EMBO reports

Developmental cell death of cortical projection neurons is controlled by a Bcl11a/Bcl6-dependent pathway

Christoph Wiegreffe, Tobias Wahl, Natalie Joos, Jerome Bonnefont, Pentao Liu, and Stefan Britsch
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(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.)

Dear Dr. Britsch

Thank you for the submission of your research manuscript to our journal. We have now received the full set of referee reports that is copied below.

As you will see, the referees acknowledge that the findings are potentially interesting but also suggest a number of experiments required to strengthen the current data and conclusions. It will be important to test whether Bcl11 controls the activity of the Bcl6 enhancer and whether Foxo1 is causal to the phenotype. A deeper analysis of the gene expression data and further cell death analysis should be provided. The data on p53 expression need verification and the choice of heterozygous Bcl11 mice as control needs to be justified. The referees also suggest testing whether the choice of Cre line could explain the milder phenotype of the Bcl6 KO and data on young neocortical neurons should be analysed.

In addition to these experiments, referee 1 suggested several further reaching experiments that will further strengthen the manuscript (ChIP-seq or ATAC-seq for Bcl11, analyse the role of COUP-TFs and the phenotype of Bcl11/Bcl6 double KO). We agree that these experiments will strengthen the conclusions, but we and the referee also recognize that some of these experiments will be time-consuming and technically challenging and it will therefore not be essential to address these experimentally.

Given these constructive comments, we would like to invite you to revise your manuscript with the understanding that the referee concerns (as detailed above and in their reports) must be fully addressed and their suggestions taken on board. Please address all referee concerns in a complete point-by-point response. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions. Please contact us if a 3-months time frame is not sufficient for the revisions so that we can discuss the revisions further.

***IMPORTANT NOTE: we perform an initial quality control of all revised manuscripts before re-review. Your manuscript will FAIL this control and the handling will be DELAYED if the following APPLIES:

1) A data availability section is missing.
2) Your manuscript contains error bars based on n=2. Please use scatter blots showing the individual datapoints in these cases. The use of statistical tests needs to be justified.

When submitting your revised manuscript, please carefully review the instructions that follow below. Failure to include requested items will delay the evaluation of your revision.***

When submitting your revised manuscript, we will require:

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. If your revised manuscript contains up to five figures, it will be published in our Reports section. In this case the Results and Discussion sections need to be combined.

2) individual production quality figure files as .eps, .tif, .jpg (one file per figure).
Please download our Figure Preparation Guidelines (figure preparation pdf) from our Author Guidelines pages https://www.embopress.org/page/journal/14693178/authorguide for more info on how to prepare your figures.

3) a .docx formatted letter INCLUDING the reviewers' reports and your detailed point-by-point responses 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 (). 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 (). Please find instructions on how to link your ORCID ID to your account in our manuscript tracking system in our Author guidelines
()

6) We replaced Supplementary Information with Expanded View (EV) Figures and Tables that are collapsible/expandable online. 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 labeled 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.

=> This applies to Supplementary Table 1. Please submit it as Dataset EV1.

7) Please list the accession number and database for the transcriptome analysis in a formal "Data Availability " section (placed after Materials & Method) that follows the model below (see also < https://www.embopress.org/page/journal/14693178/authorguide#dataavailability>). Please note that the Data Availability Section is restricted to new primary data that are part of this study.

Data availability

The datasets (and computer code) produced in this study are available in the following databases:

- RNA-Seq data: Gene Expression Omnibus GSE46843 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE46843)
- [data type]: [name of the resource] [accession number/identifier/doi] ([URL or identifiers.org/DATABASE:ACCESSION])

*** Note - All links should resolve to a page where the data can be accessed. ***

8) Figure legends and data quantification:

The following points must be specified in each figure legend:
- the name of the statistical test used to generate error bars and P values,
- the number (n) of independent experiments (please specify technical or biological replicates) underlying each data point,
- the nature of the bars and error bars (s.d., s.e.m.)
- If the data are obtained from n {less than or equal to} 2, use scatter blots showing the individual data points.
Discussion of statistical methodology can be reported in the materials and methods section, but figure legends should contain a basic description of n, P and the test applied.

See also the guidelines for figure legend preparation:
https://www.embopress.org/page/journal/14693178/authorguide#figureformat

- Please also include scale bars in all microscopy images.

9) We would also encourage you to include the source data for figure panels that show essential data. Numerical data should 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 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 .

10) 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 .

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You are able to opt out of this by letting the editorial office know (emboreports@embo.org). If you do opt out, the Review Process File link will point to the following statement: "No Review Process File is available with this article, as the authors have

chosen not to make the review process public in this case."

We would also welcome the submission of cover suggestions, or motifs to be used by our Graphics Illustrator in designing a cover.

I look forward to seeing a revised form of your manuscript when it is ready. Please use this link to submit your revision: https://embor.msubmit.net/cgi-bin/main.plex

Please let me know if you have questions or comments regarding the revision.

Yours sincerely,

Martina Rembold, PhD
Senior Editor
EMBO reports

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Referee #1:

In a previous study, authors found that the loss of Bcl11a in cortical projection neurons induces pronounced cell death in upper-layer cortical projection neurons during postnatal corticogenesis. In current study, authors explored a Bcl11a-dependent mechanism in neuronal apoptosis in developing cortex and identify that the neuronal viability requires the Bcl11a/Bcl6/(perhaps) Foxo1 transcriptional pathway. Overall, the work is well designed and executed, the results well presented. This study would thus be of great interest in cortical development topic and should a merit publication in EMBO Reports. Following suggestions would strengthen the excellence of manuscript:

1. As a systematic approach to identify direct targets of Bcl11a, author might perform Bcl11a ChIP-seq or ATAC-seq with chromatin prepared from cortical layers 2-4 to consolidate their study.

2. Does Bcl11a control transcriptional activity of the Bcl6 promoter/enhancer? Authors could examine this possibility in vivo by co-electorate CAG-Cre-GFP or CAG-GFP with a plasmid of Bcl6-Promoter/Enhancer-Luciferase into Bcl11a flox/flox brains at E15.5 and measure luciferase activity at P4-P5.

3. To provide additional evidence about Bcl6 acts a direct downstream gene of Bcl11a, authors should analyze their overlapping DE genes in RNA-seq data.

4. Since single loss of either Bcl11a or Bcl6 cause apoptosis, it is interesting to examine Bcl11a/Bcl6 dKO phenotype.

5. Bcl6cKO_Nex-Cre has quite mild apoptotic phenotype compared to Bcl11acKO_Emx1-Cre. How is about apoptotic phenotype in Bcl11acKO_Nex-Cre?

6. Authors concluded that "Bcl11a/Bcl6 to regulate DCD at least in part through Foxo1 function". Perhaps a rescue experiment by Foxo1 knock-down in either Bcl11a or Bcl6 mutants would strengthen their conclusion about neuronal viability requires the Bcl11a/Bcl6/Foxo1 transcriptional pathway.

7. Since Bcl11a regulates transcription through interaction with COUP-TF proteins. Is there any data about role of COUP-TF in cell viability in cortex? If yes, author can expand their conclusion about the neuronal viability requires the COUP-TF/Bcl11a/Bcl6/(perhaps) Foxo1 transcriptional pathway.

Referee #2:

In their manuscript the authors study an intriguing phenomenon in cerebral cortex development - developmental cell death of cortical projection neurons. The work is built upon earlier studies by the Britsch and Vanderhaeghen laboratories demonstrating diverse functions for Bcl11a and Bcl6 in developmental cell death in a variety of cell types. The authors now show that Bcl6 seems to be directly regulated by Bcl11a (at the transcriptional level). As such, Bcl6 levels are reduced in Bcl11a conditional mutant mice. The authors could then demonstrate that reintroduction of Bcl6 into Bcl11a mutant neurons rescues them from cell death in a cell-autonomous manner. Mice with postmitotic ablation of Bcl6 in turn show elevated cell death. Lastly, the authors pursued gene expression profiling in both Bcl6 and Bcl11a mutants and identified Foxo1 to be upregulated in mutant cortex as putative causal link to the cell death phenotype.

Overall the study is presented very neatly, the data conclusive and the interpretation appropriate. I have only some minor comments that could improve the clarity of the presentation.

1. The Figure S3 does not show properly in the PDF. Please make sure that the images are visible well.
2. The authors at some passages throughout their manuscript refer to interneurons but do not go into detail. In the discussion the authors mention a few cell types in other regions but do not comment on cortical interneurons much. Adding a short paragraph about the major differences in developmental cell death in cortical interneurons versus projection neurons would help to put their new findings in a broader context.
3. The gene expression profiling is a first step in the right direction although there are much more sensitive methods now on the market. Still the data is important as presented but would benefit from some more in-depth analysis. The authors should show common deregulated genes in Bcl11a and Bcl6 mutant cortex. They mention Foxo1 to be upregulated in both mutants. Are there more genes that show similar (up and down) expression pattern in their dataset?

Referee #3:

In this manuscript, the authors investigate the role of Bcl11a and Blc6 in programmed developmental cell death. Building upon previous work where they identified increased apoptosis in cortical (Emx1-Cre deleted) Bcl11a mouse mutants. The authors show that Bcl6 is downregulated in Bcl11a mutants, and identify Bcl6 as a target of Bcl11a by ChIP and co-immunolocalization in upper-layer cortical projection neurons. Cell death in Bcl11a mutants was rescued by overexpression of Bcl6 using a Neurod specific conditional construct. The authors go on to investigate cell death in Bcl6 conditional mutant mice, and identify the common target Foxo1 between the two models. Using these results and Bcl6 overexpression the authors hypothesize that Bcl6-dependent survival is mediated via FOXO1 rather than p53 (previously identified as a Bcl6-dependent cell death pathway in lymphoid cells). Overall the experiments are well performed and clearly presented.

We present a few points requiring clarification and provide some suggestions.

The authors use a heterozygous conditional mouse (Bcl11aF/+) as controls. Knowing that heterozygous (albeit) constitutive knockout of Bcl11a is associated with a phenotype, ideally the authors would demonstrate that the Bcl11aF/+;Emx1IRESCre does not show a significantly different phenotype from Bcl11a+/+;Emx1IRESCre or reference where that has been shown, or use the latter as a primary or additional control. We recognise that to obtain sufficient experimental mice from heterozygote crosses would require a significantly greater number of mice, at increased cost and welfare impact on animals. Nonetheless the authors should at a minimum discuss their choice of control considering previously published data.

Figs. 3, 4c and Supp Fig S4 - co-immunostaining for BCL11A could be showed, if not in the main figure, in a supplementary figure.

The authors limit their apoptosis analysis to caspase-3. Because in the discussion they indicate (page 11, lines 15,16) "compared to Bcl11a mutants we observed only moderate increase in apoptosis in Bcl6 mutant CPN, raising the possibility of additional signals to contribute to apoptosis in Bcl11a mutants." The paper would have benefited from looking into different cell death pathways, for eg. by immunohistochemistry or western blot for a broader range of intrinsic and extrinsic pathway proteins in Bcl11a and Bcl6 mutants.

Page 5, lines 12-14: Overall, the methods used for selection of candidate genes (outlined only in Figure 1B) is not detailed. Specifically, the authors indicate they performed a "GO overrepresentation test, which revealed genes involved in axon guidance, cell-cell adhesion, and regulation of cell communication". No detail is provided on the methods used for GO overrepresentation, nor the results of this analysis (which we suggest could be included in supplementary data).

In the supplementary data, the authors provide the gene expression data for the Bcl11a mutant (Supp. Table S1). The same should be provided for the Bcl6 mutants.

Bcl6 is expressed in deep cortical neurons, yet expression is unchanged in deep cortical layers of Bcl11aF/F;Emx1IRESCre compared to control neocortex (Suppl. Fig. 4). The authors' previous work showed mild increase in apoptosis within the deep cortical layers at P4-P5 in Bcl11a mutants. Would the authors like to discuss what may drive the specificity of the proposed Bcl11a/Bcl6-dependent pathway in upper cortical layers?

The authors did not detect changes in p53 expression in their expression analysis. We suggest confirmation of p53 expression by an orthogonal method, eg. rtPCR.

The authors restrict their analysis to the role of Bcl6 in apoptosis in Bcl11a mutants. If Bcl6 is a direct target of Bcl11a without TF redundancy, one would hypothesise that Bcl11aEmx1Cre mutants would have a broader effect via Bcl6 on notch, SHH, FGF, and Wnt pathways (per the authors' previous publication, Bonnefont et al., 2019). Do authors have gene expression data on young neocortical neurons (namely form work in Wiegreffe et al, 2015) that would support that? We appreciate it may be outside the scope of the present paper, but could warrant some discussion.

The authors suggest that FOXO1 function mediates DCD. We suggest the authors could perform FOXO1 genetic knockdown or use a small molecule inhibitor to rescue the phenotype. We appreciate this may be outside the scope of the present paper.

Minor points:
Methods: please detail the antibody conditions for IF
Fig. 1 - label with (estimate of) layers would be helpful
Page 20, line 14. Fig. 1 legend. Typo? **p>0.01, should be **p<0.01?
Page 22, line 26. Fig 4 legend. indicate arrows in legend (CC3 positive cells).

We thank the reviewers for evaluating our ms, "*Developmental cell death of cortical projection neurons is controlled by a Bcl11a/Bcl6-dependent pathway"* (EMBOR-2021-54104V1) by Wiegreffe et al., and for their critical comments, which helped us to improve the ms.

Point-to-point response to reviewers:

Reviewer Comments:

Referee #1:

In a previous study, authors found that the loss of Bcl11a in cortical projection neurons induces pronounced cell death in upper-layer cortical projection neurons during postnatal corticogenesis. In current study, authors explored a Bcl11a-dependent mechanism in neuronal apoptosis in developing cortex and identify that the neuronal viability requires the Bcl11a/Bcl6/(perhaps) Foxo1 transcriptional pathway. Overall, the work is well designed and executed, the results well presented. This study would thus be of great interest in cortical development topic and should a merit publication in EMBO Reports. Following suggestions would strengthen the excellence of manuscript:

1. As a systematic approach to identify direct targets of Bcl11a, author might perform Bcl11a ChIP-seq or ATAC-seq with chromatin prepared from cortical layers 2-4 to consolidate their study.

> We agree with reviewer #1, that additional approaches, as for example ChIP-seq or ATAC-seq on layer-specific tissue samples, to systematically identify candidate targets of Bcl11a would help to broaden our understanding of the complex functions of Bcl11a during cns development. We do, however, not believe that such analyses might necessarily help to deepen mechanistic understanding of the Bcl11a/Bcl6-dependent regulatory pathway we identified and which is the focus of this study. In an independent project we are performing ChIP-seq and ATAC-seq analyses to systematically identify Bcl11a-dependent regulatory pathways during neocortical development. According to our experience, these studies are time- as well as animal-consuming, require extensive computational analyses and verifying experiments in order to extract meaningful data. Thus, we feel that the suggested experiments are beyond the actual scope of the present study as well as beyond the available time frame for this revision.

2. Does Bcl11a control transcriptional activity of the Bcl6 promoter/enhancer? Authors could examine this possibility in vivo by co-electorate CAG-Cre-GFP or CAG-GFP with a plasmid of Bcl6-Promoter/Enhancer-Luciferase into Bcl11a flox/flox brains at E15.5 and measure luciferase activity at P4-P5.

> As requested by reviewer #1 we have carried out luciferase assays in order to further determine whether Bcl11a directly controls the transcriptional activity of the Bcl6 gene. We show that Bcl11a but not the closely related Bcl11b protein increased transcriptional activity of a luciferase reporter construct containing the conserved Bcl11a binding motif, which we identified in the first intron of the Bcl6 gene. Together with the already presented data our new findings consolidate Bcl6 to be a direct, functional downstream target of Bcl11a in regulating late DCD of cortical projection neurons. The new data are included in the revised ms in figure 2 G, and described in detail in the results part.

3. To provide additional evidence about Bcl6 acts a direct downstream gene of Bcl11a, authors should analyze their overlapping DE genes in RNA-seq data.

> As requested by reviewer #1 we systematically compared DE genes from Bcl6 and Bcl11a mutants. Only 3 DE genes including Foxo1 were overlapping in both data sets. Differential expression of all overlapping genes was verified by qRT-PCR in both Bcl11a and Bcl6 mutants as well as corresponding controls. The new data are shown on the extended figure EV5 C-E of our revised ms.

4. Since single loss of either Bcl11a or Bcl6 cause apoptosis, it is interesting to examine Bcl11a/Bcl6 dKO phenotype.

> We agree with reviewer #1 that it might have been interesting to analyze apoptosis in Bcl11a/Bcl6 compound mutants. We apologize that due to the limited breeding capacities of our animal facility, still suffering from pandemic restrictions, as well as due to low Mendelian rates for the intercrossing of three independent alleles, we were not able to generate sufficient numbers of $Bcl11a^{F/F}$; $Bcl6^{F/F}$; Nex^{Cre} animals to quantify the apoptosis phenotype in compound mutants and to compare to phenotypes occuring in single mutants.

5. Bcl6cKO_Nex-Cre has quite mild apoptotic phenotype compared to Bcl11acKO_Emx1-Cre. How is about apoptotic phenotype in Bcl11acKO_Nex-Cre?

> As requested by reviewer #1 we have determined the apoptosis phenotype in $Bcl11a^{F/F}$; Nex^{Cre} animals as well. The new data are shown in extended figure EV5 A-B of the revised ms. The apoptosis phenotype is more severe in NexCre recombined Bcl11a mutants as in Bcl6 mutants, and similar to the previously published phenotype in Emx1Cre recombined Bcl11a mutants (Wiegreffe et al., 2015). This supports our interpretation, that upstream of Bcl6, Bcl11a controls additional developmental functions in neocortex development, which may indirectly and independently of Bcl6 contribute to cell death of cortical projection neurons. For example, we and others have shown previously, that morphogenesis and connectivity of cortical projection neurons depend on Bcl11a. Impaired connectivity and morphogenesis may contribute to the apoptosis phenotype in Bcl11a mutants. We discuss this point in detail in the revised ms. Please see also response to point 7.

6. Authors concluded that "Bcl11a/Bcl6 to regulate DCD at least in part through Foxo1 function". Perhaps a rescue experiment by Foxo1 knock-down in either Bcl11a or Bcl6 mutants would strengthen their conclusion about neuronal viability requires the Bcl11a/Bcl6/Foxo1 transcriptional pathway.

> As suggested by reviewer #1 we have further analyzed the functional role of Foxo1 in Bcl11a/Bcl6-dependent developmental cell death of cortical projection neurons. In our revised ms we now show by help of *in utero* electroporation that shRNA-mediated knock-down of Foxo1 gene expression in Bcl11a mutants is sufficient to suppress, i.e rescue the Bcl11a-dependent apoptosis phenotype in cortical projection neurons *in vivo* (revised Figure 5 H-K). This provides direct experimental evidence for a functional role of Foxo1 in the Bcl11a/Bcl6 regulatory pathway we identified in our study.

7. Since Bcl11a regulates transcription through interaction with COUP-TF proteins. Is there any data about role of COUP-TF in cell viability in cortex? If yes, author can expand their conclusion about the neuronal viability requires the COUP-TF/Bcl11a/Bcl6/(perhaps) Foxo1 transcriptional pathway.

> Bcl11a has been previously demonstrated to directly interact with COUP-TFI (Nr2f1). Moreover, in a very recent study from Du et al., 2022 the authors suggest Bcl1a to directly bind to the Nr2f1 gene locus and suppress transcription raising the question whether Nr2f1 is involved in Bcl11a-dependent control of late DCD in cortical projection neurons. Several lines of evidence argue against this assumption. (i) extensive phenotype analyses of Nr2f1mutants from different labs have implicated this factor in control of cortical progenitor proliferation as well as cortical patterning, and laminar fate determination in postmitotic neurons. Yet, a direct role for Nr2f1 in control of postmitotic neuron survival has not been reported. (ii) in our study, we did not detect deregulated Nr2f1 expression in Bcl11a mutants nor in Bcl6 mutants as compared to controls. Thus, we do not have evidence for Nr2f1 to be part of the transcriptional regulatory cascade Bcl11a - Bcl6 - Foxo1. On the other hand, Bcl11a and Nr2f1 have been shown to be involved in establishing somatomotor versus somatosensory cortical area identity leading to a partial motorization of the mutant neocortex. Interestingly, wildtype Bcl6 expression is lower in the somatomotor cortex as in the somatosensory cortex. Nr2f1 might thus indirectly participate in the control of Bcl6 expression via control of cortical area identity. Whether this occurs through direct protein-interaction with Bcl11a or indirectly through mechanisms independent of Bcl11a, remains to be determined. We discuss this topic in detail, including relevant literature in our revised ms.

Referee #2:

In their manuscript the authors study an intriguing phenomenon in cerebral cortex development - developmental cell death of cortical projection neurons. The work is built upon earlier studies by the Britsch and Vanderhaeghen laboratories demonstrating diverse functions for Bcl11a and Bcl6 in developmental cell death in a variety of cell types. The authors now show that Bcl6 seems to be directly regulated by Bcl11a (at the transcriptional level). As such, Bcl6 levels are reduced in Bcl11a conditional mutant mice. The authors could then demonstrate that reintroduction of Bcl6 into Bcl11a mutant neurons rescues them from cell death in a cell-autonomous manner. Mice with postmitotic ablation of Bcl6 in turn show elevated cell death. Lastly, the authors pursued gene expression profiling in both Bcl6 and Bcl11a mutants and identified Foxo1 to be upregulated in mutant cortex as putative causal link to the cell death phenotype.

Overall the study is presented very neatly, the data conclusive and the interpretation appropriate. I have only some minor comments that could improve the clarity of the presentation.

1. The Figure S3 does not show properly in the PDF. Please make sure that the images are visible well.

> This issue has been fixed in the revised ms.

2. The authors at some passages throughout their manuscript refer to interneurons but do not go into detail. In the discussion the authors mention a few cell types in other regions but do not comment on cortical interneurons much. Adding a short paragraph about the major differences in developmental cell death in cortical interneurons versus projection neurons would help to put their new findings in a broader context.

> As requested by reviewer #2 we have added a paragraph discussing DCD in cortical interneurons to the revised ms.

3. The gene expression profiling is a first step in the right direction although there are much more sensitive methods now on the market. Still the data is important as presented but would benefit from some more in-depth analysis. The authors should show common deregulated genes in Bcl11a and Bcl6 mutant cortex. They mention Foxo1 to be upregulated in both mutants. Are there more genes

that show similar (up and down) expression pattern in their dataset?

> As requested by reviewer #2 we systematically compared DE genes from Bcl6 and Bcl11a mutants. Only 3 DE genes including Foxo1 were overlapping in both data sets. Differential expression of all overlapping genes was verified by qRT-PCR in both Bcl11a and Bcl6 mutants as well as corresponding controls. The new data are shown on the extended figure EV5 C-E of our revised ms. (Please see also response to reviewer #1, point 3)

Referee #3:

In this manuscript, the authors investigate the role of Bcl11a and Blc6 in programmed developmental cell death. Building upon previous work where they identified increased apoptosis in cortical (Emx1-Cre deleted) Bcl11a mouse mutants. The authors show that Bcl6 is downregulated in Bcl11a mutants, and identify Bcl6 as a target of Bcl11a by ChIP and co-immunolocalization in upper-layer cortical projection neurons. Cell death in Bcl11a mutants was rescued by overexpression of Bcl6 using a Neurod specific conditional construct. The authors go on to investigate cell death in Bcl6 conditional mutant mice, and identify the common target Foxo1 between the two models. Using these results and Bcl6 overexpression the authors hypothesize that Bcl6-dependent survival is mediated via FOXO1 rather than p53 (previously identified as a Bcl6-dependent cell death pathway in lymphoid cells). Overall the experiments are well performed and clearly presented.

We present a few points requiring clarification and provide some suggestions.

The authors use a heterozygous conditional mouse (Bcl11aF/+) as controls. Knowing that heterozygous (albeit) constitutive knockout of Bcl11a is associated with a phenotype, ideally the authors would demonstrate that the Bcl11aF/+;Emx1IRESCre does not show a significantly different phenotype from Bcl11a+/+;Emx1IRESCre or reference where that has been shown, or use the latter as a primary or additional control. We recognise that to obtain sufficient experimental mice from heterozygote crosses would require a significantly greater number of mice, at increased cost and welfare impact on animals. Nonetheless the authors should at a minimum discuss their choice of control considering previously published data.

> Dias and co-workers have nicely demonstrated in their 2016 (Am J Hum Genet) study that Bcl11a haploinsufficiency causes defects in nervous system development, and we agree with reviewer #3 on the importance to include analyses of wildtype controls into every knock-out study. Our group has longstanding experimental experience in analyzing mutations of the Bcl11a during cns development in mice (for review please refer to Simon et al., 2020). We routinely include comparison of wildtype versus heterozygous mutant mice into phenotype analyses. So far, for pre-/postnatal development of the neocortex we did not observe significant structural differences between heterozygous and wildtype mice. This does not, of course, exclude subtle changes on the levels of gene expression or behavior. To further corroborate this, we reassessed apoptosis rates in upper layers of the neocortex of $Bcl11a^{F/+}$; $Emx1^{IRESCre}$ and $Bcl11a^{+/+}$; $Emx1^{IRESCre}$ mice by quantification of CC3+ cells. We did not observe significant differences between both genotypes. The new data are included in the revised ms (Appendix Fig. S4 and methods).

Figs. 3, 4c and Supp Fig S4 - co-immunostaining for BCL11A could be showed, if not in the main figure, in a supplementary figure.

> Throughout the study we have used previously established experimental strategies to delete Bcl11a. In Wiegreffe et al., 2015, we demonstrated efficient recombination of the conditional Bcl11a allele by Emx1Cre, NexCre, DeleterCre as well as in utero electroporation of DNA plasmids carrying Cre recombinase and refer to this publication. In addition, the $Bcl11a^{flox}$ mouse line we use in our study, and which has originally been generated by Pengtao Liu is frequently used by other labs, that have

also shown efficient recombination of the Bcl11a genomic locus in the nervous system (for example, Tolve et al., 2021; Du et al., 2022). Therefore, we feel that it might not be mandatory to repeat these experiments in the present study.

The authors limit their apoptosis analysis to caspase-3. Because in the discussion they indicate (page 11, lines 15,16) "compared to Bcl11a mutants we observed only moderate increase in apoptosis in Bcl6 mutant CPN, raising the possibility of additional signals to contribute to apoptosis in Bcl11a mutants." The paper would have benefited from looking into different cell death pathways, for eg. by immunohistochemistry or western blot for a broader range of intrinsic and extrinsic pathway proteins in Bcl11a and Bcl6 mutants.

> We absolutely agree with reviewer #3 that the different extent of apoptosis observed in Bcl11a versus Bcl6 mutant mice suggests additional Bcl11a-dependent mechanisms to be involved in cell survival control. In the revised ms we completely reorganized and extended the results and discussion part with respect to this aspect (please see also comment below on p53 expression).

Page 5, lines 12-14: Overall, the methods used for selection of candidate genes (outlined only in Figure 1B) is not detailed. Specifically, the authors indicate they performed a "GO overrepresentation test, which revealed genes involved in axon guidance, cell-cell adhesion, and regulation of cell communication". No detail is provided on the methods used for GO overrepresentation, nor the results of this analysis (which we suggest could be included in supplementary data).

> As requested by reviewer #3 we have included more comprehensive information and descriptions of this part of our study in the revised ms (Fig. EV2 and revised methods section).

In the supplementary data, the authors provide the gene expression data for the Bcl11a mutant (Supp. Table S1). The same should be provided for the Bcl6 mutants.

> As suggested by reviewer #3 we have included a complete list of DE genes for Bcl6 mutants in the revised ms (Dataset EV2).

Bcl6 is expressed in deep cortical neurons, yet expression is unchanged in deep cortical layers of Bcl11aF/F;Emx1IRESCre compared to control neocortex (Suppl. Fig. 4). The authors' previous work showed mild increase in apoptosis within the deep cortical layers at P4-P5 in Bcl11a mutants. Would the authors like to discuss what may drive the specificity of the proposed Bcl11a/Bcl6-dependent pathway in upper cortical layers?

> There is emerging evidence from the literature including our work that the transcriptional activity and specificity of Bcl11a is context-dependent (for review see Simon et al., 2020). The underlying molecular mechanisms are largely undetermined. As discussed above (please see response to reviewer #1, point 7), Bcl11a, for example interacts with COUP-TF proteins, which may modify its transcriptional activity and specificity depending on cell type and differentiation state. Moreover, there is compelling experimental evidence that Bcl11a exerts its functions at least in part as a component of the BAF multi-protein complex. Interestingly, the composition of BAF has been demonstrated to differ between progenitors and postmitotic cortical neurons. Such mechanisms might contribute to the differential activity of the Bcl11a/Bcl6 pathway in upper- versus deep-layer cortical neurons. This, however, remains to be experimentally determined.

The authors did not detect changes in p53 expression in their expression analysis. We suggest confirmation of p53 expression by an orthogonal method, eg. rtPCR.

> As requested by reviewer #3 confirmation of p53 expression by qRT-PCR has been added to the revised ms (Fig. EV5).

The authors restrict their analysis to the role of Bcl6 in apoptosis in Bcl11a mutants. If Bcl6 is a direct target of Bcl11a without TF redundancy, one would hypothesise that Bcl11aEmx1Cre mutants would have a broader effect via Bcl6 on notch, SHH, FGF, and Wnt pathways (per the authors' previous publication, Bonnefont et al., 2019). Do authors have gene expression data on young neocortical neurons (namely form work in Wiegreffe et al, 2015) that would support that? We appreciate it may be outside the scope of the present paper, but could warrant some discussion.

> Several studies including our own work suggest interactions of Bcl6- and Bcl11a-dependent downstream pathways with Wnt-, Notch- and other signaling systems. We systematically explored changes of genes known to be involved in these pathways. Indeed, we detected Wnt7b, Fzd7 and Frzb to be deregulated in our transcriptomic analyses of Bcl11a mutant neocortex (Dataset EV1). However, none of these genes show up in Bcl6 mutants (Dataset EV2). While our study does not provide an in-depth analysis of this question, our current data do not support that Bcl11a, through transcriptional regulation of Bcl6 expression interferes with the mentioned signaling pathways. This does not exclude interactions during earlier stages in cortical neuron development.

The authors suggest that FOXO1 function mediates DCD. We suggest the authors could perform FOXO1 genetic knockdown or use a small molecule inhibitor to rescue the phenotype. We appreciate this may be outside the scope of the present paper.

> As suggested by reviewer #3 as well as reviewer #1 we have further analyzed the functional role of Foxo1 in Bcl11a/Bcl6-dependent developmental cell death of cortical projection neurons. In our revised ms we show that knock-down of Foxo1 gene expression by in utero electroporation of sh-RNA constructs in Bcl11a mutants is sufficient to suppress, i.e rescue the Bcl11a-dependent apoptosis phenotype in cortical projection neurons *in vivo* (revised Figure 5 H-K). This provides direct experimental evidence for a functional role of Foxo1 in the Bcl11a/Bcl6 regulatory pathway we identified for the first time in our study.

Minor points:
Methods: please detail the antibody conditions for IF
Fig. 1 - label with (estimate of) layers would be helpful
Page 20, line 14. Fig. 1 legend. Typo? **p>0.01, should be **p<0.01?
Page 22, line 26. Fig 4 legend. indicate arrows in legend (CC3 positive cells).

> All minor points have been fixed in the revised ms.

Dear Dr. Britsch

Thank you for the submission of your revised manuscript to EMBO reports. We have now received the full set of referee reports that is copied below.

As you can see, the referees find that the study is significantly improved during revision and recommend publication. Before I can accept the manuscript, I need you to address some minor points below:

- Please update the 'Conflict of interest' paragraph to our new 'Disclosure and competing interests statement'. For more information see
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- We now use CRediT to specify the contributions of each author in the journal submission system. CRediT replaces the author contribution section. Please use the free text box to provide more detailed descriptions. See also guide to authors.

- Abstract: please describe your findings in present tense.

- Please note that we request that all data relevant to the conclusion must be shown in the manuscript. Therefore, please show the control data mentioned on page 18 (data not shown).

- Please add callouts to Fig EV4A-C, Appendix Fig S3A-C, S4A+B.

- Please remove the EV table and Dataset legends from the manuscript file.

- Please add the name and legends for the EV tables to the same page as the table.

- Appendix: Please add page numbers to the table of content.

- Appendix Figure S3: Please specify whether n = 3 refers to biological/independent transfections or technical replicates. A statistical analysis is only recommended for independent experiments.

- "Experimental Procedures" should be corrected to 'Materials and Methods'.

- The figure legends should come after the Reference section.

- Finally, EMBO reports papers are accompanied online by A) a short (1-2 sentences) summary of the findings and their significance, B) 2-3 bullet points highlighting key results and C) a synopsis image that is 550x200-600 pixels large (width x height) in .png format. You can either show a model or key data in the synopsis image. Please note that the size is rather small and that text needs to be readable at the final size. Please send us this information along with the revised manuscript.

We look forward to seeing a final version of your manuscript as soon as possible.

With kind regards,

Martina Rembold, PhD
Senior Editor
EMBO reports

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Referee #1:

The revised manuscript has been significantly improved with clarification to the text. I have no further concerns.

Referee #3:

The authors have addressed all reviewer comments where feasible. I recommend acceptance in its current form.

The authors have addressed all minor editorial requests.

Stefan Britsch
University of Ulm
institute of Molecular and Cellular Anatomy
Ulm 89081
Germany

Dear Dr. Britsch,

Thank you for implementing the final minor changes. I am now very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

At the end of this email I include important information about how to proceed. Please ensure that you take the time to read the information and complete and return the necessary forms to allow us to publish your manuscript as quickly as possible.

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Martina Rembold, PhD
Senior Editor
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Please note that a copy of this checklist will be published alongside your article.

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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.
- ideally, figure panels should include only measurements that are directly comparable to each other and obtained with the same assay.
- plots 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. Any statistical test employed should be justified.
- Source Data should be included to report the data underlying figures according to the guidelines set out in the authorship guidelines on Data Presentation.

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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.

Please complete ALL of the questions below.
Select "Not Applicable" only when the requested information is not relevant for your study.

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Newly Created Materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
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