EMBO reports

Chromatin remodeler CHD7 is required for GABAergic neuron development by promoting PAQR3 expression

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DOI: 10.15252/embr.202050958

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Review Timeline:

Submission Date:	30th Sep 20
Editorial Decision:	13th Nov 20
Revision Received:	11th Feb 21
Editorial Decision:	1st Mar 21
Revision Received:	7th Mar 21
Accepted:	15th Mar 21

Editor: Esther Schnapp

Transaction Report:

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1st Editorial Decision 13th Nov 2020

Dear Dr. Patten,

Thank you for your patience while your manuscript was peer-reviewed at EMBO reports. We have now received the enclosed comments from both referees.

As you will see, the referees acknowledge that the findings are interesting and have potential therapeutic implications. However, they also point out that the mechanistic aspects are rather weak and should be strengthened. I think all concerns raised make sense and should be addressed. Please let me know if you disagree, so that we can discuss the revisions further, also per video chat, if you like.

I would thus like to invite you to revise your manuscript with the understanding that the referee concerns 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 major 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 this further.

Regarding data quantification, please specify the number "n" for how many independent experiments were performed, the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values in the respective figure legends. This information must be provided in the figure legends. Please also include scale bars in all microscopy images.

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 providing access to data deposited in public databases is missing. If you have not deposited any data, please add a sentence to the data availability section that explains that.
2) Your manuscript contains statistics and error bars based on n=2. Please use scatter blots in these cases. No statistics should be calculated if n=2.

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.

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).
See https://wol-prod-cdn.literatumonline.com/pb-assets/embo-site/EMBOPress_Figure_Guidelines_061115-1561436025777.pdf for more info on how to prepare your figures.

3) 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: <https://www.embopress.org/page/journal/14693178/authorguide#expandedview>

- 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.

4) 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.

5) a complete author checklist, which you can download from our author guidelines <https://www.embopress.org/page/journal/14693178/authorguide>. Please insert information in the checklist that is also reflected in the manuscript. The completed author checklist will also be part of the RPF.

6) Please note that all corresponding authors are required to supply an ORCID ID for their name upon submission of a revised manuscript (<https://orcid.org/>). Please find instructions on how to link your ORCID ID to your account in our manuscript tracking system in our Author guidelines <https://www.embopress.org/page/journal/14693178/authorguide#authorshipguidelines>

7) 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/14693178/authorguide#datadeposition). Please remember to provide a reviewer password if the datasets are not yet public. The accession numbers and database should be listed in a formal "Data Availability" section placed after Materials & Method (see also https://www.embopress.org/page/journal/14693178/authorguide#datadeposition). Please note that the Data Availability Section is restricted to new primary data that are part of this study. * Note - All links should resolve to a page where the data can be accessed. *
If your study has not produced novel datasets, please mention this fact in the Data Availability Section.

8) 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 at <https://www.embopress.org/page/journal/14693178/authorguide#sourcedata>.

9) Our journal also 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 https://www.embopress.org/page/journal/14693178/authorguide#referencesformat

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

As part of the EMBO publication's Transparent Editorial Process, EMBO reports publishes online a Review Process File (RPF) to accompany accepted manuscripts. This File will be published in conjunction with your paper and will include the referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript.

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I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

Kind regards,
Esther

Esther Schnapp, PhD
Senior Editor
EMBO reports

Referee #1:

Summary:

This is a very nice paper examining the role of the gene CDH7, which is associated with CHARGE syndrome and autism. Authors created a CDH7 knockout zebrafish and examined its role in neurodevelopment and behavior. Both the writing and experiments are clear and thorough. It is a multi-disciplinary work: the authors use available data from ENCODE to identify paqr3b as a direct target of CDH7, and they use zebrafish, c. elegans, and human cell culture to confirm their findings, explain how CDH7 works on a molecular level, and ultimately even rescue GABAergic defect phenotypes using ephedrine. The use of multiple organisms and drug screening, in addition to a classic dissection of the neurodevelopment phenotype in zebrafish, gives this paper breadth and increases its appeal to the large audience of researchers interested in neurodevelopmental disorders. The experiments are careful and thorough, such as including a check of the mRNA expression level in the zebrafish mutants. The introduction and discussion are informative, as the authors avoid unnecessary distractions and are not missing any key pieces of information. I offer some minor suggestions to improve the writing and have a couple of questions about missing details that should be included in the materials and methods.

Major Comments:

- Were p-Erk mutants stained together in the same tube as the controls? We do a lot of phospho-Erk whole-mount 6 dpf staining in my lab, and that can be important comparing between samples (we stain and genotype afterwards). I am bit surprised by the level of p-Erk staining in the +/+ example in Fig. 3E, as our standard stain usually looks brighter, but we use a different cat no. antibody from Cell Signaling. Most importantly, I couldn't find the staining conditions in the materials and methods (the "TUNEL and Fluorescence immunochemistry" section includes only the pH3 staining), so that is an oversight that needs to be corrected (also check all other staining are described in this section). I do not doubt the data and do not expect any new experiments to be added, but would like the methods to be complete.

- Line 250: what was the amount injected for overexpression? I also could not find this detail in the methods, so methods should be updated. Were there negative effects of this overexpression on the health and viability of the larvae?

- It could be interesting to do social behavior assays with the heterozygous animals. While not necessary for publication, it could be informative given the connection to autism.

Minor Comments:

Line 150: Add A before hyperactivity
Line 152: remove a from before similar
Line 153: remove is occurring and replace with occur
Line 165: the name is based on dpf in EV Fig. 1E, but hpf here. Better to keep consistent labeling, particularly for non-zebrafish readers.
Line 193: add s after nucleosome
Line 194: add a before positive
Line 303: change this to these or defects to defect
Line 305: comma after ephedrine and change that to which
Line 338: replace inhibiting with inhibition
Line 340: remove are before may
Line 341: remove s in investigations
Line 348: comma after AR and after (ARs), have instead of has
Line 349: add s to neonate
Line 379: remove thus or additionally
Lines 445 and 450: replace commas with periods for decimals
Lines 483 and 486: there should be space between every and day
Lines 483 and 487: replace till with until
Line 537: space before parenthesis
Lines 546, 547, 568, 569, 570: use prime symbols for primers, not apostrophes
Figure 4 - a new graphic generated by the authors would be better than screenshot of UCSC genome browser

Referee #2:

The manuscript by Jamadagni et al. describes an interesting set of experiments on homozygous

Chd7-/- zebrafish and C. elegans models. They provide convincing evidence for reduced generation of GABAergic interneurons, an increased propensity to hyperactivity and seizure behaviors. They provide a potential mechanism for this phenotype by showing downregulation of the direct CHD7 target gene paqr3b and upregulation of ERK signalling, validating the latter in a CHD7 haploinsufficient human LCL line, suggesting that the dysregulation of ERK signaling might be clinically relevant. Screening for compounds to rescue this phenotype in C. elegans identified ephedrine, which was then validated in the zebrafish model.

Over-all, this manuscript provides important new insights into the role of Chd7 in brain development and has important translational implications. The fact that chd7+/- fish, the model with most relevant construct validity to CHARGE syndrome, a CHD7-haploinsufficient condition, needs to be commented on. An attempt is made to validate the molecular findings in a human CHD7 haploinsufficient cellular system. The mechanistic links between chd7, pERK dysregulation, the GABAergic phenotype and ephedrine rescue remain somewhat tenuous and experiments to strengthen these links will significantly strengthen the mechanistic conclusions of the manuscript.

Specific comments and recommendations:
1) The authors describe a new zebrafish model with exon 17, encoding the helicase domain is targeted by CRISPR/Cas9, a line carrying a frameshift mutation terminating 8 amino acids after he targeting site. A better characterization of this mutation is needed. A reduction in Chd7 mRNA is reported (EVFig1B) - where are the qPCR primers located, upstream or downstream of the target site i.e. is mRNA degraded by nonsense-mediated decay in the mutant? Do the mutant fish express a truncated protein or is the protein completely absent by for example Western blot using an antibody against the N-terminal portion of the protein?

2) They report apparent blindness (line 144) of the fish - how was this assessed?

3) The authors examine the GABAergic population using a GABAergic specific transgenic line and state: 'Compared to controls, chd7-/- larvae had a significant reduction in the density of GFP-positive GABAergic cells in the brain at 5 dpf (Fig. 2B)' and quantify this in Figure 2D. The distribution of GABAergic interneurons appears to be altered in the region of the OT in Chd7-/- zebrafish. Mispositioning appears to lead to expansion of the GABAergic interneuron population in the OT. It seems important to determine if there has been any alteration in the identity of brain regions, region-specific effects on proliferation/differentiation or migration defects to explain this phenotype.

4) They identify interesting changes in the dynamics of progenitor proliferation by pH3 staining (EV Fig. 1). However, the identity of these progenitors are not known - they need to co-stain with specific markers of GABAergic and glutamatergic progenitors to distinguish between these, especially at the earlier stages when no apparent differences in proliferation or apoptosis are seen. Furthermore, more rigorous experiments are needed to show evidence of premature cell cycle exit/differentiation by for example BrdU/EdU labeling of proliferating progenitors a day or so earlier and co-staining for EdU/BrdU, differentiation and proliferation markers at 5dpf.

5) They report a reduction in GABAergic progenitors in 5 dpf fish, is this phenotype still present in adult fish?

6) They chose to perform RNAseq at 5 dpf, when an overt phenotype was already present - can they justify why this stage was chosen?

7) The U0126 treatments yielded very interesting results. It is not clear to me from the methods when the treatment was initiated and how long it was maintained for. These details need to be provided, as well as the rationale for choosing this treatment regime. Is this treatment sufficient to normalize pERK levels? Similarly, the phenotypic rescue by overexpressing Paqr3b (Fig. 5) is very compelling, can they show that this is sufficient to rescue pERK levels, thereby making a more direct mechanistic connection between paqr3b downregulation, pERK dysregulation and GABArgic interneuron numbers?

8) The data presented in Fig. 4 requires a more rigorous explanation of the methods used for qPCR and associated statistical analyses. Fig. 4A compares 4 control data points with 4 patient samples - what do these data points represent? Are these independently generated lines, independently performed experiments? Why are the 4 data points in the controls identical? Was each control compared to a mutant in 4 independent experiments and this is the data plotted? What method was used to compare expression, DDCt? In contrast to Fig. 4A, Fig. 4C now has 6 control samples and only 3 patient samples. Can the authors explain the reason for this and provide details in the methods and figure legends so that statistical rigor can be assessed?

9) Can the data presented in Fig. 6G-I be quantified?

10) The ephedrine rescue experiments are very interesting. To draw a more direct mechanistic link between ephedrine treatment, pERK normalization and phenotypic rescue, can the authors activate pERK in ephedrine-treated embryos and show that they no longer rescue the phenotype?

11) Finally, I can't find any details in the methods on how the cell counts were performed (for example for Fig. 3F,G) - can this be provided?

12) Some figure legends (e.g. Fig. 13, 4, 5, 6) do not state the statistical test performed for each analysis, these need to be stated.

Minor comments:

Abstract: "hyperactivity disorder", shouldn't this be ADHD (attention deficit hyperactivity disorder)?
Line 41: direct target gene of the paqr3b
Line 45: restore normal levels of MAPK/ERK signaling
Line 46: that this network - define what network: gene network, signaling network?
Line 104: It is worth noting that cerebellar hypoplasia as a result of Chd7 deficiency in cerebellar granule neuron progenitores was eliminated as a likely cause of autism-like behaviors in Whittaker et al.
Line 111-123: I found the summary of the results in the Introduction too long and detailed, can this be shortened to a few sentences rather than summarizing the manuscript in great detail?
Figure1C. the figure legend does not match the figure.
Line 168: we did not observe a change in either...or..
Line 177: connectivity is not the correct term here
Line 193: CHD7 remodels chromatin by translocating nucleosomes, it does not remodel nucleosomes
Line 702: No dotted line in figure 1E as indicated in the legend
Line 801: EV figure 1L the wrong data is shown relative to the figure legend.

Point-by point response to the reviewers:

We would like to begin by thanking the reviewers for their constructive comments. We have made substantial changes to the manuscript to address their concerns, with all significant changes tracked in the revised Ms Word document. A point-by-point response to the reviewers' comments is provided below.

Reviewer 1

This is a very nice paper examining the role of the gene CDH7, which is associated with CHARGE syndrome and autism. Authors created a CDH7 knockout zebrafish and examined its role in neurodevelopment and behavior. Both the writing and experiments are clear and thorough. It is a multi-disciplinary work: the authors use available data from ENCODE to identify paqr3b as a direct target of CDH7, and they use zebrafish, c. elegans, and human cell culture to confirm their findings, explain how CDH7 works on a molecular level, and ultimately even rescue GABAergic defect phenotypes using ephedrine. The use of multiple organisms and drug screening, in addition to a classic dissection of the neurodevelopment phenotype in zebrafish, gives this paper breadth and increases its appeal to the large audience of researchers interested in neurodevelopmental disorders. The experiments are careful and thorough, such as including a check of the mRNA expression level in the zebrafish mutants. The introduction and discussion are informative, as the authors avoid unnecessary distractions and are not missing any key pieces of information. I offer some minor suggestions to improve the writing and have a couple of questions about missing details that should be included in the materials and methods.

- We wish to thank the reviewer for the positive comments and appreciation of the significance of our work.

1) Were p-Erk mutants stained together in the same tube as the controls? We do a lot of phospho-Erk whole-mount 6 dpf staining in my lab, and that can be important comparing between samples (we stain and genotype afterwards). I am bit surprised by the level of p-Erk staining in the +/+ example in Fig. 3E, as our standard stain usually looks brighter, but we use a different cat no. antibody from Cell Signaling. Most importantly, I couldn't find the staining conditions in the materials and methods (the "TUNEL and Fluorescence immunochemistry" section includes only the pH3 staining), so that is an oversight that needs to be corrected (also check all other staining are described in this section). I do not doubt the data and do not expect any new experiments to be added, but would like the methods to be complete.

-Thank you for this comment. For the p-ERK staining experiments, the fish were divided by genotypes prior to the immunostainings and of note p-ERK staining results were consistent between two independent trainees. We thank the reviewer for bringing this to our attention and making us realize that this staining methodology was missing in the original submission- our apologies. We now provide complete details about the pERK immunostainings in the materials and methods (Line 738-743). Additionally, we went over the methodology of all the other staining (TUNEL assay and pH3 staining) and have expanded them to make sure they are now more thorough (Line 714-736).

2) Line 250: what was the amount injected for overexpression? I also could not find this detail in the methods, so methods should be updated. Were there negative effects of this overexpression on the health and viability of the larvae?

- For the rescue experiments, we injected 1nl of paqr3b mRNA (40 ng/µl). We updated the methods accordingly to provide the requested information (Line 554). No, neither the gross morphology nor the survival rate of the larvae was affected upon overexpression of paqr3b mRNA. We now include this information in this revised manuscript (Line 357-358; EV Fig. 5D)

3) It could be interesting to do social behavior assays with the heterozygous animals. While not necessary for publication, it could be informative given the connection to autism.

- We agree with the reviewer and it is indeed part of our future plans to analyze heterozygous animals further in a separate study, including performing social behaviour assays. To at least take the reviewer's point into consideration, we now include in the discussion section of this revised manuscript a note about heterozygous animals and potential future work (Line 456-457).

4) Line 150: Add A before hyperactivity
5) Line 152: remove a from before similar
6) Line 153: remove is occurring and replace with occur
7) Line 165: the name is based on dpf in EV Fig. 1E, but hpf here. Better to keep consistent labeling, particularly for non-zebrafish readers.
8) Line 193: add s after nucleosome
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20) Lines 483 and 487: replace till with until
21) Line 537: space before parenthesis
22) Lines 546, 547, 568, 569, 570: use prime symbols for primers, not apostrophes

- We thank the reviewer for catching these errors (points 4-22; below), which we have now corrected.

23) Figure 4 - a new graphic generated by the authors would be better than screenshot of UCSC genome browser

- We agree with the reviewer. We have significantly improved the quality and visuals of Figure 4B. However, we have to keep the saved imaged from the UCSC genome browser to show where the ENCODE data for the ChIP-seq peaks and signal come from.

Reviewer 2

The manuscript by Jamadagni et al. describes an interesting set of experiments on homozygous Chd7-/- zebrafish and C. elegans models. They provide convincing evidence for reduced generation of GABAergic interneurons, an increased propensity to hyperactivity and seizure behaviors. They provide a potential mechanism for this phenotype by showing downregulation of the direct CHD7 target gene paqr3b and upregulation of ERK signalling, validating the latter in a CHD7 haploinsufficient human LCL line, suggesting that the dysregulation of ERK signaling might be clinically relevant. Screening for compounds to rescue this phenotype in C. elegans identified ephedrine, which was then validated in the zebrafish model.

Over-all, this manuscript provides important new insights into the role of Chd7 in brain development and has important translational implications. The fact that chd7+/- fish, the model with most relevant construct validity to CHARGE syndrome, a CHD7-haploinsufficient condition, needs to be commented on. An attempt is made to validate the molecular findings in a human CHD7 haploinsufficient cellular system. The mechanistic links between chd7, pERK dysregulation, the GABAergic phenotype and ephedrine rescue remain somewhat tenuous and experiments to strengthen these links will significantly strengthen the mechanistic conclusions of the manuscript.

- We thank the reviewer for the positive comments and the valuable suggestions on how to improve our manuscript. We believe that the experiments carried out during the revision substantially strengthened the manuscript. Additionally, we have included in the discussion section of this revised manuscript a note about heterozygous animals (Line 449-457).

1) The authors describe a new zebrafish model with exon 17, encoding the helicase domain is targeted by CRISPR/Cas9, a line carrying a frameshift mutation terminating 8 amino acids after he targeting site. A better characterization of this mutation is needed. A reduction in Chd7 mRNA is reported (EVFig1B) - where are the qPCR primers located, upstream or downstream of the target site i.e. is mRNA degraded by nonsense-mediated decay in the mutant? Do the mutant fish express a truncated protein or is the protein completely absent by for example Western blot using an antibody against the N-terminal portion of the protein?

- ***Thank you for this comment. We have revised the manuscript to clarify these points and to provide a better the characterization of the frameshift mutation; leading to a premature stop-codon and the degradation of mRNA via non-sense-mediated decay in chd7 mutant fish (Line 133-136). As we showed by qPCR, mutant chd7 transcript underwent nonsense-mediated***

decay. The presence of a truncated protein in mutant fish would have resulted in no obvious loss (decrease) in the relative abundance of the chd7 transcript in mutant fish (Noel et al, 2020). The lack of an antibody recognizing the N-terminal portion of zebrafish chd7 precluded us from additionally validating the complete loss of chd7 protein in mutant fish by Western blot.

2) They report apparent blindness (line 144) of the fish - how was this assessed?

- ***We are guessing that the reviewer meant <u>no</u> apparent blindness here: (Original manuscript, line 144-145): "….but with less pronounced cardiac defects and <u>no apparent</u> blindness, thereby making it an..". This statement was based on the ability of mutant fish to (a) respond to a visual motor response (Fig. 1F; a motor response triggered by light on (light-cycle)); and (b) to the very obvious normal food-seeking behaviour in their living environment.***

3) The authors examine the GABAergic population using a GABAergic specific transgenic line and state: 'Compared to controls, chd7-/- larvae had a significant reduction in the density of GFP-positive GABAergic cells in the brain at 5 dpf (Fig. 2B)' and quantify this in Figure 2D. The distribution of GABAergic interneurons appears to be altered in the region of the OT in Chd7-/- zebrafish. Mispositioning appears to lead to expansion of the GABAergic interneuron population in the OT. It seems important to determine if there has been any alteration in the identity of brain regions, region-specific effects on proliferation/differentiation or migration defects to explain this phenotype.

- ***Thank you for this comment. We have assessed for any differences in brain regions between chd7+/+ and chd7-/- zebrafish larval brains (Line 663-670). We now report in this revised manuscript that despite the small head phenotype and reduced number of GABAergic neurons, the brain regions are well-preserved in mutant fish (EV. Fig 1C; Line140-141). We also found that the reduced number of GABAergic neurons in OT is due to impaired neurogenesis in chd7-/- zebrafish larval brains (EV. Figs 2-4; see point 4).***

4) They identify interesting changes in the dynamics of progenitor proliferation by pH3 staining (EV Fig. 1). However, the identity of these progenitors are not known - they need to co-stain with specific markers of GABAergic and glutamatergic progenitors to distinguish between these, especially at the earlier stages when no apparent differences in proliferation or apoptosis are seen. Furthermore, more rigorous experiments are needed to show evidence of premature cell cycle exit/differentiation by for example BrdU/EdU labeling of proliferating progenitors a day or so earlier and co-staining for EdU/BrdU, differentiation and proliferation markers at 5dpf.

- ***We agree with the reviewer. We have performed co-staining of pH3 and NeuroD1 (neuronal progenitor marker) (EV Fig. 2). We have also performed a BrdU 24h pulse-labelling and co-stained brain sections for BrdU and NeuroD1, HuC/D (neuronal marker) or dlx5a/6a-GFP (GABAergic neuron marker) (EV. Figs. 3-4; Line 630-662). Importantly, data from these extensive experimentations confirmed an increase in proliferation (BrdU-positive cells) in 5 dpf chd7-/- zebrafish brain (EV. Fig 3). We also observed a significant decrease in BrdU-HuC/D , BrdU-NeuroD1 and BrdU- dlx5a/6a-GFP double positive cells in 5 dpf chd7-/- (EV. Fig 3,4; Line 233-269). These findings strongly indicate suppressed neurogenesis and***

impaired GABAergic neuronal differentiation in chd7-/- fish. Altogether, these results strengthen the mechanistic conclusions of the manuscript.

We have attempted to perform combined BrdU/nestin (NSC/progenitor), BrdU with pH3 (using a different BrdU antibody from that used in the above-listed co-staining studies to avoid cross-reaction) and pH3/pax6 (glutamatergic progenitors) co-staining on the brain sections and had difficulty getting these to work likely due to that some of these antibodies do not work in fish. Generating chd7-/- fish with GFP-labelled NSC/progenitors (gfap, nestin), mature neurons (HuC/D) and glutamatergic neurons (Vglut2a) would circumvent the technical issues faced but the production of these stable transgenic lines and their analysis would take over a year to complete. Additionally, with the above in mind, we do not see how the results from these experiments, if they had worked, would add substantially to our main conclusions.

5) They report a reduction in GABAergic progenitors in 5 dpf fish, is this phenotype still present in adult fish?

- ***Given mutations in CHD7 are associated with human developmental disorders (Bouazoune & Kingston, 2012; Zentner et al, 2010) , we focused our analyses at the early stages of zebrafish development. It can certainly be interesting to evaluate phenotypes in adult fish but we believe that evaluating adult phenotype is non-trivial and is outside the scope of this manuscript. Additionally, this kind of experiment will require an animal ethic protocol for the use of adult fish and would unreasonably delay publication.***

6) They chose to perform RNAseq at 5 dpf, when an overt phenotype was already present - can they justify why this stage was chosen?

- ***Thanks for this comment. This age was chosen to perform an exhaustive analysis of the molecular phenotype because it corresponds to a stage when the behavioural phenotype is distinct. This stage also is critical in zebrafish tectal development (the brain region where we found a highly reduced number of GABAergic neurons) (Avitan et al, 2017; DeMarco et al, 2020; Hoffman et al, 2016; Robles et al, 2011). We now justify why this stage was chosen in the text results of this revised manuscript (Line 297-299).***

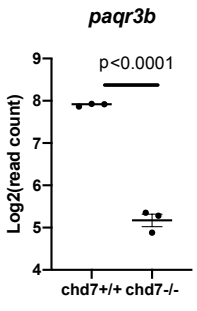

Image response 1

Of note, we have RNA sequenced zebrafish at an earlier developmental stage (2 dpf) for another study. We find that at both 2 dpf and 5 dpf, the majority of genes that are dysregulated are very similar. Importantly, the main dysregulated gene in focus in this manuscript, paqr3b, is strongly downregulated at 2 dpf (Image response 1). This is not surprising given that paqr2b is already highly expressed in 2 dpf fish (EV. Fig. 5A). However, we prefer not to create an over-complexity of the manuscript with 2 dpf transcriptomic data and to keep this 2 dpf RNAseq dataset for another manuscript in preparation.

7) The U0126 treatments yielded very interesting results. It is not clear to me from the methods when the treatment was initiated and how long it was maintained for. These details need to be provided, as well as the rationale for choosing this treatment regime. Is this treatment sufficient to normalize pERK levels? Similarly, the phenotypic rescue by overexpressing Paqr3b (Fig. 5) is very compelling, can they show that this is sufficient to rescue pERK levels, thereby making a more direct mechanistic connection between paqr3b downregulation, pERK dysregulation and GABArgic interneuron numbers?

- ***Thanks for this comment. We apologize that the methods for the U0126 treatments (treatment regime with supported citations etc..) was not clearly stated in our original manuscript and have added this clarification to the revised text (Line 681-682). The specificity of U0126 as a MEK1/2 inhibitor in decreasing the level of pERK is already well established by many studies (Murakami et al, 2000; Naska et al, 2004) including in zebrafish (Guo et al, 2015; Hawkins et al, 2008; Hong et al, 2006; Huang et al, 2012). We thank the reviewer for his/her very good point in evaluating the levels of pERK upon overexpression of paqr3b. We have performed this experiment and showed that overexpression of paqr3b in chd7-/- fish significantly recovered pERK level to basal wild-type level (Fig. 5E; Line 356-357). These new findings provide a convincing mechanistic link between paqr3b downregulation, pERK dysregulation and GABAergic neuron development. These data significantly strengthen the main mechanistic conclusion of the manuscript.***

8) The data presented in Fig. 4 requires a more rigorous explanation of the methods used for qPCR and associated statistical analyses. Fig. 4A compares 4 control data points with 4 patient samples - what do these data points represent? Are these independently generated lines, independently performed experiments? Why are the 4 data points in the controls identical? Was each control compared to a mutant in 4 independent experiments and this is the data plotted? What method was used to compare expression, DDCt? In contrast to Fig. 4A, Fig. 4C now has 6 control samples and only 3 patient samples. Can the authors explain the reason for this and provide details in the methods and figure legends so that statistical rigor can be assessed?

- We apologize that the methods for the qPCR analysis of LCLs were missing in our original manuscript. We have added a detailed description of the methods (Line 804-810) and statistical analyses used for the qPCR analysis in this revised manuscript. The relative transcript levels of the PAQR3 analyzed by reverse qPCR, was calculated according to the 2 −ΔΔCt method, using HPRT1 and RPS1 as housekeeping genes for normalization. All data are expressed as mean fold change ± SD across four independent experiments, with parental control values in each experiment set to 1. We have updated the Fig. 4a to appropriately represent the data. We have also adjusted the figure legend of Fig. 4 accordingly to include complete details on the experiments performed and the statistical analyses used. The two experiments (qPCR-Fig. 4A and ChIP-Fig.4C) are independent experiments and they were performed in two different labs and institutes.

9) Can the data presented in Fig. 6G-I be quantified?

- In the original manuscript, Fig. 6G-I were representative images of GABAergic defects in chd7 mutant worms and they were actually quantified in the set of panels of Fig. 6D-F. We have changed the order of presentation to facilitate the report of the data, by now presenting the images first in Fig. 6D-F and the quantification in 6G-I.

10) The ephedrine rescue experiments are very interesting. To draw a more direct mechanistic link between ephedrine treatment, pERK normalization and phenotypic rescue, can the authors activate pERK in ephedrine-treated embryos and show that they no longer rescue the phenotype?

- We agree with the reviewer's point. We have attempted to activate pERK using an optogenetic approach in zebrafish larvae but increasing pERK levels during early developmental stages is lethal (Image response 2).

There is no specific agonists allowing specific activation of ERK. On the other hand, constitutive active ERK signaling (via expression of mutant ERK) in the zebrafish embryo leads to abnormal convergence and extension movements that cause stretching of the normally spherical yolk along the anterior-to-posterior axis (Patel et al, 2019; Rian et al, 2013). The result is an elongated embryo at 6 hpf and lead to developmental arrest and death. To overcome these drawbacks, we thought of using a photoswitching mutant MEK activity (psMEK^{E203K}) to control ERK signalling in vivo in zebrafish (Patel et al., 2019) and activate p-ERK at drug treatment time (8 hpf). We injected psMEK^{E203K} mRNA (zebrafish optimized psMEK^{E203K} plasmid (Patel et al., 2019) was a kind gift from Dr. Shvartsman) at 1-cell stage and the embryos were illuminated at 8 hpf with 500 nm light to activate pERK. Zebrafish mutant embryos exposed to 500 nm light exhibit a high rate of mortality by 24 hpf compared to controls that were not illuminated (Image response 2). The lack of tools (or specific chemical agonists) that can allow specific activation of pERK without lethality precluded the possibility of successfully carrying out the requested experiments at present.

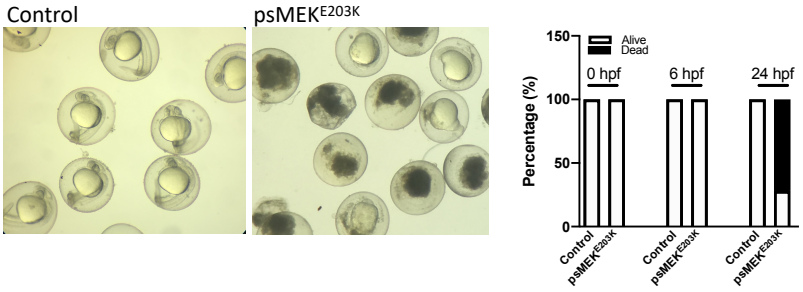

Image Response 2

11) Finally, I can't find any details in the methods on how the cell counts were performed (for example for Fig. 3F,G) - can this be provided?

- We have improved the description of the methods related to how the cell counts were performed (Line 606-614).

12) Some figure legends (e.g. Fig. 13, 4, 5, 6) do not state the statistical test performed for each analysis, these need to be stated.

-Thanks for this comment. We have ensured that all the statistical test performed are stated in the figure legends.

Minor comments:

Abstract: "hyperactivity disorder", shouldn't this be ADHD (attention deficit hyperactivity disorder)?
- Corrected, thanks

Line 41: direct target gene of the paqr3b
- Corrected, thanks

Line 45: restore normal levels of MAPK/ERK signaling
- Corrected, thanks

Line 46: that this network - define what network: gene network, signaling network?
- We have specified what network we are referring to.

Line 104: It is worth noting that cerebellar hypoplasia as a result of Chd7 deficiency in cerebellar granule neuron progenitores was eliminated as a likely cause of autism-like behaviors in Whittaker et al.
- We now report this observation from Whittaker et al. in the Introduction (Line 103-104).

Line 111-123: I found the summary of the results in the Introduction too long and detailed, can this be shortened to a few sentences rather than summarizing the manuscript in great detail?
- We agree with the reviewer and we have significantly shortened this part of the Introduction.

Figure1C. the figure legend does not match the figure.
- Corrected, thanks.

Line 168: we did not observe a change in either...or..
- Corrected, thanks

Line 177: connectivity is not the correct term here
- We have replace the term "connectivity" with a more appropriate term.

Line 193: CHD7 remodels chromatin by translocating nucleosomes, it does not remodel nucleosomes
- Corrected, thanks

Line 702: No dotted line in figure 1E as indicated in the legend
- Corrected, thanks

Line 801: EV figure 1L the wrong data is shown relative to the figure legend.
- Corrected, thanks. Of note, this panel has been moved to EV Fig. 4E

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Dear Dr. Patten,

Thank you for the submission of your revised manuscript. We have now received the enclosed report from the referee that was asked to assess it. I am happy to say that referee 2 only has one more minor suggestion that I would like you to incorporate before we can proceed with the official acceptance of your manuscript.

A few other editorial requests also need to be addressed:

- Please reduce the number of keywords to 5.

- Please be consistent with the author initials, either SP or K(S)P. The corresponding author name should be the same throughout. In our online system and the ORCID it's Kessen (Shunmoogum) Patten, but in the manuscript it is Shunmoogum (Kessen) Patten.

- Please enter the funding information in our online manuscript handling system when you upload the final version of the manuscript.

- Fig 2A callout is missing. Fig 4E callout is missing. Fig 6B-D panel callouts are missing. Please add.

- The EV figure callouts need to be corrected to "Figure EV#".

- Of the 3 "Appendix Tables", the first table should be called "Dataset EV1", the second table should be called "Table EV1" and the third table "Table EV2". Please also correct the callouts of these tables in the manuscript text.

- Please upload all figures in portrait format as per journal policy.

- Please move the figure legends to after the references in the manuscript file.

- I attach to this email a related manuscript file with comments by our data editors. Please address all comments in the final manuscript.

I would like to suggest a few minor changes to the title and abstract. Please let me know whether you agree with the following:

Chromatin remodeler CHD7 is required for GABAergic neuron development by promoting PAQR3 expression

Mutations in the chromatin remodeler-coding gene CHD7 cause CHARGE syndrome (CS). CS features include moderate to severe neurological and behavioural problems, clinically characterized by intellectual disability, attention-deficit/hyperactivity disorder and autism spectrum disorder. To investigate the poorly characterized neurobiological role of CHD7, we here generate a zebrafish chd7-/- model. chd7-/- mutants display defects in the number of GABAergic neurons and exhibit a hyperactivity behavioural phenotype. The GABAergic neuron defect is at least in part due to downregulation of the CHD7 direct target gene, paqr3b, and subsequent upregulation of MAPK/ERK signalling, a regulatory axis that is also dysregulated in CHD7 mutation-positive human cells. Through a phenotype-based screen in chd7-/- zebrafish and C. elegans, we further identify

ephedrine as a small molecule able to restore normal levels of MAPK/ERK signalling and improve both GABAergic defects and behavioural anomalies. We conclude that chd7 promotes paqr3b expression, and that this is required for normal GABAergic network development. This work provides insight into the neuropathogenesis associated with CHD7 deficiency and identifies a promising compound for further preclinical studies.

EMBO press 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 exactly 550 pixels wide and 200-600 pixels high (the height is variable). You can either show a model or key data in the synopsis image. Please note that text needs to be readable at the final size. Please send us this information along with the revised manuscript.

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

Best regards,
Esther

Esther Schnapp, PhD
Senior Editor
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Referee #2:

The authors have made every effort to address all my comments on the original manuscript. I am particularly happy that they managed to show that PAQR3 over expression restores normal P-ERK activation, representing an important mechanistic addition to the manuscript. The revised manuscript represents and important addition to the field and will in my opinion be of interest to a wide readership. I recommend publication in EMBO Reports.

I only have one minor comment: Figure 6 in the revised manuscript is incorrectly labelled as Fig. 5.

Point-by point response to the reviewers:

Reviewer 2

The authors have made every effort to address all my comments on the original manuscript. I am particularly happy that they managed to show that PAQR3 over expression restores normal P-ERK activation, representing an important mechanistic addition to the manuscript. The revised manuscript represents and important addition to the field and will in my opinion be of interest to a wide readership. I recommend publication in EMBO Reports.

I only have one minor comment: Figure 6 in the revised manuscript is incorrectly labelled as Fig. 5.

- We wish to thank the reviewer for his/her comments and appreciation of the significance of our work. We have correctly labelled the Figure 6 now.

2nd Revision - Editorial Decision 15th Mar 2021

Dr. Shunmoogum (Kessen) Patten
INRS
531 Boul des Prairies
Laval, Quebec H7V1B7
Canada

Dear Dr. Patten,

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

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Best regards,
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Corresponding Author Name: Shunmoogum A. Patten
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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.
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- ➔ 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;
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- ➔ 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;
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  - 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.

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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?	Sample size was determined based on previous experience with similar behavioural and imaging experiments.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	The number of samples was determined empirically.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No data were exluded from the 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.	All samples and animals for the experiments were randomly selected.
For animal studies, include a statement about randomization even if no randomization was used.	All samples and animals for the experiments were randomly selected.
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.	Blinding was not performed in the stuides as we needed to select transgenics and compared them to control groups.
4.b. For animal studies, include a statement about blinding even if no blinding was done	Blinding was not performed in the stuides as we needed to select transgenics and compared them to control groups.
5. For every figure, are statistical tests justified as appropriate?	Yes- Reported in statistically sections.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Graphpad PRISM software automatically assessed the data prior to test recommendations.
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Is the variance similar between the groups that are being statistically compared?	Significance was determined using either Student's t-test or One-way ANOVA followed by multiple comparisons test. A Tukey post-hoc multiple comparisons test was used for normally distributed and equal variance data. Kruskal-Walllis ANOVA and Dunn's method of comparison were used for non-normal distributions.

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).	We provided catalog number for all antibodies used in this study.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	NA

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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.	Provided in the Methods section
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11. Identify the committee(s) approving the study protocol.	Provided in the Methods section
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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	We provide a data availability section.
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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).	NA
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