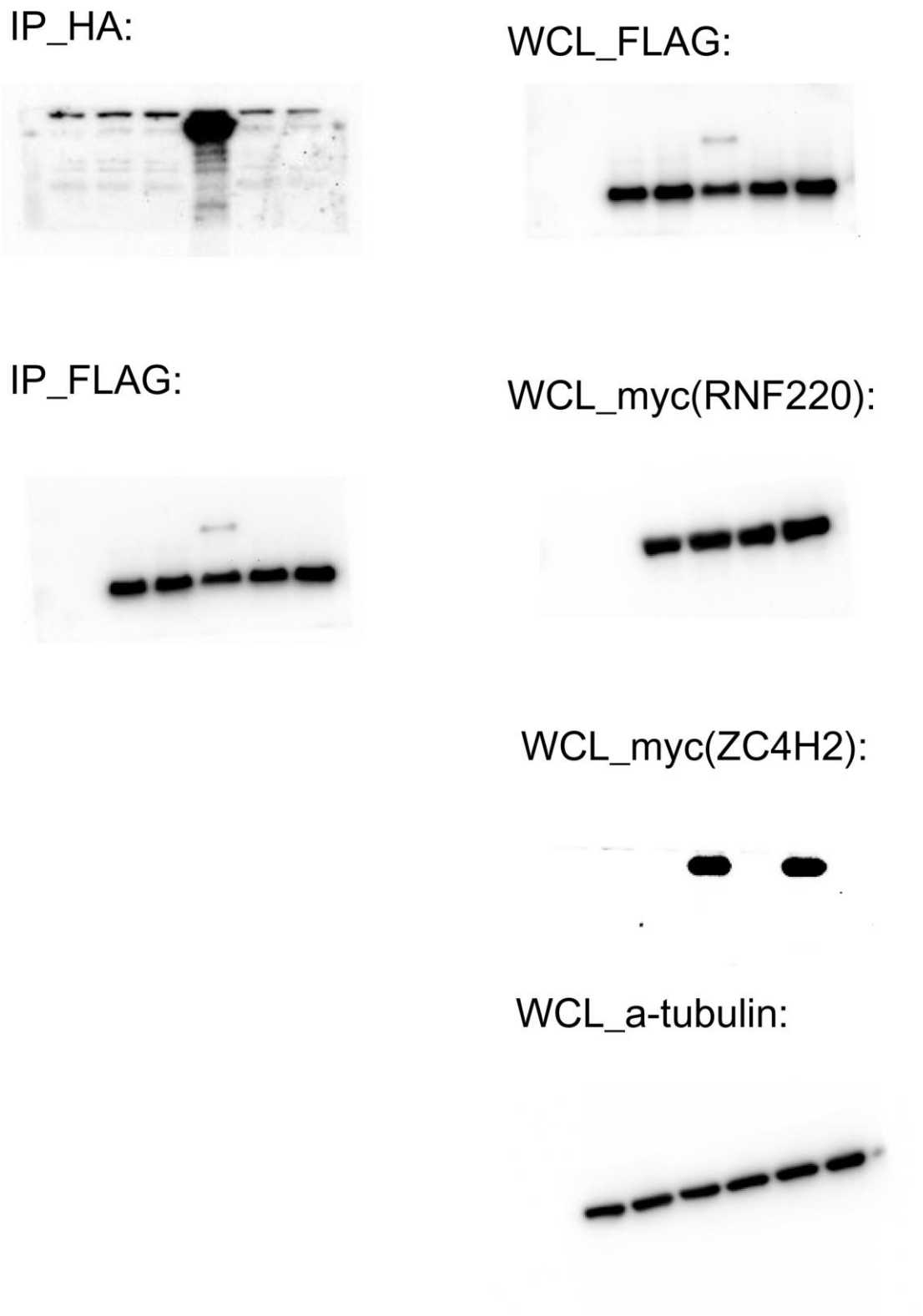

Figure S5e

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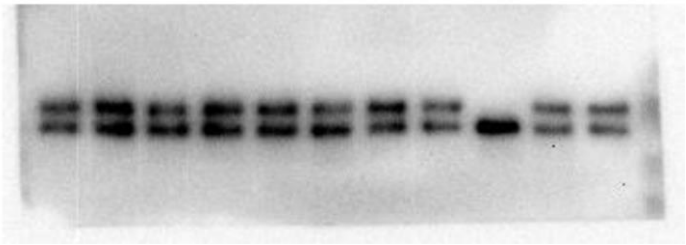

Figure S5f

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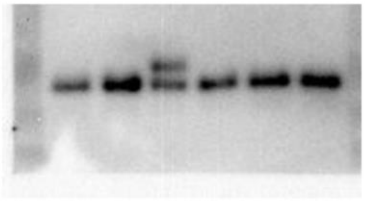

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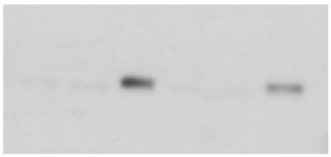

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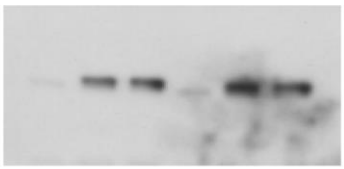
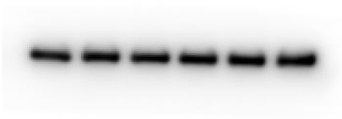


a-tubulin:



Second decision letter

MS ID#: DEVELOP/2019/185199

MS TITLE: RNF220/ZC4H2-mediated monoubiquitination of Phox2 is required for noradrenergic neuronal development

AUTHORS: Ning-Ning Song, Pengcheng Ma, Qiong Zhang, Lei Zhang, Longlong Zhang, Liang Zhu, Chun-Hui He, Bingyu Mao, Yu-Qiang Ding, and Huishan Wang

I have now received the reports of two of the referees who reviewed the earlier version of your manuscript and I 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 remaining comments of the referees 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

As previously

Comments for the author

The authors have made a number of improvements to this manuscript, making it suitable for publication. One remaining minor issue is that in the chick overexpression experiments, they do not control for overexpression of the proteins or mRNA. Basically, only GFP and DBH are used. Not a standard set up.

Reviewer 2

Advance summary and potential significance to field

The authors have addressed most of the issues I raised, however, there are still some points which need to be addressed:

Comments for the author

1) Molecular mechanisms: The authors present new data from DNA binding and reporter gene assays but some information is missing on these experiments. The schematic in Fig. 6B is not explained in the figure legend but it appears that there are three potential Phox2 binding sites. Were all three sites tested in the bandshift or only one? The authors should also provide information in the Methods section which region of the DBH promoter was used for the reporter gene assay. They also claim that co-expression of RNF220/ZC4H2 enhances DNA binding of Phox2a/b but this has not been quantified. Moreover, it seems that the bandshift figures for Phox2a (Fig. 6A) and Phox2b (SupFig. 5C) are very similar or even identical. The authors should confirm that two non-identical figures were used or replace one of the figures. Finally, the bandshift and reporter gene assay provide important information, but it is still unclear whether mono-ubiquitination affects Phox2a/b binding to target genes in vivo. In contrast to the author’s claim, this reviewer found a previous study which included Phox2a chromatin immunoprecipitation experiments (Kim et al., 2015), so Phox2a antibodies suitable for this type of experiment are available.

2) There are still problems with quantification and statistics. The authors provide quantification of the numbers of DBH+ neurons but not of the Western blots I had originally asked for. Also, the new bandshift data was not quantified (see comment above). The manuscript also lacks information whether a posthoc test was used following the ANOVA test and which posthoc test this was.

3) There are a number of problems with English, especially in the newly added sections. There are not only typos but some sentences, for example lines 246-248, are not comprehensible.

Second revision

Author response to reviewers' comments

Response Letter

Reviewer 1 Advance Summary and Potential Significance to Field: As previously

Reviewer 1 Comments for the Author:
The authors have made a number of improvements to this manuscript, making it suitable for publication.
One remaining minor issue is that in the chick overexpression experiments, they do not control for overexpression of the proteins or mRNA. Basically, only GFP and DBH are used. Not a standard set up.

Thank you for your comment. Indeed, we examined the expression of all the constructs by *in situ* hybridization or immunofluorescence in chick neural tube. As shown in Figure R1, the presence of mRNAs (*Phox2a*, *Phox2b* and *RNF220*) and protein (ZC4H2) were detected in the electroporated neural tube. We added this description in the revised manuscript. The results were not included but as described as "data not shown" in the revised manuscript.

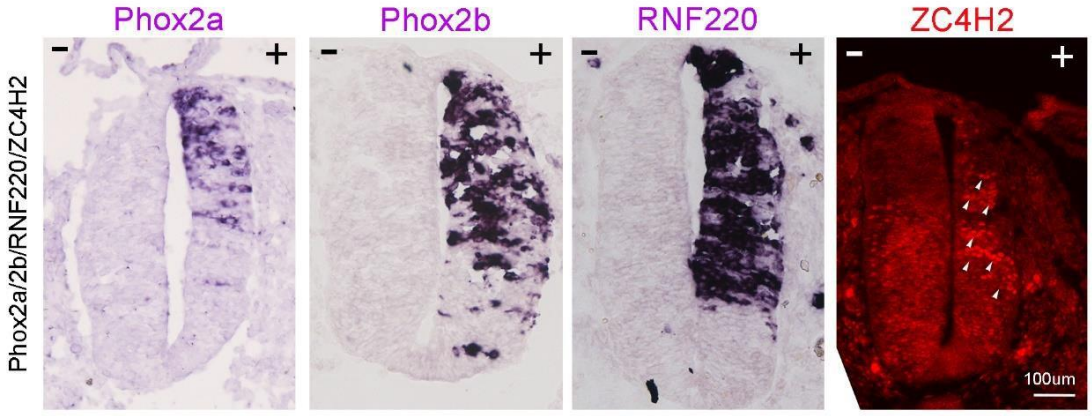


Figure R1 Overexpression of Phox2a/Phox2b/RNF220/ZC4H2 in the chick neural tubes.
The presence of Phox2a, Phox2b and RNF220 mRNAs in the tube is examined by *in situ* hybridization, and ZC4H2 (white arrows) is revealed by immunohistochemistry.

Reviewer 2 Advance Summary and Potential Significance to Field:
The authors have addressed most of the issues I raised, however, there are still some points which need to be addressed:

Reviewer 2 Comments for the Author:
1) Molecular mechanisms:
The authors present new data from DNA binding and reporter gene assays but some information is

missing on these experiments. The schematic in Fig. 6B is not explained in the figure legend but it appears that there are three potential Phox2 binding sites. Were all three sites tested in the bandshift or only one? The authors should also provide information in the Methods section which region of the DBH promoter was used for the reporter gene assay.

Thank you for your comments. We added more information about the reporter construct in the related figure legend. Indeed, we tied all the three potential Phox2 binding sites (PBD1, PBD2 and PBD3) of human DBH promoter and take them as whole Phox2 binding site in both our reporter and bandshift assays. This information was add and clearly described in the revised manuscript.

They also claim that co-expression of RNF220/ZC4H2 enhances DNA binding of Phox2a/b but this has not been quantified.

Thank you for your suggestion. All the mentioned EMSA experiments were carried out 3 times, and we added the statistical data of all EMSA results in the revised Figure 6 and Supplementary Figure 5.

Moreover, it seems that the bandshift figures for Phox2a (Fig. 6A) and Phox2b (SupFig. 5C) are very similar or even identical. The authors should confirm that two non-identical figures were used or replace one of the figures.

Thank you for your comment. The panels for Figure 6A and Supplementary Figure 5C is indeed similar, and we confirmed that they are two non-identical figures. As shown in the Supplementary Information for reviewers, we supplied the two repeated original blots for your review.

Finally, the bandshift and reporter gene assay provide important information, but it is still unclear whether mono-ubiquitination affects Phox2a/b binding to target genes in vivo. In contrast to the author's claim, this reviewer found a previous study which included Phox2a chromatin immunoprecipitation experiments (Kim et al., 2015), so Phox2a antibodies suitable for this type of experiment are available.

Thank you for your comments. Indeed, in the previous study by Kim (Kim et. al., 2015, PLoS Genet 11(10): e1005560), they successfully carried out Phox2a chromatin immunoprecipitation experiments as shown in their Figure 3A. However, we were confused about the discrepancy between their figures and materials, and even we were not sure which antibody they used in their experiments. They showed the Phox2a Chip-seq data in Figure 3A but the only Phox2b antibody information is provided in the materials. Also, the antibody they used for chromatin immunoprecipitation experiments is not a commercial one. We tried at least two commercially-available Phox2a antibodies and one Phox2b antibody but they all failed in ChIP experiments in our hands. Although it is a very important evidence, which we could not get in the near future, to support our conclusion *in vivo*, we think we have supplied a body of evidence to support it. We hope you support our work.

2) There are still problems with quantification and statistics.
The authors provide quantification of the numbers of DBH+ neurons but not of the Western blots I had originally asked for. Also, the new bandshift data was not quantified (see comment above).

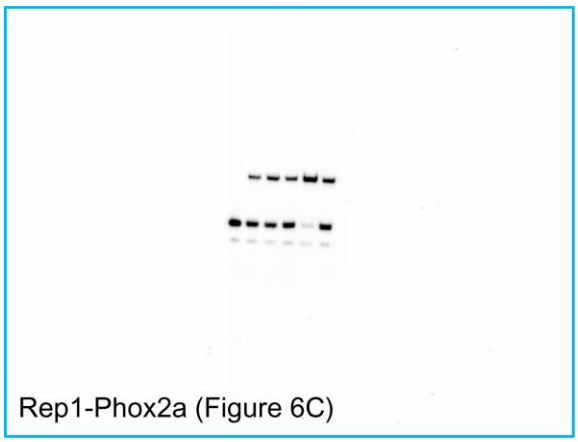
Thank you for your suggestion. All the mentioned EMSA experiments were carried out 3 times. The statistical data of Western blot and EMSA results were shown in the revised Figure 6 and Supplementary Figure 5.

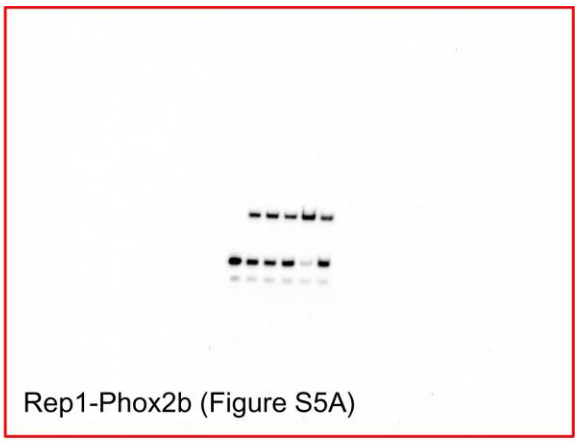
The manuscript also lacks information whether a posthoc test was used following the ANOVA test and which posthoc test this was.

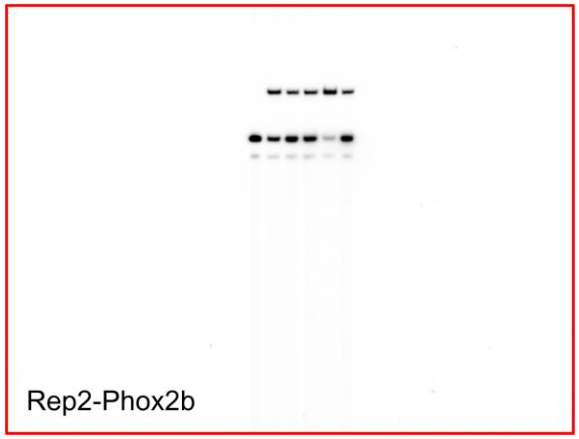
Indeed, we have replaced all the *t*-test evaluation with one way ANOVA evaluation with posthoc Student-Newman-Keuls test for the statistics in the revised Figure 6 and Supplementary Figure 5. We revised the description again.

3) There are a number of problems with English, especially in the newly added sections. There are not only typos but some sentences, for example lines 246-248, are not comprehensible.

Thank you for your careful reading. We carefully revised the whole manuscript to avoid such errors.







Third decision letter

MS ID#: DEVELOP/2019/185199

MS TITLE: RNF220/ZC4H2-mediated monoubiquitination of Phox2 is required for noradrenergic neuronal development

AUTHORS: Ning-Ning Song, Pengcheng Ma, Qiong Zhang, Lei Zhang, Longlong Zhang, Liang Zhu, Chun-Hui He, Bingyu Mao, Yu-Qiang Ding, and Huishan Wang
ARTICLE TYPE: Research Article

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