THE EMBO JOURNAL

Mitotic WNT signalling orchestrates neurogenesis in the developing neocortex

Christof Niehrs, Fabio Da Silva, Kaiqing Zhang, Anneline Pinson, Edoardo Fatti, Michaela Wilsch-Bräuninger, Jessica Herbst, Valerie Vidal, Andreas Schedl, and Wieland Huttner
DOI: 10.15252/embj.2021108041

Corresponding author: Christof Niehrs (niehrs@dkfz-heidelberg.de)

Review Timeline:

Submission Date:	17th Feb 21
Editorial Decision:	1st Apr 21
Revision Received:	16th Jun 21
Editorial Decision:	28th Jul 21
Revision Received:	5th Aug 21
Accepted:	10th Aug 21

Editors: Ieva Gailite / David del Alamo

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

1st Editorial Decision 1st Apr 2021

Dear Christof,

Thank you for submitting your manuscript for consideration by The EMBO Journal. We have now received three referee reports on your manuscript, which are included below for your information.

As you will see from the comments, all reviewers find the topic of interest, while also indicating a number of concerns that would have to be addressed and clarified before they can support publication of the manuscript. In particular, reviewer #1 indicates that the evidence for the specific function of WNT/STOP signalling has to be strengthened (point 2), and this issue is echoed in the comments by the other two reviewers. Furthermore, the reviewers also raise concerns regarding the presented rescue experiments with Sox4/11 overexpression. In addition, all reviewers indicate various concerns regarding the experimental approach, data analysis and presentation that would need to be addressed.

Based on the interest expressed by the reviewers, I would like to invite you to address the concerns raised by both reviewers in a revised manuscript. While addressing point 1 by reviewer 1 certainly would significantly strengthen the manuscript, it will not be required for consideration of the revised version.

I should add that it is The EMBO Journal policy to allow only a single major round of revision and that it is therefore important to resolve the main concerns at this stage. We are aware that many laboratories cannot function at full efficiency during the current COVID-19/SARS-CoV-2 pandemic, and I would be happy to discuss the revision in more detail via email or phone/videoconferencing.

We have extended our 'scooping protection policy' beyond the usual 3 month revision timeline to cover the period required for a full revision to address the essential experimental issues. This means that competing manuscripts published during revision period will not negatively impact on our assessment of the conceptual advance presented by your study. Please contact me if you see a paper with related content published elsewhere to discuss the appropriate course of action.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: https://www.embopress.org/page/journal/14602075/authorguide#transparentprocess. Please also see the attached instructions for further guidelines on preparation of the revised manuscript.

Please feel free to contact me if you have any further questions regarding the revision. Thank you for the opportunity to consider your work for publication. I look forward to receiving the revised manuscript.

Best regards,

Ieva

Ieva Gailite, PhD
Scientific Editor
The EMBO Journal

Meyerhofstrasse 1
D-69117 Heidelberg
Tel: +4962218891309
i.gailite@embojournal.org

When submitting your revised manuscript, please carefully review the instructions below and include the following items:

1) a .docx formatted version of the manuscript text (including legends for main figures, EV figures and tables). Please make sure that the changes are highlighted to be clearly visible.

2) individual production quality figure files as .eps, .tif, .jpg (one file per figure).

3) a .docx formatted letter INCLUDING the reviewers' reports and your detailed point-by-point response to their comments. As part of the EMBO Press transparent editorial process, the point-by-point response is part of the Review Process File (RPF), which will be published alongside your paper.

4) a complete author checklist, which you can download from our author guidelines (https://wol-prod-cdn.literatumonline.com/pb-assets/embo-site/Author Checklist%20-%20EMBO%20J-1561436015657.xlsx). Please insert information in the checklist that is also reflected in the manuscript. The completed author checklist will also be part of the RPF.

5) Please note that all corresponding authors are required to supply an ORCID ID for their name upon submission of a revised manuscript.

6) Before submitting your revision, primary datasets produced in this study need to be deposited in an appropriate public database (see https://www.embopress.org/page/journal/14602075/authorguide#datadeposition). Please remember to provide a reviewer password if the datasets are not yet public. Please note that the Data Availability Section is restricted to new primary data that are part of this study. If no data deposition in external databases is needed for this paper, please then state in this section: This study includes no data deposited in external repositories.
*** Note - All links should resolve to a page where the data can be accessed. ***

7) Our journal encourages inclusion of *data citations in the reference list* to directly cite datasets that were re-used and obtained from public databases. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records from which the data can be accessed. In the main text, data citations are formatted as follows: "Data ref: Smith et al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference. Further instructions are available at .

8) We would also encourage you to include the source data for figure panels that show essential data. Numerical data can be provided as individual .xls or .csv files (including a tab describing the data). For 'blots' or microscopy, uncropped images should be submitted (using a zip archive or a single pdf per main figure if multiple images need to be supplied for one panel). Additional information on source data and instruction on how to label the files are available at .

9) We replaced Supplementary Information with Expanded View (EV) Figures and Tables that are collapsible/expandable online (see examples in https://www.embopress.org/doi/10.15252/embj.201695874). A maximum of 5 EV Figures can be typeset. EV Figures should be cited as 'Figure EV1, Figure EV2" etc. in the text and their respective legends should be included in the main text after the legends of regular figures.

- For the figures that you do NOT wish to display as Expanded View figures, they should be bundled together with their legends in a single PDF file called *Appendix*, which should start with a short Table of Content. Appendix figures should be referred to in the main text as: "Appendix Figure S1, Appendix Figure S2" etc. See detailed instructions regarding expanded view here: .

- Additional Tables/Datasets should be labelled and referred to as Table EV1, Dataset EV1, etc. Legends have to be provided in a separate tab in case of .xls files. Alternatively, the legend can be supplied as a separate text file (README) and zipped together with the Table/Dataset file.

10) When assembling figures, please refer to our figure preparation guideline in order to ensure proper formatting and readability in print as well as on screen: http://bit.ly/EMBOPressFigurePreparationGuideline

Please remember: Digital image enhancement is acceptable practice, as long as it accurately represents the original data and conforms to community standards. If a figure has been subjected to significant electronic manipulation, this must be noted in the figure legend or in the 'Materials and Methods' section. The editors reserve the right to request original versions of figures and the original images that were used to assemble the figure.

Further information is available in our Guide For Authors: https://www.embopress.org/page/journal/14602075/authorguide

Please click on the link below to submit the revision online. The resubmission deadline is currently set to 30th Jun 2021. Please contact us if you would need and extension.

Link Not Available

Referee #1:

Wnt-dependent signaling is generally accepted as a major regulator of embryonic neurogenesis and mammalian cortex development. A major unresolved issue is, however, through which signaling pathways / downstream effectors Wnts control distinct steps in neurogenesis / cortex development. Multiple and often contradictory functions have, for example, been ascribed to the Wnt/LRP6/GSK3beta/beta-catenin signaling cascade (canonical Wnt-signaling). Such contradictions may stem from the fact that Wnt-signaling through LRP6 can also activate beta-catenin-independent signaling pathways. In this manuscript, Da Silva and colleagues analyse cyclin Y and cyclin Y-like 1 (Ccny/l1) double mutant mice. These two proteins have previously been found to be regulators of the beta-catenin independent Wnt/LRP6/GSK3beta WNT/STOP signaling pathway. The authors focus in their analyses on cortical neurogenesis. Given the early embryonic

lethality of the double KO mutants starting at embryonic day 14.5 (E14.5), the authors analyze the cortical neurogenesis phenotype at E13.5. their main findings are:
1. Substantial neurogenesis defects (thinner neocortical wall thickness with increased ventricular zone (VZ) size and decreased subventricular VZ and intermediate zone (IZ) + cortical plate (CP). Correspondingly, the authors describe a slight increase in apical progenitors (APs), and a substantial decrease in basal progenitors (BPs) and neurons.
2. Immunohistochemistry suggests expression of Ccny/l1 at the apical cell cortex in the VZ and in a subset of BPs. The authors also provide evidence that phosphorylated LRP6 is present in BPs. Moreover the authors provide biochemical evidence that levels of pLRP6 are lower in Ccny/l1 double knockouts (dKO).
3. The authors provide evidence that duration of mitosis of BPs but not of APs is prolonged in dKOs. Moreover, they provide evidence that the mode of division of APs is altered in dKO. Knockdown of Ccny/l1 via in utero electroporation was consistent with the dKO data and showed reduction in BPs and neurons at E17.5.
4. dKO derived cortical neural precursors show decreased neuronal differentiation
5. Evidence that the transcription factors Sox4 and 11 are phosphorylated by GSK3beta, that Sox4&11 levels are decreased in the E13.5 dKO cortex and that Sox4 and 11 can be ubiquitinated.
6. Overexpression of Sox4&11 augments expression of the neuronal marker Tuj1 in dKO derived cortical neural precursors.
The manuscript addresses in principle a very interesting and central question in neurodevelopmental biology, i.e., how Wnt-signaling coordinates the complex process of neurogenesis and controls multiple steps in neurogenesis.

The main concern with the manuscript is that the conclusions that finally lead up to the model on Wnt-function (summarized in Figure 6D) are premature.

1. The authors observe a complex phenotype in dKO mutants that affects the neurogenesis from APs to neurons. There are two major concerns: firstly, because the knockout is not conditional the observed phenotypes may in principle stem from "non-cell autonomous" functions of Ccny/l1. The shRNA approaches do to a certain extent suggest a "cell autonomous" origin, but here the authors did not examine the impact on APs. Secondly, the non-specific knockout of Ccny/l1 does not allow to determine e.g. whether the phenotypes in BPs and in neurons are the consequence of Ccny/l1 effects in the preceding developmental stage / whether the phenotype in APs is the consequence of feedback mechanisms from BPs or neurons. In the best case scenario the authors would target the knockout specifically to APs/ BPs/neurons and compare the phenotypes. In principle, the tools are available as the ko mouse lines have the potential for conditional knockout. I do recognize that this represents a substantial amount of work that is likely to go beyond a simple revision. However, the phenotype seems too multifaceted / complex to be satisfactorily tackled by a non-conditional approach. The reductionist approach using cortical neural progenitors suggests that the neuronal differentiation deficit is cell autonomous but fails to provide information on the effects in BPs and APs.
2. The authors convincingly show that a double knockout of Ccny/l1 affects cortical neurogenesis. The (conceptually highly important) conclusion that a) WNT/STOP signaling controls the described aspects of corticogenesis and that b) WNT/beta-catenin signaling controls symmetric division / proliferation of APs is premature. The authors do not provide convincing in vivo evidence that a) increased GSK3beta activity and/or decreased LRP6 activity are causally linked to the neurogenesis phenotype. The stainings for LRP6 are suggestive but at present correlative. To establish a causal link of the Ccny/l1 phenotype with the LRP6 / GSK3beta axis in vivo, the authors should consider to conduct rescue experiments that specifically target LRP6 / GSK3beta activity. The authors provide evidence that the Ccny/l1 knockout does not affect Axin2 levels, which is a

good indicator for sustained WNT-betacatenin signaling acivity. This finding, however, does not exclude that WNT-beta catenin signaling serves functions beyond the regulation of AP proliferation. To substantiate the claim of "division of labor" between distinct WNT pathways (which undoubtedly would be very exciting) the authors will have to e.g. study the effects of additional Wnt/beta-catenin signaling loss of function.
3. In Figure 1H and S1G the authors use immunofluorescence to identify Ccny/l1 expressing cells. Such analysis is important to understand the knockout phenotype. Assigning the signal to a specific cell type, however, is not convincing. The staining for Ccny/l1 is patchy; but even more problematic is the fact that the outline of cells cannot be traced. One potential solution would be to use fluorescent reporter electroporated brains and to use the expression of the reporter combined with a cell type specific marker to study the expression of Ccny/l1 in a specific cell type.
4. The authors make qualitative statements regarding the expression of Ccny/l1 and pLRP6. For understanding of the phenotype it would be important to provide a quantification of what the fraction of APs / BPs / neurons / pHH3+ cells is that express Ccny/l1 and pLRP6.
5. The authors suggest Sox4 and Sox11 as targets of GSK3beta phosphorylation-dependent destabilization, ubiquitination and degradation. The decrease in Sox4 and Sox11 protein levels in the embryonic cortex is suggestive of decreased stability but this reduction is also easily explained by the fact that BP and neuron numbers are reduced. The authors overexpress Sox4 and Sox11 to test their ability to rescue the Ccny/l1 dKO phenotype. Overexpression results in higher fraction of Tuj1 expressing cells but whether this constitutes a rescue of the neuronal differentiation phenotype remains questionable. The authors score all Tuj1+ cells as neurons even if these cells do not feature the typical bipolar neuronal morphology. This is mentioned in the manuscript, but as Tuj1 is a direct target of SoxC proteins (Bergsland et al 2006), induction of Tuj1 expression by itself is not sufficient to claim a rescue of the Ccny/l1 dKO phenotype and to causally link Ccny/l1 and SoxC function. Moreover, the execution and quantification of the experiment are unclear. The authors describe that they use Flag-Sox4/11 in a pLENTI-CAG-IRES-GFP vector. How do the authors express Sox4 and Sox11 simultaneously? Is this construct tricistronic? Neuronal numbers should be normalized to the number of transduced cells based on GFP expression. The lack of increased neuronal differentiation in the control group following Sox11 / Sox4 overexpression is surprising as Sox11 / Sox4 overexpression has been reported to potently induce neuronal differentiation. Finally, the baseline differentiation of NPCs with 20% Tuj1 positive cells in WT NPCs is rather low compared to other reports.
6. SoxC factor ubiquitination (Chiang et al 2021), phosphorylation (Balta et al. 2018 a,b) and sumoylation (Chang et al. 2017) have been described in the context of neurodevelopment. The authors may want to consider to include these papers in their discussion as these papers provide further evidence that the function of this transcription factor family is controlled by post-translational modifications.
7. The observations regarding altered kinetics of mitosis in BPs and altered mode of division in APs are very interesting but remain underexplored in terms of mechanism. As the neuronal differentiation deficit is potentially only the culmination / consequence of altered AP and BP behavior, it would be important to mechanistically understand the role of Ccny/l1 in these cell types.

Referee #2:

Mammalian corticogenesis is a complex process where various molecules play intricate roles. Wnt signaling pathways are previously considered as one of the key regulators in neurogenesis, although there are both sides of evidence that Wnt signaling maintains proliferation or promotes

differentiation. In this study, the authors focused on a novel non-canonical Wnt pathway involved in the stabilization of protein (here they term the WNT/STOP pathway) by analyzing Cyclin Y (Ccny) and Cyclin Y-like 1 (Ccnyl1) double knockout (DKO) mice. Through detailed in vivo and invitro analyses, the authors identified Sox4 and Sox11 as direct targets of Gsk3 and concluded that Wnt/STOP signaling drives neuronal differentiation whereas that Wnt/b-catenin signaling regulates self-renewal of neural progenitor cells.

Overall, the analyses seemed to be carefully performed with multiple approaches, and the data are logically presented. The topic will attract the eyes of researchers in developmental neurobiology. However, this reviewer considers the following issues should be considered before publication:

Major points:
1) Quantitative data should be given for Fig. 1A, 1J, 3I, 4G, 4H, 4I, 4J, 5E, 5F, 5G, 5J, S1I, S3B, S5A, S5E, and S5G. Relatedly, the number of samples and areas of the neocortical areas should be indicated. More detailed descriptions of the methodology for quantitative analyses are needed. For example, which of the cortical areas are chosen and how many of the cells are counted for Figs. 1B, D-F?
2) In Fig. 1B, DKO shows a wider space between CP and the meninges. Is it a phenotype?
3) In Fig. 1C, only the rational values are shown. It would be better to describe specific values regarding the difference between Co and DKO for Fig 1C and the text (page7, lines 15 to 19) as for Fig 2H. How many percentages are decreased/increased in DKO?
4) Regarding the expression levels of pLRP6 and Axin2, what kind of cells are measured? Are they APs or BPs?
5) It is not clear to this reviewer that "WNT/LRP6 signaling peaks during mitosis in APs and BPs." (p9, line 20-21). What is the evidence for this statement?
6) It would be better to perform qPCR using RNA extracted from E13.5 embryonic "dorsal" telencephalon to eliminate the effects from "ventral" telencephalon for obtaining data of Figs. 1L, S5F, and S5L. In addition, if the authors show the data for N-myc, Sox4 and Sox11 expression, they should state the results in the text.
7) Is it a phenotype that DKO has no Tbr2+ cells in the ventricular zone in Fig. 2D?
8) According to a previous article (i.e., Martynoga et al., Dev Biol, 2005), Ts was calculated by the following formula:
Ts=Ti (Lcells/Scells)
Ti is 1.5h
Lcells= IdU+/BrdU-, Scells=IdU+/BrdU+
For example, if the authors calculate APs in Co with reference to the above equation, it will result in 4.1, which seems to be different from the value in the graph in Fig. 2I. What is the reason for this discrepancy? If they use a different formula, please indicate by citing a reference. It would also be necessary to describe the formula for calculating the length of S-phase, total cell cycle, and mitosis so that we can understand in appendix method page 14 line 28-40.
9) Regarding data of Fig. 3I, why NPCs from E13.5 Ccny-/-; Ccnyl1-/- forebrains were used instead of from Ccny+/-; Ccnyl1-/- (page 14, lines 11)? What kind of control was used here? It would also be better to explain the length of Tuj1+ dendrites and axons in Fig. 3I (page 14, lines 16). It would be suitable if they are based on the quantitative analysis.
10) Regarding data for Sox4, what is the evidence for "In DKO embryos, we could not detect an obvious decrease in Sox4 staining intensity in Tbr2+ cells" (p.17, line 25-p18, line 1)? It would be better to measure Sox4 in APs.
11) It would be better to examine the ratio of mature neurons (asterisks) and immature neurons (arrowheads) in 4 groups (Co, Co+Sox4/11, DKO, DKO+Sox4/11) in Fig. 6B.
12) What is the evidence to state "The reason why Lrp6 mutants do not also manifest the

proliferation defects of β-catenin mutants is unclear but may be due to compensation from LRP5."

Minor points:
1) It would be better to show the figure numbers separatory in figures and graphs of the following figures such as Fig 1D-F, I-K (figures and graphs), S1E (Ccny and Ccnyl1), S3A (βIII-tublin, Doublecortin, and Sox2), S3C (Ccny and pLLRP6 (S1490)), S3D (Sox4, Sox4; Tbr2 and Sox4; Tbr2; pHH3), and S3M (CCNY and pLRP6).
2) Scale bars are needed for Fig S1F-G, 4B-C.
3) The angles of the images should be similar between Co and DKO for Figs. 1D, 1E, 2A, and 2D.
4) The number of embryos should be indicated in Fig. 3D.
5) For the terminology, it would be better to use "forebrain" rather than "whole brain" (regarding Fig. 5J and its related text).
6) It is better to state that Ccny+/-; Ccny11+/- was used as a control in the main text (page 26, line 11-12).
7) It is useful for the reader if the authors provide product information for the 70 μm cell strainer; TRIzol; and MG132 (page 28, line 6; text, page30, line6; and text, page 31, line 13).
8) There are many typos that should be edited in the revised text.

Referee #3:

Several studies have implicated the Wnt signaling pathway in mouse cortical neurogenesis but the results are heterogeneous and difficult to reconcile. In this manuscript, Da Silva and colleagues attempt to do so by calling into play the WNT-stabilization of proteins pathway (Wnt/STOP). This pathway is β-catenin independent and involves the activity of cyclin Y and cyclin Y-like 1. The authors generate a mouse line mutant for both Ccny and Ccnyl1 genes, which is embryonic lethal at E13.5. Taking advantage of this line, in vivo knock-down experiments (to cover later stages developmental stages), in vitro studies with neural stem cells and different biochemical approaches, the authors show that WNT/STOP controls basal progenitor generation and their asymmetrical division, which gives rise to differentiated cells. They further show that Sox4 and Sox11 are targets of the pathway and that their activity is needed to promote neuron generation. The propose a model in which WNT/β-catenin signaling would be the main driver of apical progenitor proliferation, whereas the WNT/STOP pathway would be in charge of controlling the asymmetric proliferation of basal progenitors and their differentiation into neurons through Sox4 and Sox11 activity.

This is an interesting and well performed study which provides novel ideas on the control of neurogenesis in the mammalian cortex. The conclusions are based on a large set of experimental approaches that support much of the authors conclusions. The manuscript is also in general well written. Nevertheless, there are a few aspects that could be improved or better explained given that the manuscript is rather dense and non-experts may have difficulties in following some of the conclusions.

1. Figure 2J and M. Can the authors explain in methods and results how they have extrapolated the values of the length of the cell cycle and the duration of mitosis? This is currently poorly explained in the results and it is not mentioned in the methods.
2. Figure 3E, F. Additional high-power images of the electroporated cells will help to appreciate the triple labelling, at the moment impossible to see.
3. Figure 3I. Staining with Tuj1 cannot differentiate axon and dendrites so, the description of

process in culture should simply refer to neurites and neuritic length.
4. Figure 6B. Experiments here are confusing. Are all the cells transduced with Sox4/11? Double staining for Sox4/11 and Tuj1 would help to understand the results. The Tuj1+ in the DKO cells looks rather fibroblast-like. The use of additional neuronal markers would help to support the idea that Sox4/11 indeed rescue the phenotype even if partially. This is not very convincing at the moment. This partial rescue should be discussed further.
5. The model proposed in Fig 6D indicate that canonical WNT/β-catenin signaling promotes AP self-renewal. It is unclear exactly how the authors have reached the conclusion.

Overview of Revision

Item	Panel	Contents	Reviewer
Figure 1	**NEW** Panel Q	qPCR analysis of *Axin2* and *N-myc* expression levels in RNA extracted from dorsal telecephalon of control and DKO embryos.	#2
Figure 3	**NEW** Insets for panels E-F	Insets with magnified IF images from IUE shRNA knockdown of Ccny/l1 in E15.5 (E) and E17.5 (F) embryos.	#3
NEW Figure 6	**NEW** Panels A, B, C, D, E, F	**A:** Updated schematic for Sox4/11 rescue in DKO NPCs. **B-C:** Repetition of Sox4/11 rescue experiment with normalization to transduced cells (IF and quantification). **D:** Schematic for CHIR rescue in DKO NPCs. **E-F:** CHIR rescue of DKO NPC differentiation defect (IF and quantification).	#1 & #3
NEW Figure 7	**NEW** schematic	Schematic of main findings in study moved from Fig 6 to Fig 7.	Editorial change
Figure EV1	**NEW** Panels G and H	Co-IF for Ccny/l1 plus GFP and Sox2 (APs) (G) or GFP and Tbr2 (BPs) (H) in E15.5 embryos electroporated with GFP plasmid at E13.5. Assigns Ccny/l1 immunoreactivity to APs and BPs.	#1
Figure EV5	**NEW** Panels E, G, M	**E:** Quantification of Sox4 pixel intensity (determined by IF) in non-mitotic BPs of control and DKO neocortex. **G, M**: qPCR analysis of *Sox4* (G) and *Sox11* (M) expression levels in RNA extracted from dorsal telecephalon of control and DKO embryos.	#2
NEW Appendix Fig S1	**NEW** Panels A, B, C, D, E, F	**A-D:** Quantification of VZ (A), SVZ (B), IZ-CP (C), and total layer thickness (D) in control and DKO neocortex. Results shown are specific values from Fig 1C. **E-F:** Quantification of $IdU^{+}BrdU^{+/-}$ APs and BPs. Results shown are specific values from Fig 2H.	#2
Appendix Figure S2 (previous Appendix figure S6)	**NEW** Panels E and F	**E:** IF for GFP in control NPCs transduced with empty CAG-IRES-EGFP lentivirus. **F:** IF for FLAG in control NPCs transduced with *Flag-Sox4/11* overexpressing lentiviruses.	#1
NEW Appendix Fig S3	**NEW** Panels A, B and C	**A-B:** IF for Map2 and GFP/FLAG (A) and quantification of $Map2^{+}$ cells for Sox4/11 rescue experiments in DKO NPCs (B). **C:** Quantification of mature and immature neurons for Sox4/11 DKO NPC rescue experiments. Related to Fig 6C.	#2

Response to the Referees

Referee#1:

Wnt-dependent signaling is generally accepted as a major regulator of embryonic neurogenesis and mammalian cortex development. A major unresolved issue is, however, through which signaling pathways / downstream effectors Wnts control distinct steps in neurogenesis / cortex development. Multiple and often contradictory functions have, for example, been ascribed to the Wnt/LRP6/GSK3beta/beta-catenin signaling cascade (canonical Wnt-signaling). Such contradictions may stem from the fact that Wnt-signaling through LRP6 can also activate beta-catenin-independent signaling pathways. In this manuscript, Da Silva and colleagues analyse cyclin Y and cyclin Y-like 1 (Ccny/l1) double mutant mice. These two proteins have previously been found to be regulators of the beta-catenin independent Wnt/LRP6/GSK3beta WNT/STOP signaling pathway. The authors focus in their analyses on cortical neurogenesis. Given the early embryonic lethality of the double KO mutants starting at embryonic day 14.5 (E14.5), the authors analyze the cortical neurogenesis phenotype at E13.5. their main findings are:

1. Substantial neurogenesis defects (thinner neocortical wall thickness with increased ventricular zone (VZ) size and decreased subventricular VZ and intermediate zone (IZ) + cortical plate (CP). Correspondingly, the authors describe a slight increase in apical progenitors (APs), and a substantial decrease in basal progenitors (BPs) and neurons.

2. Immunohistochemistry suggests expression of Ccny/l1 at the apical cell cortex in the VZ and in a subset of BPs. The authors also provide evidence that phosphorylated LRP6 is present in BPs. Moreover the authors provide biochemical evidence that levels of pLRP6 are lower in Ccny/l1 double knockouts (dKO).

3. The authors provide evidence that duration of mitosis of BPs but not of APs is prolonged in dKOs. Moreover, they provide evidence that the mode of division of APs is altered in dKO. Knockdown of Ccny/l1 via in utero electroporation was consistent with the dKO data and showed reduction in BPs and neurons at E17.5.

4. dKO derived cortical neural precursors show decreased neuronal differentiation

5. Evidence that the transcription factors Sox4 and 11 are phosphorylated by GSK3beta, that Sox4&11 levels are decreased in the E13.5 dKO cortex and that Sox4 and 11 can be ubiquitinated.

6. Overexpression of Sox4&11 augments expression of the neuronal marker Tuj1 in dKO derived cortical neural precursors.

The manuscript addresses in principle a very interesting and central question in

neurodevelopmental biology, i.e., how Wnt-signaling coordinates the complex process of neurogenesis and controls multiple steps in neurogenesis.

The main concern with the manuscript is that the conclusions that finally lead up to the model on Wnt-function (summarized in Figure 6D) are premature.

We thank Reviewer 1 for the detailed summary and for stating that our manuscript addresses a very interesting and central question. We have included data from new experiments that address many of the Reviewer's concerns, especially concerning the connection between the DKO neurogenesis phenotype and WNT signaling.

1. The authors observe a complex phenotype in dKO mutants that affects the neurogenesis from APs to neurons. There are two major concerns: firstly, because the knockout is not conditional the observed phenotypes may in principle stem from "non-cell autonomous" functions of Ccny/l1. The shRNA approaches do to a certain extent suggest a "cell autonomous" origin, but here the authors did not examine the impact on APs. Secondly, the non-specific knockout of Ccny/l1 does not allow to determine e.g. whether the phenotypes in BPs and in neurons are the consequence of Ccny/l1 effects in the preceding developmental stage / whether the phenotype in APs is the consequence of feedback mechanisms from BPs or neurons. In the best case scenario the authors would target the knockout specifically to APs/ BPs/neurons and compare the phenotypes. In principle, the tools are available as the ko mouse lines have the potential for conditional knockout. I do recognize that this represents a substantial amount of work that is likely to go beyond a simple revision. However, the phenotype seems too multifaceted / complex to be satisfactorily tackled by a non-conditional approach. The reductionist approach using cortical neural progenitors suggests that the neuronal differentiation deficit is cell autonomous but fails to provide information on the effects in BPs and APs.

We agree that a conditional deletion approach would be informative. However, characterization of such a conditional KO mouse is a project in itself and hence beyond the scope of this paper. Moreover, as indicated by the Reviewer, our acute shRNA knockdown approaches (IUE and cultured NPCs) address many of these issues.

2. The authors convincingly show that a double knockout of Ccny/l1 affects cortical neurogenesis. The (conceptually highly important) conclusion that a) WNT/STOP signaling controls the described aspects of corticogenesis and that b) WNT/beta-catenin signaling controls symmetric division / proliferation of APs is premature. The authors do not provide convincing in vivo evidence that a) increased GSK3beta activity and/or decreased LRP6 activity are causally linked to the neurogenesis phenotype. The stainings for LRP6 are suggestive but at present correlative. To establish a causal link of the Ccny/l1 phenotype with the LRP6 / GSK3beta axis in vivo, the authors should consider to conduct rescue experiments that specifically target LRP6 / GSK3beta activity. The authors provide evidence that the Ccny/l1 knockout does not affect Axin2 levels, which is a good indicator for sustained WNT-beta catenin signaling activity. This finding, however, does not exclude that WNT-beta catenin signaling serves functions beyond the regulation of AP proliferation. To substantiate the claim of "division of labor" between distinct WNT pathways (which undoubtedly would be very exciting) the

authors will have to e.g. study the effects of additional Wnt/beta-catenin signaling loss of function.

To strengthen the link between Ccny/l1 and WNT signaling we have conducted the requested rescue experiments. We treated cultured DKO NPCs with the GSK3 inhibitor CHIR99021, which robustly rescued the DKO differentiation phenotype in a dose-dependent manner. This result (new Fig 6E-F) corroborates causality between Ccny/l1 and WNT signaling in cultured NPCs.

In regards to *in vivo* rescue experiments, these would be technically challenging and time consuming. We would also like to reiterate that DKO embryos display reduced LRP6 phosphorylation both by immunofluorescence (IF) (Fig 1L) and immunoblot (Fig 1N), and that LRP6 phosphorylation is a direct and faithful indicator of active WNT signaling (Davidson et al., 2005; Davidson et al., 2009). Moreover, the lack of changes in *Axin2* expression (Fig 1 O-Q) and dephosphorylated β-catenin (a.k.a. active β-catenin) (Fig EV1L) in the DKO neocortex rule out the possibility that the Ccny/l1 mutant neurogenesis defects are due to canonical WNT signaling. Hence, we do provide *in vivo* evidence that WNT signaling is reduced in the DKO neocortex at the receptor level, but not at the transcriptional level. This is consistent with previously reported *in vivo* roles of the WNT/STOP pathway in germ cell development (Koch et al., 2015, Huang et al., 2015). Finally, we note that our *Ccny/l1* mutant phenotype is strikingly similar to that of *Lrp6* knock out mice (Zhou *et al*, 2006).

3. In Figure 1H and S1G the authors use immunofluorescence to identify Ccny/l1 expressing cells. Such analysis is important to understand the knockout phenotype. Assigning the signal to a specific cell type, however, is not convincing. The staining for Ccny/l1 is patchy; but even more problematic is the fact that the outline of cells cannot be traced. One potential solution would be to use fluorescent reporter electroporated brains and to use the expression of the reporter combined with a cell type specific marker to study the expression of Ccny/l1 in a specific cell type.

We thank Reviewer 1 for this insightful suggestion. We have performed IF using antibodies against Ccny/l1 plus Sox2 (radial glia marker) and GFP (to detect APs), or Tbr2 and GFP (to detect BPs) on E15.5 control embryos electroporated with GFP plasmid at E13.5. This revealed clear and high Ccny/l1 immunoreactivity at the apical membrane in $Sox2^+$ APs, while in $Tbr2^+$ BPs, Ccny/l1 appeared as single puncta. These data, which are displayed in new Fig EV1G-H, effectively assign the Ccny/l1 signals to specific cell types (e.g. APs and BPs) as requested by Reviewer 1.

4. The authors make qualitative statements regarding the expression of Ccny/l1 and pLRP6. For understanding of the phenotype it would be important to provide a quantification of what the fraction of APs / BPs / neurons / pHH3+ cells is that express Ccny/l1 and pLRP6.

To address this concern, we have performed IF for Ccny/l1 plus pHH3 and Tbr2 on E13.5 neocortex sections and quantified the number of $Ccny/l1^+$ BPs, $Ccny/l1^+$ mitotic BPs

(Tbr2$^+$pHH3$^+$), and Ccny/l1$^+$ mitotic APs (pHH3$^+$ cells lining ventricle). We have also performed IF for pLRP6 (using both S1490 and T1479 antibodies) plus Tbr2 and pHH3, and quantified the number of pLRP6$^+$ mitotic BPs (Tbr2$^+$pHH3$^+$) and APs (pHH3$^+$ cells lining ventricle). The percentages of positive cells have been included in paragraph 5 of the results section. Details on the quantification methods used have been added to the Appendix material (pg. 8). We did not detect Ccny/l1 immunoreactivity in post-mitotic neurons. In regards to APs, Ccny/l1 is detected throughout the entire apical membrane in the neocortex, suggesting that most APs are positive for Ccny/l1.

5. The authors suggest Sox4 and Sox11 as targets of GSK3beta phosphorylation-dependent destabilization, ubiquitination and degradation. The decrease in Sox4 and Sox11 protein levels in the embryonic cortex is suggestive of decreased stability but this reduction is also easily explained by the fact that BP and neuron numbers are reduced.

Maybe a misunderstanding: First, the most striking decreases in Sox4/11 were observed in mitotic BPs by IF, and the quantification of Sox4/11 immunoreactivity was performed on individual cells (Fig 5B-C (Sox4) and Fig 5H-I (Sox11)). Hence, the overall decrease in BPs and neurons observed in the DKO neocortex would not influence this method of quantification. Second, in regards to the immunoblot analyses on forebrain lysates, levels of Sox11, which were decreased in DKO embryos, were normalized to Tuj1 and not GAPDH (Fig 5G), thereby taking into account the reduction in newly formed neurons observed in DKOs. Sox4 total protein levels were normalized to Tbr2 protein levels, although no significant differences were observed in forebrain lysates (Fig EV5F).

The authors overexpress Sox4 and Sox11 to test their ability to rescue the Ccny/l1 dKO phenotype. Overexpression results in higher fraction of Tuj1 expressing cells but whether this constitutes a rescue of the neuronal differentiation phenotype remains questionable. The authors score all Tuj1+ cells as neurons even if these cells do not feature the typical bipolar neuronal morphology. This is mentioned in the manuscript, but as Tuj1 is a direct target of SoxC proteins (Bergsland et al 2006), induction of Tuj1 expression by itself is not sufficient to claim a rescue of the Ccny/l1 dKO phenotype and to causally link Ccny/l1 and SoxC function. Neuronal numbers should be normalized to the number of transduced cells based on GFP expression.

The Reviewer is correct in pointing out that analyzing Tuj1 levels alone is not sufficient to claim a rescue as βIII-tubulin (Tuj1) is indeed a direct SoxC target (Bergsland *et al*, 2006). To address this issue we have repeated the experiments and quantified the number of neurons using two different markers: Tuj1 and Map2 (microtubule-associated protein 2). We have also normalized neuron number to the number of transduced cells (identified by IF for FLAG (Sox4/11) or GFP (empty vector)), as suggested by the Reviewer. *Sox4/11* overexpression once again rescued the DKO neurogenesis phenotype as shown by IF for Tuj1 (Fig 6B-C), and results with Map2 (Appendix Fig S3A-B) were nearly identical to Tuj1. Moreover, the transduction efficiencies of the Sox4/11-expressing lentiviruses were very high (typically over 90%) (Appendix Fig S2F), which explains the similar rescue efficiency observed when compared to previous experiments where neuronal numbers were not normalized to the number of transduced cells.

Moreover, the execution and quantification of the experiment are unclear. The authors describe that they use Flag-Sox4/11 in a pLENTI-CAG-IRES-GFP vector. How do the authors express Sox4 and Sox11 simultaneously? Is this construct tricistronic?

For the Sox4/11 rescue experiments two separate viruses, one overexpressing *Flag-Sox4* and one overexpressing *Flag-Sox11*, were both transduced simultaneously. We apologize for not explaining this clearly. More experimental details have been added to the Results, Materials and Methods, Figure Legends, and Figure panels.

The lack of increased neuronal differentiation in the control group following Sox11 / Sox4 overexpression is surprising as Sox11 / Sox4 overexpression has been reported to potently induce neuronal differentiation.

As to why Sox4/11 overexpression does not, as previously reported, increase neuronal differentiation in control cells is unclear, but may be related to differences in the experimental set up, length/efficiency of transduction etc. In support of our findings, we would like to state that the experiment was repeated four separate times, and all repetitions led to similar results.

Finally, the baseline differentiation of NPCs with 20% Tuj1 positive cells in WT NPCs is rather low compared to other reports.

A differentiation efficiency of around 20%, although lower than in some studies, has been reported elsewhere (see Hirabayashi *et al*, 2004). We would also like to point out that the control NPCs used were heterozygous for Ccny and either Ccnyl1-deficient (Fig 3I) or heterozygous (Fig 5B-C). This may explain the slightly lower than anticipated differentiation efficiency.

6. SoxC factor ubiquitination (Chiang et al 2021), phosphorylation (Balta et al. 2018 a,b) and sumoylation (Chang et al. 2017) have been described in the context of neurodevelopment. The authors may want to consider to include these papers in their discussion as these papers provide further evidence that the function of this transcription factor family is controlled by post-translational modifications.

We would like to thank the Reviewer for pointing out these papers. We have now included a sentence in paragraph 5 of the Discussion that addresses the implications of Sox11 post-transcriptional modifications in the context of neocortex development.

7. The observations regarding altered kinetics of mitosis in BPs and altered mode of division in APs are very interesting but remain underexplored in terms of mechanism. As the neuronal differentiation deficit is potentially only the culmination / consequence of altered AP and BP behavior, it would be important to mechanistically understand the role of Ccny/l1 in these cell types.

Regarding the altered mode of AP division, we respectfully disagree with the Reviewer's statement that this phenotype is *"underexplored in terms of mechanism"*. We show in Figure 3A-D and describe in the text that the increase in symmetric AP divisions in DKO embryos is associated with an increase specifically in apical-basal, but not central, astral microtubules. Apical-basal astral microtubules were previously shown by one of us (W.B.H.) to exert a key role in governing symmetric vs. asymmetric AP divisions. High levels of apical-basal astral microtubules ensure symmetric, self-renewing AP divisions by enforcing a perfectly vertical cleavage plane, whereas reducing the level of apical-basal astral microtubules allows greater variability in cleavage plane orientation and leads to asymmetric, BP-genic/neurogenic AP divisions (Mora-Bermúdez *et al*, 2014). Hence, our data showing the increase specifically in apical-basal, but not central, astral microtubules in DKO embryos (Figure 3C-D) not only provide

a mechanistic explanation for the increase in symmetric AP divisions in DKO embryos (Figure 3A-B), but also for the decrease in the level of BPs (Figure 1E) and in neuron numbers (Figure 1F) in these embryos, as is discussed in the text.

In terms of the altered kinetics observed in DKO BPs, the Reviewer is correct in pointing out that we do not provide a mechanistic explanation. However, such an analysis would involve identifying and validating new WNT/GSK3 targets. Identifying and functionally characterizing such a protein would constitute a study in itself and is beyond the scope of this paper.

Referee #2:

Mammalian corticogenesis is a complex process where various molecules play intricate roles. Wnt signaling pathways are previously considered as one of the key regulators in neurogenesis, although there are both sides of evidence that Wnt signaling maintains proliferation or promotes differentiation. In this study, the authors focused on a novel non-canonical Wnt pathway involved in the stabilization of protein (here they term the WNT/STOP pathway) by analyzing Cyclin Y (Ccny) and Cyclin Y-like 1 (Ccnyl1) double knockout (DKO) mice. Through detailed in vivo and invitro analyses, the authors identified Sox4 and Sox11 as direct targets of Gsk3 and concluded that Wnt/STOP signaling drives neuronal differentiation whereas that Wnt/b-catenin signaling regulates self-renewal of neural progenitor cells.

Overall, the analyses seemed to be carefully performed with multiple approaches, and the data are logically presented. The topic will attract the eyes of researchers in developmental neurobiology. However, this Reviewer considers the following issues should be considered before publication:

Thank you!

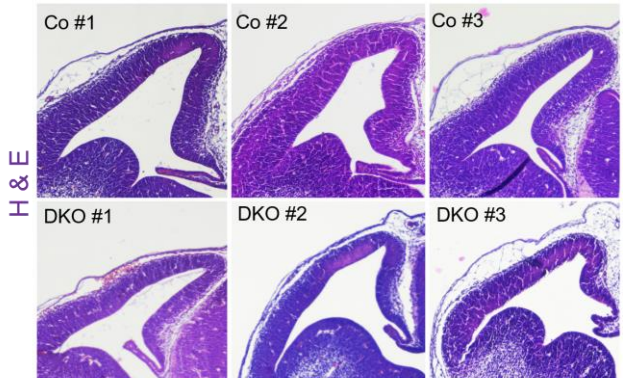
Major points:
1) Quantitative data should be given for Fig 1A, 1J, 3I, 4G, 4H, 4I, 4J, 5E, 5F, 5G, 5J, S1I, S3B, S5A, S5E, and S5G. Relatedly, the number of samples and areas of the neocortical areas should be indicated. More detailed descriptions of the methodology for quantitative analyses are needed. For example, which of the cortical areas are chosen and how many of the cells are counted for Figs. 1B, D-F?

Quantitative data for Fig 1A and 3I, was already provided in the image panels as numbers ± SEM, and the number of embryos/samples analyzed, as well as the experimental details, are provided in the Figure Legends and Methods. For the numbers in Fig 1A, we added asterisks to indicate statistical significance. For the remaining panels, which are all immunoblots, quantification was provided in the form of numbers above the blots with the details described as above. For the immunoblot quantification data, we have added ‘numbers above blot’ before the description of the quantitative data to further clarify. Bar graphs were not provided for these data due to space constraints.

For Figs 1B-F, we have added information on the number of cells counted and the cortical areas chosen. This data is included in the Appendix Methods (pg. 8).

2) In Fig 1B, DKO shows a wider space between CP and the meninges. Is it a phenotype?

The Reviewer is correct in pointing out that the space between the CP and meninges in Fig 1B is wider. However, this is not a phenotype since the wider meningeal space between DKOs and controls is not consistently observed (see below). Instead, it is more likely an artifact of the tissue processing/cutting, which can occasionally lead to the meninges peeling off from the CP, giving the impression that there is a wider space.



The meningeal space is not consistently wider in the DKO neocortex.
H & E staining in the E13.5 neocortex from 3 separate control and DKO embryos.

3) In Fig 1C, only the rational values are shown. It would be better to describe specific values regarding the difference between Co and DKO for Fig 1C and the text (page 7, lines 15 to 19) as for Fig 2H. How many percentages are decreased/increased in DKO?

We have added data with the specific values for Fig 1C in the form of bar graphs to Appendix Fig S1A-D. We kept the rational values in Fig 1C to indicate the relative thickness of each layer compared to the total thickness of the neocortex. Paragraph 2 of the results section has been updated to include the percentage changes of the rational values for VZ, SVZ, IZ-CP thickness, as requested. We have also added data on the specific values for Fig 2H to Appendix Fig S1 (E-F).

4) Regarding the expression levels of pLRP6 and Axin2, what kind of cells are measured? Are they APs or BPs?

For the pLRP6 (T1479) IF analysis, both mitotic APs (division at the apical membrane / cell cortex) and mitotic BPs (SVZ division) were measured. The number of APs and BPs measured is now indicated on pg. 8 of the Appendix Methods. For the *Axin2 RNAscope* analysis, APs and BPs were not measured separately since we could not use cell-type specific markers. Instead, we measured the entire *Axin2*$^{+}$ area per section.

5) It is not clear to this Reviewer that "WNT/LRP6 signaling peaks during mitosis in APs and BPs." (p9, line 20-21). What is the evidence for this statement?

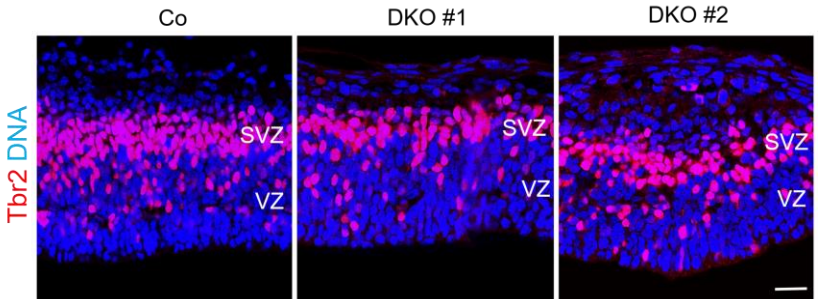
WNT/LRP6 signaling in this context is measured by LRP6 phosphorylation at the S1490 (priming and active phosphorylation) and the T1479 (active phosphorylation) sites, both of which peak in pHH3^{+} APs (Fig 1Jc; EV1I - left panel) and BPs (Fig 1Kc; Ev1I - right panel), as shown by IF.

6) It would be better to perform qPCR using RNA extracted from E13.5 embryonic "dorsal" telencephalon to eliminate the effects from "ventral" telencephalon for obtaining data of Figs. 1L, S5F, and S5L. In addition, if the authors show the data for N-myc, Sox4 and Sox11 expression, they should state the results in the text.

We have repeated the qPCR experiments using RNA extracted only from the dorsal telencephalon and replaced the panels in Figs. 1L, S5F, and S5L with the new data. The results were similar to those obtained from the whole forebrain RNA extractions in that *Axin2*, *N-myc*, *Sox4* and *Sox11* expression are not significantly altered in DKO embryos. We have added p-values for these results to the text as requested by the reviewer.

7) Is it a phenotype that DKO has no Tbr2+ cells in the ventricular zone in Fig 2D?

No. The image of the DKO embryo shown in Fig 2D is atypical in that regard. As shown below in two different DKO embryos, Tbr2^{+} cells in the VZ are reduced but not absent.



DKO embryos display Tbr2^{+} cells in the VZ.
IF for Tbr2 in the neocortex of one control and two DKO embryos shows reduced but not absent Tbr2+ cells in the DKO VZ.

8) According to a previous article (i.e., Martynoga et al., Dev Biol, 2005), Ts was calculated by the following formula:

Ts=Ti (Lcells/Scells)
Ti is 1.5h
Lcells= IdU+/BrdU-, Scells=IdU+/BrdU+

For example, if the authors calculate APs in Co with reference to the above equation, it will result in 4.1, which seems to be different from the value in the graph in Fig 2I. What is the reason for this discrepancy? If they use a different formula, please indicate by citing a reference.

It would also be necessary to describe the formula for calculating the length of S-phase, total cell cycle, and mitosis so that we can understand in Appendix method page 14 line 28-40.

We did not calculate S-phase length (T_S) according to the formula cited by the Reviewer and published by Martynoga *et al*, in Dev. Biol. in 2005. Rather, we used our own reasoning and previous experience in cell cycle analyses (for example, Arai *et al*, 2011; Turrero García *et al*, 2016) for this calculation, as is explained below and now described in greater detail in the Results and Appendix Methods (pg. 8-9) sections. Thus, the percentage of the total IdU-labeled progenitors that no longer incorporate BrdU 1.5 hours later (IdU^+BrdU^-; 27%, 22%, 29%, 19% in Fig 2H) obviously indicates the percentage of progenitors that have left S-phase 1.5 hours after the IdU labeling. If it takes 1.5 hours for 27%, 22%, 29% or 19% of the progenitors to leave S-phase, then by dividing 1.5 hours by 27, 22, 29 or 19 and then multiplying the resulting numbers with 100 (to get the 100% values) will give the times until all IdU-labeled progenitors will have left S-phase, that is, the length of S-phase (5.6, 6.8, 5.2, 7.9 hours, Fig 2I).

$$T_S = 1.5 \text{ hours} \Big/ \frac{IdU^+BrdU^- \text{ progenitors}}{IdU^+ \text{ progenitors}}$$

One of the differences between the Martynoga *et al*, 2005 formula and our formula is that in the denominator, we use the total number of IdU^+ progenitors counted 2 hours after the start of IdU labeling (rather than only the IdU^+BrdU^+ progenitors then still present), which in our humble opinion is the correct way of calculation.

Given these S-phase length values, total cell cycle length (T_C) was calculated as follows. If 30.7% and 38.8% of the APs (Fig 2C) and 23.5% and 21.2% of the BPs (Fig 2F) are in S-phase at steady state as determined by a BrdU pulse, then dividing the S-phase length values (Fig 2I) by these percentage values (30.7, 38.8, 23.5, 21.2) and then multiplying the resulting numbers with 100 (to get the 100% values) will give the total length of the cell cycle of the various progenitor populations (18.1, 17.6, 22.1, 37.3 hours; Fig 2J).

$$T_C = T_S \Big/ \frac{BrdU^+ \text{ progenitors}}{\text{total progenitors}}$$

It is worth noting that the total cell cycle length values for E13.5 wt APs (18.1 hours) and E13.5 wt BPs (22.1) determined in the present study fit quite well to those determined previously at E14.5 by cumulative EdU labeling (Arai et al. 2011; 19.1 hours for APs, 26.5 hours for BPs; cell cycle length is known to increase at later developmental stages, i.e. E14.5 vs. E13.5).

Finally, the total cell cycle length values were used to calculate the length of mitosis (T_M), as follows. First, the percentages of progenitors in mitosis were determined by calculating the percentages of cycling ($Ki67^+$) APs (Tbr2–) and BPs (Tbr2+) that were positive for the mitotic marker phospho-histone H3 ($pHH3^+$; 5.06%, 6.18%, 3.26%, 4.12%; Fig 2K,L). Multiplying the

total cell cycle length values (Fig 2J) with the latter percentage values (Fig 2L) and dividing the resulting numbers by 100 yielded the length of mitosis (0.92, 1.09, 0.72, 1.54 hours; Fig 2M).

$$T_M = T_C \times \frac{pHH3^+Ki67^+ \text{ progenitors}}{Ki67^+ \text{ progenitors}}$$

Please find a more detailed description of our calculations in the revised Results and Appendix Methods sections, including the formulas used (in Appendix Methods pg. 9).

9) Regarding data of Fig 3I, why NPCs from E13.5 Ccny-/-; Ccnyl1-/- forebrains were used instead of from Ccny+/-; Ccnyl1-/- (page 14, lines 11)? What kind of control was used here? It would also be better to explain the length of Tuj1+ dendrites and axons in Fig 3I (page 14, lines 16). It would be suitable if they are based on the quantitative analysis.

For the analysis in Fig 3I we opted for an acute knockdown approach by lentiviral delivery of shRNA against *Ccny* in *Ccny*+/- *Ccnyl1*-/- cultured NPCs. The purpose of the acute knockdown was to rule out early onset or non-cell autonomous causes of the DKO differentiation phenotype. To clarify, we have changed the description above the left panel in Fig 3I from "*Ccnyl1* deficient" to "*Ccny*+/-; *l1*-/-; sh*Ccny*". The controls used were cultured NPCs from *Ccn*y+/-; *Ccnyl1*-/- embryos transduced with a sh*Control*-expressing lentivirus. This information has been added to Fig 3I and the corresponding Figure legend.

We have also performed a quantitative analysis of neurite length for Fig 3I, as requested by the Reviewer. This revealed a highly significant reduction in mutant NPCs. This data is presented as numbers ± SEM in paragraph 15 of the results section. Experimental details of the quantification method used are included in pg. 10 of the Appendix Methods.

10) Regarding data for Sox4, what is the evidence for "In DKO embryos, we could not detect an obvious decrease in Sox4 staining intensity in Tbr2+ cells" (p.17, line 25-p18, line 1)? It would be better to measure Sox4 in APs.

We have now measured Sox4 staining intensity in non-mitotic BPs on sections of the neocortex and included these data in new Fig EV5E. No significant differences were detected between control and DKO embryos. In regards to measuring Sox4 expression in APs, this would be difficult since the protein levels of Sox4 in mitotic APs are very low and the IF signal for Sox4 is only slightly above background fluorescence levels.

11) It would be better to examine the ratio of mature neurons (asterisks) and immature neurons (arrowheads) in 4 groups (Co, Co+Sox4/11, DKO, DKO+Sox4/11) in Fig 6B.

We thank the Reviewer for this suggestion. The measurement of the ratio of mature to non-mature neurons for the newly performed Sox4/11 rescue experiments (see response to Reviewer 1 point 5) is now included in new Appendix Fig S3C. As shown, only the number of immature neurons are rescued in DKO NPCs upon Sox4/11 overexpression.

12) What is the evidence to state "The reason why Lrp6 mutants do not also manifest the proliferation defects of β-catenin mutants is unclear but may be due to compensation from LRP5."

Functional redundancy between Lrp5 and Lrp6 in transducing Wnt/β-catenin signaling in several biological processes is well documented (Kelly *et al*, 2004; Zhong *et al*, 2011, Goel *et al*, 2012; Liu *et al*, 2019). Hence, it can be assumed that Lrp6 mutants do not display the same proliferation phenotype in the neocortex as β-catenin mutants because Lrp5 compensates for the loss of Lrp6. Whether or not Lrp5 can also compensate for Lrp6 in WNT/STOP signaling, particularly in the context of neocortex development, is unclear, but based on our observations in the DKO neocortex, this does not seem to be the case since Lrp6 mutants display a remarkably similar phenotype in the neocortex to our DKOs (Zhou *et al*, 2006). However, since there is no direct evidence of this, we do not claim this to be true, we merely suggest it as a possibility.

Minor points:
1) It would be better to show the figure numbers separatory in figures and graphs of the following figures such as Fig 1D-F, I-K (figures and graphs), S1E (Ccny and Ccnyl1), S3A (βIII-tublin, Doublecortin, and Sox2), S3C (Ccny and pLLRP6 (S1490)), S3D (Sox4, Sox4; Tbr2 and Sox4; Tbr2; pHH3), and S3M (CCNY and pLRP6).

This has been corrected.

2) Scale bars are needed for Fig S1F-G, 4B-C.

These have been added.

3) The angles of the images should be similar between Co and DKO for Figs. 1D, 1E, 2A, and 2D.

The angles of the images have been corrected as suggested by the Reviewer.

4) The number of embryos should be indicated in Fig 3D.

This information has been added.

5) For the terminology, it would be better to use "forebrain" rather than "whole brain" (regarding Fig 5J and its related text).

The term “whole brain” has been replaced by “forebrain” in the text as requested.

6) It is better to state that Ccny+/-; Ccny11+/- was used as a control in the main text (page 26, line 11-12).

This has been corrected.

7) It is useful for the reader if the authors provide product information for the 70 μm cell strainer; TRIzol; and MG132 (page 28, line 6; text, page30, line6; and text, page 31, line 13).

The product information for the 70 μm cell strainer and MG132 has been added. For the new dorsal telencephalon RNA extractions, we used an RNA isolation kit instead of TRIzol. Hence,

all references to TRIzol in the materials and methods section have been removed since no experiments in which TRIzol was used are included in the revised version of the manuscript.

8) There are many typos that should be edited in the revised text.

The typos have been corrected.

Referee #3:

Reviewer's comment:

Several studies have implicated the Wnt signaling pathway in mouse cortical neurogenesis but the results are heterogeneous and difficult to reconcile. In this manuscript, Da Silva and colleagues attempt to do so by calling into play the WNT-stabilization of proteins pathway (Wnt/STOP). This pathway is β-catenin independent and involves the activity of cyclin Y and cyclin Y-like 1. The authors generate a mouse line mutant for both Ccny and Ccnyl1 genes, which is embryonic lethal at E13.5. Taking advantage of this line, in vivo knock-down experiments (to cover later stages developmental stages), in vitro studies with neural stem cells and different biochemical approaches, the authors show that WNT/STOP controls basal progenitor generation and their asymmetrical division, which gives rise to differentiated cells. They further show that Sox4 and Sox11 are targets of the pathway and that their activity is needed to promote neuron generation. The propose a model in which WNT/β-catenin signaling would be the main driver of apical progenitor proliferation, whereas the WNT/STOP pathway would be in charge of controlling the asymmetric proliferation of basal progenitors and their differentiation into neurons through Sox4 and Sox11 activity.

This is an interesting and well performed study which provides novel ideas on the control of neurogenesis in the mammalian cortex. The conclusions are based on a large set of experimental approaches that support much of the authors conclusions. The manuscript is also in general well written. Nevertheless, there are a few aspects that could be improved or better explained given that the manuscript is rather dense and non-experts may have difficulties in following some of the conclusions.

Thank you!

1. Figure 2J and M. Can the authors explain in methods and results how they have extrapolated the values of the length of the cell cycle and the duration of mitosis? This is currently poorly explained in the results and it is not mentioned in the methods.

Given the S-phase length values (see Fig 2I), total cell cycle length (T_C) was calculated as follows. If 30.7% and 38.8% of the APs (Fig 2C) and 23.5% and 21.2% of the BPs (Fig 2F) are in S-phase at steady state as determined by a BrdU pulse, then dividing the S-phase length values (Fig 2I) by these percentage values (30.7, 38.8, 23.5, 21.2) and then multiplying the resulting numbers with 100 (to get the 100% values) will give the total length of the cell cycle of the various progenitor populations (18.1, 17.6, 22.1, 37.3 hours; Fig 2J).

$$\text{The formula used is: } T_C = T_S \,/\, \frac{BrdU^+ \text{ progenitors}}{\text{total progenitors}}$$

The total cell cycle length values were used to calculate the duration of mitosis (T_M), as follows. First, the percentages of progenitors in mitosis were determined by calculating the percentages of cycling ($Ki67^+$) APs (Tbr2–) and BPs (Tbr2+) that were positive for the mitotic marker phosphohistone H3 ($pHH3^+$; 5.06%, 6.18%, 3.26%, 4.12%; Fig 2K,L). Multiplying the total cell cycle length values (Fig 2J) with the latter percentage values (Fig 2L) yielded the length of mitosis (0.92, 1.09, 0.72, 1.54 hours; Fig 2M).

$$\text{The formula used is: } T_M = \frac{pHH3^+Ki67^+ \text{ progenitors}}{Ki67^+ \text{ progenitors}} \times T_C$$

We admit that the previous description of these calculations in Results and Methods was perhaps not sufficiently detailed. A more detailed description of our calculations is now provided in the revised Results and Appendix Methods sections, including the formulas used (in Appendix Methods pg. 9).

2. Figure 3E, F. Additional high-power images of the electroporated cells will help to appreciate the triple labelling, at the moment impossible to see.

These have been added. We thank the Reviewer for this excellent suggestion.

3. Figure 3I. Staining with Tuj1 cannot differentiate axon and dendrites so, the description of process in culture should simply refer to neurites and neuritic length.

We fully agree and the terms “axons” and “dendrites” have been replaced by “neurites”.

4. Figure 6B. Experiments here are confusing. Are all the cells transduced with Sox4/11? Double staining for Sox4/11 and Tuj1 would help to understand the results. The Tuj1+ in the DKO cells looks rather fibroblast-like. The use of additional neuronal markers would help to support the idea that Sox4/11 indeed rescue the phenotype even if partially. This is not very convincing at the moment. This partial rescue should be discussed further.

For the Sox4/11 rescue experiments two separate viruses, one overexpressing *Flag-Sox4* and one overexpressing *Flag-Sox11*, were both transduced simultaneously. We apologize for not explaining this clearly. This has been corrected. We have also repeated the experiments with an additional marker, Map2, this time normalizing to the number of transduced cells (FLAG IF for Sox4/11 overexpression and GFP IF for empty vector). The number of neurons was once again rescued by Sox4/11 overexpression both with Tuj1 (new Fig 6B-C) and Map2 (Appendix Fig

S3A-B) quantification. As with previous experiments, only the number of immature neurons was rescued (Tuj1 and Map2), once again suggesting Sox4/11 can only rescue the initial stages of the neurogenesis defect in cultured DKO NPCs.

5. The model proposed in Fig 6D indicate that canonical WNT/β-catenin signaling promotes AP self-renewal. It is unclear exactly how the authors have reached the conclusion.

The conclusion that WNT/β-catenin signaling promotes AP self-renewal is not based on experimental evidence from this manuscript, but from a plethora of previous publications all demonstrating the same phenomenon: WNT/β-catenin signaling, as determined by β-catenin loss or gain of function, promotes AP self-renewal in the developing neocortex (Machon *et al*, 2003; Mutch *et al*, 2010; Woodhead *et al*, 2006; Chenn & Walsh, 2002; Machon *et al*, 2007; Wrobel *et al*, 2007; Kim *et al*, 2009; Draganova *et al*, 2015). These papers are cited in the introduction (paragraph 2) and re-analyzed in the context of this manuscript in paragraphs 2 and 4 of the discussion.

References:

Arai Y, Pulvers JN, Haffner C, Schilling B, Nüsslein I, Calegari F, Huttner WB (2011) Neural stem and progenitor cells shorten S-phase on commitment to neuron production. *Nat. Commun* 2:154.

Chenn A, Walsh CA (2002) Regulation of cerebral cortical size by control of cell cycle exit in neural precursors. *Science* 297: 365–369.

Draganova K, Zemke M, Zurkirchen L, Valenta T, Cantù C, Okoniewski M, Schmid MT, Hoffmans R, Götz M, Basler K, Sommer L (2015). Wnt/β-catenin signaling regulates sequential fate decisions of murine cortical precursor cells *Stem Cells* 33: 170–182.

Goel S, Chin EN, Fakhraldeen SA, Berry SM, Beebe DJ, Alexander CM (2012) Both LRP5 and LRP6 receptors are required to respond to physiological Wnt ligands in mammary epithelial cells and fibroblasts. *J Biol Chem* 287: 16454–16466.

Hirabayashi Y, Itoh Y, Tabata H, Nakajima K, Akiyama T, Masuyama N, Gotoh Y (2004) The Wnt/β-catenin pathway directs neuronal differentiation of cortical neural precursor cells. *Development* 131: 2791–2801.

Huang YL, Anvarian Z, Döderlein G, Acebron SP, Niehrs C (2015) Maternal Wnt/STOP signaling promotes cell division during early Xenopus embryogenesis. *Proc Natl Acad Sci USA* 112: 5732–5737.

Kelly OG, Pinson KI, Skarnes WC (2004) The Wnt co-receptors Lrp5 and Lrp6 are essential for gastrulation in mice. *Development* 131: 2803–2815.

Kim WY, Wang X, Wu Y, Doble BW, Patel S, Woodgett JR, Snider WD (2009) GSK-3 is a master regulator of neural progenitor homeostasis. *Nat Neurosci* 12: 1390–1397.

Koch S, Acebron SP, Herbst J, Hatiboglu G, Niehrs C (2015) Post-transcriptional Wnt signaling governs epididymal sperm maturation. *Cell* 163: 1225–1236.

Liu J, Cui Z, Wang F, Yao Y, Yu G, Liu J, Cao D, Niu S, You M, Sun Z, *et al* (2019) Lrp5 and Lrp6 are required for maintaining self-renewal and differentiation of hematopoietic stem cells. *FASEB J* 33: 5615–5625.

Machon O, Van Den Bout CJ, Backman M, Kemler R, Krauss S (2003) Role of β-catenin in the developing cortical and hippocampal neuroepithelium. *Neuroscience* 122: 129–143.

Machon O, Backman M, Machonova O, Kozmik Z, Vacik T, Andersen L, Krauss S (2007) A dynamic gradient of Wnt signaling controls initiation of neurogenesis in the mammalian cortex and cellular specification in the hippocampus. *Dev Biol* 311: 223–237.

Mutch CA, Schulte JD, Olson E, Chenn A (2010) Beta-catenin signaling negatively regulates intermediate progenitor population numbers in the developing cortex. *PLoS One* 5(8): e12376.

Turrero García M, Chang Y, Arai Y, Huttner WB (2016) S-phase duration is the main target of cell cycle regulation in neural progenitors of developing ferret neocortex. *J Comp Neurol* 524: 456–470.

Woodhead GJ, Mutch CA, Olson EC, Chenn A (2006) Cell-autonomous β-catenin signaling regulates cortical precursor proliferation. *J Neurosci* 26: 12620–12630.

Wrobel CN, Mutch CA, Swaminathan S, Taketo MM, Chenn A (2007) Persistent expression of stabilized β-catenin delays maturation of radial glial cells into intermediate progenitors. *Dev Biol* 309: 285–297.

Zhong Z, Baker JJ, Zylstra-Diegel CR, Williams BO (2012) Lrp5 and Lrp6 play compensatory roles in mouse intestinal development. *J Cell Biochem* 113: 31–38.

Zhou CJ, Borello U, Rubenstein JLR, Pleasure SJ (2006) Neuronal production and precursor proliferation defects in the neocortex of mice with loss of function in the canonical Wnt signaling pathway. *Neuroscience* 142: 1119–1131.

1st Revision - Editorial Decision 28th Jul 2021

Dear Dr Niehrs,

Thank you for the submission of your revised manuscript to The EMBO Journal and please accept my apologies for the delay in responding. As you will see below, your article was seen by the original referees, who now consider that you have properly dealt with most of their main concerns originally raised in the review process. There are however a few points, mainly referring to the presentation of data in the paper that will need to be addressed before it can be accepted for publication

In addition, please address the following editorial points:

- The format of the references is correct, but numbers need to be removed.
- The text in the Appendix must be black.
- Please provide a valid e-mail address for Edoardo Fatti. The one provided seems to be incorrect.
- Please find additional comments made by the quality check team at Wiley in the Word document attached (see comments in the figure legend section).

Please let me know if you have any further questions regarding any of these points. Thank you again for giving us the chance to consider your manuscript for The EMBO Journal. I look forward to receiving the final version.

Yours sincerely,

David del Alamo
Editor
The EMBO Journal

Further information is available in our Guide For Authors:
https://www.embopress.org/page/journal/14602075/authorguide

Please click on the link below to submit the revision online:

Link Not Available

Referee #1:

The authors have addressed all my concerns and comments with one exception: the authors now convincingly show that the WNT/STOP pathway contributes to the regulation of the mode of division of apical progenitors as well as to the regulation of neuronal fate determination of basal progenitors. While these findings uncover a new level of complexity on the role of Wnt-signaling in cortex development, they do not resolve the longstanding question of the precise function of WNT-beta catenin signaling in cortex development. What the authors observe is that inhibition of WNT/STOP signaling via knock-out of Ccny/l1 results in defects in AP and BP function in the absence of alterations of stabilized b-catenin levels and axin2 levels. The latter findings indicate

that the knock-out of Ccny/l1 does not affect WNT-beta catenin signaling but does not demonstrate that WNT-beta catenin signaling has no/little function in these processes. In my view it is therefore important to revise the manuscript in the relevant parts.

Referee #2:

Revision Comments
The authors responded to the comments raised by the reviewers, and the revised manuscript has been much improved. However, this reviewer is still concerned about some of the issues including the followings:

Major points:
1) Regarding Fig. 1A, it is more proper to indicate quantitative data of the cortical thickness as bar graphs, not within the images, together with the sample size (maybe as Appendix Figure). It would be more informative to add a similar graph for the length of the neocortical wall (red arrows).
The same is applied for Fig. 3I. However, this suffers inconsistency with the previous version of the figure indicating three asterisks, whereas in the revised version it has been reduced to two. Was this a simple mistake? If so, it should be indicated in the point-by-point responses.
In some figures of immunoblotting (e.g. Fig. 1N), it would be better to use average{plus minus}SEM and the same significant digits.

2) Images of DKO in Fig 1B should be replaced with more typical one, as in Fig. 1A. Otherwise, it is misleading.

4) In Fig 1L-P, it is assumed that the authors used ROI on pLRP6 or Axin2 positive single cells and measured the average intensity with Fiji. Procedures should be explained more in detail by mentioning, for example, how ROI was drawn. The same is applied for other methods such as calculation of brightness values.

7) The figures used in the "Response to the Referees" is very helpful. It would be better to include the data as Appendix Figure.

8) It would be suitable to refer previous studies on cell cycle analyses (Arai et al, 2011; Turrero García et al, 2016) in the Appendix Method.
It would also be helpful for the reader if the following statements are added to the Appendix Method:
"It is worth noting that the total cell cycle length values for E13.5 wt APs (18.1 hours) and E13.5 wt BPs (22.1) determined in the present study fit quite well to those determined previously at E14.5 by cumulative EdU labeling (Arai et al. 2011; 19.1 hours for APs, 26.5 hours for BPs; cell cycle length is known to increase at later developmental stages, i.e. E14.5 vs. E13.5)."

Minor points:
8) It would be better to carefully check typos and font errors in throughout the manuscript (including Appendix). For example, "Kosodo et al. 2004" should be "Kosodo et al, 2004".

Referee #3:

The authors have addressed my concerns and I believe that the manuscript woll represent and important addition for the those interested in cortical development and Wnt signalling

The authors performed the requested editorial changes.

2nd Revision - Editorial Decision 10th Aug 2021

Dear Dr. Niehrs,

I am pleased to inform you that your manuscript has been accepted for publication in the EMBO Journal.

If you have any questions, please do not hesitate to contact me. Thank you for your contribution to The EMBO Journal.

Yours sincerely,

David del Alamo
Editor
The EMBO Journal

Please note that it is EMBO Journal policy for the transcript of the editorial process (containing referee reports and your response letter) to be published as an online supplement to each paper. If you do NOT want this, you will need to inform the Editorial Office via email immediately. More information is available here:
https://www.embopress.org/page/journal/14602075/authorguide#transparentprocess

Your manuscript will be processed for publication in the journal by EMBO Press. Manuscripts in the PDF and electronic editions of The EMBO Journal will be copy edited, and you will be provided with page proofs prior to publication. Please note that supplementary information is not included in the proofs.

Please note that you will be contacted by Wiley Author Services to complete licensing and payment information. The 'Page Charges Authorization Form' is available here:
https://www.embopress.org/pb-assets/embo-site/tej_apc.pdf

Should you be planning a Press Release on your article, please get in contact with embojournal@wiley.com as early as possible, in order to coordinate publication and release dates.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Christof Niehrs
Journal Submitted to: EMBO Journal
Manuscript Number: EMBOJ-2021-108041R

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

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

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	No power analyses were performed to determine sample size. Sample size was based on previously published studies and pilot studies.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	Due to the uncertainty of the number of control and mutant embryos obtained from pregnant mice, for all experiments carried out with embryos we used embryos derived from more than 1 litter (litter number indicated in figure legends). For many statistical analyses (indicated in figure legends), we compared mutant embryos to their respective control embryos from the same litter to account for litter to litter variability.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	Mutant embryos that showed gross morphological defects were excluded from analyses.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	In all experiments, both wildtype and transgenic animals/samples were allocated to the same treatment.
For animal studies, include a statement about randomization even if no randomization was used.	N/A
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	All analyses utilizing the Fiji cell counter were performed blindly (without knowing the genotype of the sample). Analyses with user-defined Fiji macros were not performed blindly.
4.b. For animal studies, include a statement about blinding even if no blinding was done	All analyses utilizing the Fiji cell counter were performed blindly (without knowing the genotype of the sample). Analyses with user-defined Fiji macros were not performed blindly.
5. For every figure, are statistical tests justified as appropriate?	Yes, the statistical tests used are indicated in the Materials and Methods and Figure Legends.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes. Data normality was tested using Shapiro-Wilk normality test. Normally distributed data were subjected to Student's t-test.
Is there an estimate of variation within each group of data?	Yes, variation within each group of data was reported in each group of data as SEM.

USEFUL LINKS FOR COMPLETING THIS FORM

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

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

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

http://datadryad.org

http://figshare.com

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

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

http://biomodels.net/

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

Is the variance similar between the groups that are being statistically compared?	Yes, F test was used to test homogeneity of variances.

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Antibodies used in this study are listed in the Methods, with related information including species, catalog number, company, and, where applicable, citations. We refer to the manufacturer's instructions for antibody validation. Our homemade Sox4-phospho antibody was validated in Fig EV5J-K.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	HEK293T cells are routinely tested for mycoplasma.

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	Ccny; Ccnyl1 mutant mice were kept on a mixed genetic background. C57BL/6N mice were used for IUE experiments. Age and genetic modification status are reported in the Materials and Methods and Figure Legends. Gender was not taken into consideration for embryos. All animals used for this study were kept in standardized pathogen-free conditions at the Central Animal Laboratory of DKFZ or the Biomedical Services Facility of the MPI-CBG with free access to food and water. Animals were kept with the following light/dark cycle: 12 h / 12h (mice)
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	All animal work was conducted according to national and international guidelines and was approved by the state review board of Baden-Württemberg (protocol no. G-123/18) and the Landesdirektion Sachsen (licenses: mouse TVV 5/2015 and TVV 20/2020).
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	All relevant aspects of animal studies are adequately reported.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	N/A
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	N/A
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	N/A
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	N/A
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	N/A
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	N/A
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	N/A

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	No datasets were generated in this study.
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	N/A
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	N/A
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	N/A

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	N/A