

A human-specific enhancer fine-tunes radial glia potency and corticogenesis

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Version 0:

Reviewer comments:

Referee #1

(Remarks to the Author)

The authors of this manuscript study HARE5 and its functional contribution to species-specific cerebral cortex development. HARE5 is an evolutionarily accelerated genomic region that this lab previously showed has gene enhancer activity during brain development, including the cerebral cortex. They also previously showed that this enhancer activity occurs in patterns that are different for the human, non-human primate and mouse sequence of HARE5, suggesting the possibility of having a different influence on brain development. So here they study the effect of HARE5 activity on cerebral cortex development, with a focus on differences between the human version (Hs-HARE5) and the mouse (Mm) and chimpanzee (Pt) versions.

The authors report that Hs-HARE5 fine-tunes early event of cortical development by regulating the proliferation and neurogenic balance of neural progenitor cells, with long term consequences on neuron number and brain size. They first show that HARE5 is particularly active in NPCs compared to neurons, where it regulates the levels of Fzd8 transcription, and via genetic shuffling of human and mouse HARE5 they study species-specific effects on multiple parameters of cortical NPCs dynamics and function. They conclude that Hs-HARE5 specific enhancer activity promotes NPC proliferation and amplification early on, and later in development it drives neurogenesis, finally leading to increased brain size.

The study is extremely interesting and timely, nailing down how specific modifications of non-coding gene regulatory elements of the genome affect particular aspects of brain development, and contributed to brain expansion and complexity during evolution. The authors use a large variety of experimental and analytical tools, including several transgenic mouse lines and genetically-edited iPSC lines, cell and tissue culture, single cell and bulk transcriptomic and epigenomic analysis, in a remarkable effort to elucidate the specific innovative aspects of Hs-HARE5 on brain development. In my opinion, the story merits publication in Nature, but unfortunately the manuscript in its present form has a number of caveats and issues that need to be addressed before it is ready for publication.

Overall, the manuscript is quite difficult to read and with many points of confusion, which only upon the second or third read become vaguely clear. Description of results is frequently confusing and needs significant improvement. Insertion of comments about data from previous studies into the description of new results, plus spreading the new results between main and supplementary figures, makes it very confusing and difficult to follow.

Specific points:

1- The authors use CRISPRi to target and manipulate HARE5. How were these sgRNAs validated? Were off-target effects tested? If FZD8 had robust expression after iCRISPR, how does this indicate that HARE5 is an active enhancer?

2- "These data indicate human-specific expression patterns of FZD8 in the developing neocortex." - This is inaccurate, as the patterns were not specific, only the relative expression levels.

3- Panels Fig. 1e-1g really belong to Fig. 2, and should be moved there for coherence with the presentation of results in the main text.

4- "These data demonstrate that the ancestral mouse HARE5 enhancer is essential for cortical size, whereas human

HARE5, due to stronger activity, has the opposite impact to increase cortical size.” - This is confusing: it seems that both human and mouse HARE5 promote brain size, so why they have an opposite impact?

5- The authors show an increase in brain size of mice with Hs-HARE5 compared to controls, carrying the endogenous Ms-HARE5. Quantitatively this is an extremely mild defect of about 2-3%. They then compare this to mutant mice with one or both copies of endogenous Ms-HARE5 missing, finding a brain size reduction of 5-10%, which they define as profound microcephaly. Clearly, given the very small extent of these changes, the authors must tone-down their remarks.

6- “Quantification of NeuN positive cells revealed significantly more mature neurons in the Mm-HARE5Hs/Hs mouse cortex compared to controls at both stages (Fig. 2e,f)” – This is strictly not true, as their quantification is of proportion of all cells that are neurons, not absolute amount of neurons, nor neuron density per piece of cortex.

Rather, they measure % of DAPI cells that are neurons. Greater % of DAPI cells that are neurons correspondingly indicates lower % of cells that are not neurons. Is there a loss of astrocytes? Other glia types? If so, given that these are generated after neurons, does it mean a premature exhaustion of progenitor cells?

Related to the phenotype of functional connectivity, it would be very informative to measure if there is a difference in tissue density of neurons because that would be indicative of changes in neuropile, hence maybe in dendritic or axonal arborizations and connectivity.

The analysis of functional connectivity reveals decreased interareal activity, suggesting loss of connectivity... perhaps due to neuropile defects?

7- “In Mm-HARE5Hs/Hs there were significantly more NeuN cells in upper layer bins at P21 (bins 6, 8-10) while P0 showed no significant change (Extended Data Fig.2c,d).” - Why are there no differences at P0 when the analysis is binned, while altogether there is an even greater difference (22%) than at P21?

8- “However, we observed a striking increase in mitotic RGCs in Mm-HARE5Hs/Hs cortices (Fig.3d,f).” - Were these excess mitoses apical? or located elsewhere?

9- For clarity, in Figure 3 panel f should go next to d, and panel g should go next to current e.

10- “these data demonstrate that Hs-HARE5 promotes proliferative capacity and self-renewal of early stage RGCs” - What about late stage? Given the increased number of neurons at P21, the authors should investigate what happens later on to this excess of Sox2+ cells. Was there increased cell cycle exit later? When? Which cortical layer/types of neurons are being generated?

11- “we assessed whether Hs- HARE5 impacts progenitor composition at peak stages of neurogenesis. We quantified RGCs and IPs by staining for SOX2 and TBR2, respectively, in E14.5 cortices (Fig.3h,i).” – This quantification, relative to DAPI+ cells, is ill defined and seems rather wrong. According to the result, Sox2+ cells (presumptive RGCs) account for only 35-40%, which most likely means the quantification was done over the entire cortical thickness. If so, this is wrong because many confounding factors may change this abundance, such as change in neuron abundance (excitatory or inhibitory), etc. Instead, the authors must quantify exclusively within germinal layers.

Alternatively, the analysis would greatly benefit from quantification of the density of Sox2+ and Tbr2+ cells per unit length of ventricular surface. This will reveal differences in absolute density, independent from amount of DAPI cells outside of germinal layers, and will take into account potential changes in density of cells within the germinal layers, which goes unnoticed when calculating % to DAPI cells. Moreover, IPs are here considered as all Tbr2+ cells, which is wrong as newborn neurons still are Tbr2+. Tbr2+Ki67+ cells should be counted instead, or else refer to these only as Tbr2+ cells.

12- Given the problematic use of Tbr2 to selectively mark IPs, the authors should combine this with quantification of basal PH3+Tbr2+ mitoses, a defining feature of IPs in mouse.

13- The authors use PhVim stains to study oRG abundance. The example shown for oRG in Fig 3i is definitely non-convincing. While a basal mitosis positive for Pax6 is a good indicator of oRG, the definite evidence is the presence of a basal process, which cannot be really seen in Fig. 3i. I am not sure what the arrowheads point at in the high mag, but if this is their best example, I really worry about how they quantified this.

14- “brains contained significantly fewer BrdU+PAX6+ cells, indicating a higher proportion of RGCs exit the cell cycle (Fig.3o).” - This is incorrect, as RGCs incorporating BrdU at E13.5 may have become Tbr2+ IPs at E14.5, without exiting cell cycle.

15- “In comparison, there was no notable change in cell cycle exit of IPs (Extended Data Fig.4i).” - This statement is definitely wrong. According to the y-axis legend, they measured proportion of BrdU+ cells at E14.5 that are Tbr2+, and this does not indicate cell cycle exit of IPs. BrdU+ cells at E14.5 include neurons born at E13.5 after BrdU labeling, plus cycling RGCs and cycling IPs. This analysis is invalid for this purpose. The same is true for the results in Fig. 3o.

16- “These data demonstrate that Hs-HARE5 promotes RGC cell cycle exit at E14.5, resulting in production of more excitatory neurons.” - This conclusion requires the analyses indicated above to have a solid basis. Also, for greater clarity, the authors must clearly define the timeline of cell cycle exit experiments at early and later stages. Was the histological analysis done at E12.5 and E14.5? Or at E13.5 and then E14.5. There is a big difference in timing, and this is important for the conclusions on the underlying mechanism.

17- "Hs-HARE5 promotes proliferation and self-renewal of RGCs at the onset of neurogenesis and continues to expand the precursor pool while amplifying neuronal and progenitor generation as development proceeds." - This statement is not accurate, because it is in the same developmental stage (E14.5) that they observe (if confirmed with the analyses proposed in my above comments) both progenitor amplification and increased neurogenesis / cell cycle exit. Instead, the authors should be able to explain how these two aspects can occur simultaneously.

18- "the proportion of G2/M RGCs was increased relative to other cell cycle phases in the Mm-HARE5Hs/Hs cortex (Fig.3s)." - This increase is really very small (1-2% difference), much smaller than the above histological analyses. Is it statistically significant?

19- "these scRNA-seq data provide orthogonal evidence that Hs-HARE5 promotes expression of Fzd8 in RGCs, with changes in cell cycle composition of both RGCs and IPs." - This analysis is puzzling: Hs-HARE5 mice had many more cluster 8 cells and fewer cluster 0 cells, but both are in G1. In fact, cluster 8 contains cells in G1, S and G2M, so it is confusing what are they, and what does it mean an increase in the G2M population. IP cluster 5 is importantly increased in Hs, and is a G2M cluster. It is also strange the dramatically smaller proportion of cells in S compared to G2M, considering that in mouse S is much longer than G2M. The conclusion refers to RGCs and IPs, but changes in cell cycle are only shown for IPs.

20- "These data demonstrate that Hs-HARE5 causes RGCs to directly produce more IPs, which is also consistent with more newborn IPs (Fig.3m)." - This analysis is really nice and smart, but progenitor cells in this experiment are completely out of cellular context, without lateral inhibition by Notch signaling, for example. Therefore, conclusions should be toned down at this point. The next set of experiments in slice are just fabulous and conclusions are much stronger then. This result contradicts the increased cell cycle exit in vivo at E14.5.

21- The result shown in Fig. 3o-p seems perfectly parallel to above analyses (Fig. 3k) and should be better presented together, before the single cell transcriptomics.

22- "Expression of 1645 transcripts were specifically changed in the HARE5Hs/Hs cortex from E12.5 to E14.5," - Figure S6c indicates 1641 genes. This disparity should be corrected.

23- "These molecular changes are consistent with observations of the developmental impact of Hs-HARE5 upon RGCs, with reduced cell cycle exit at E12.5 and increased neural differentiation at E14.5." - This conclusion seems wrong, as downregulated genes involved in cell cycle and mitosis are downregulated at E14.5 compared to E12.5; thus, cortex at E12.5 expresses more cell cycle genes than at E14.5, just like in wild-type brains. To reach the conclusion here stated, the authors must show that GO of these changes specific to HsHs is different from GO of changes specific to MmMm.

24- "This suggests that increased neural differentiation by Hs-HARE5 is associated with reduced canonical WNT signaling." - This is again very confusing. If HARE5 increases Fzd8 expression, shouldn't Hs-HARE5 increase Wnt signaling, and this block neural differentiation? These results suggest that Hs-HARE5 acts on different pathways at early and late stages, to produce its different effects.

25- "Our data using mouse models indicate that Hs-HARE5 activates RGC expansion and neuron production." - How HARE5 increases RGC expansion if it decreases canonical Wnt-bCat signaling, consistent with neuron differentiation? The temporal dynamics of HARE5 must be presented much more clearly, or else it is very difficult to follow the multiple functions that the authors attribute to it.

26- The results related to ASD are very nice, but it is totally unclear what is the genetic relationship between ASD and HARE5 driving gene expression. For example, is ASD also associated with decreased expression of genes under HARE5 regulation? I don't see this clearly explained, and its relevance is very confusing.

27- "This finding demonstrates that Hs-HARE5 is essential for promoting proliferation of NPCs, consistent with observations in mice." - Maybe the authors could also test the late pro-neurogenic effect of HARE5 in this system. It is not essential to the story, but it would nicely clarify whether this effect reported here is specific or rather a general effect of HARE5 no matter what.

28- "just 4 chimpanzee specific variants within human cells reduces proliferative capacity of progenitors." - This is not accurate, as SOX2 stains do not reveal proliferation, but only cortical progenitor cell identity. Pt/Pt cells may proliferate more but produce neurons, hence reducing the overall % of cells that are Sox2+. Proliferative activity should be revealed by PH3 frequency and/or cell cycle re-entry rate, which these authors actually do in the following experiments.

29- "These findings are consistent with studies showing that during in vitro neural differentiation of human NPCs and in vivo mouse cortical neurogenesis, canonical WNT signaling decreases." - If HARE5 increases Fzd8 expression, shouldn't this increase Wt signaling?

Referee #2

(Remarks to the Author)

Liu et al., perform functional characterization of HARE5, a conserved non-coding sequence with an accelerated nucleotide substitution rate in humans. The non-coding element had previously been shown to interact with the Fzd8 promoter and drive reporter gene expression during cortical development. Compared to the chimpanzee element, the human sequence when driving to Fzd8 expression more strongly increased proliferation. However, previous studies had not directly evaluated the function of the human sequence at the endogenous locus. In the present study, Liu et al., demonstrate that HARE5 regulates FZD8 expression in human, chimpanzee, and mouse cells and can increase cortex size (~6%) and neuron number (12.5-21.9%) by increasing the radial glia progenitor pool initially and then by increasing intermediate progenitor producing divisions and neurogenesis later in development, with the effects likely driven by 4 nucleotide substitution that each act to increase enhancer activity.

The study represents an important step for modeling human-specific changes affecting brain development. Previous studies have modulated the expression of candidate genes by ectopic overexpression, but direct engineering of the mutations at the endogenous locus to identify a functional consequence has been lacking. The authors make excellent use of orthogonal models to test the effects of gain and loss of function. Orthogonal evidence includes quantitative differences in cortical area and neuron number and adult neural calcium dynamics in adult mice, analysis of underlying developmental mechanisms by histology, scRNA and bulk RNA-seq and timelapse imaging, and analysis of genetic changes by luciferase assays and by measuring developmental cell behavior in engineered human and chimpanzee organoids. I applaud the authors for combining so many techniques to evaluate the consequences of HARE5.

My remaining comments that I hope will be constructive are listed below:

Major comments

I want to make sure the proposed developmental mechanism is clearly articulated and that there are not any missing experiments.

Specifically, why would increased Fzd8 expression switch from promoting proliferation at E12.5 (consistent with previous HARE5 Fzd8 study and other studies of Wnt activation) to promoting more cell cycle exit and neuronal differentiation at E14.5? The argument seems to be that HS-HARE5 causes more IP generating divisions and possibly that HS-HARE5 decreases canonical Wnt activation later in development.

This result of decreased Wnt activation at E14.5 was a surprise to me - the authors suggest that this reduction may reflect the increased neural differentiation driven by HS-HARE5, citing other studies showing decreased Wnt signaling during differentiation. Can the authors clarify the argument:

-Is the suggestion that increased Fzd8 expression directly reduces Wnt signaling to drive differentiation (which I did not expect) as mentioned in the discussion ("...Hs-HARE5 inhibits canonical signaling at E14.5 correlates with the developmental impact of this enhancer from promoting RGC self-renewal to differentiation.")

-Or is the suggestion that increased Fzd8 expression first increases canonical Wnt signaling at E12.5 (which could/should be tested? and would be consistent with the effect of Wnt on radial glia), which first increases RGC proliferation and then increases IP-producing divisions and neural differentiation later, with reduced Wnt signaling as a secondary/indirect consequence?

Minor comments

-Although the chimpanzee allele was not also engineered into the mouse models for direct in vivo comparisons, the figure 5 data sufficiently address this issue by indicating that mouse and chimp alleles drive similar levels of reporter gene expression and also by examining the chimp and human alleles in the human and chimp organoid context. In addition, the mouse CKO data also support the claims.

-What was the FOXG1 threshold for excluding organoids - the text states "samples were excluded based on the extremely not comparable FOXG1 level from each differentiation," and Extended Data Fig 8 shows some variation by RT-qPCR. The authors could examine whether variation in patterning efficiency and FOXG1/PAX6 levels correlates with some measured phenotypes like FZD8 expression (or explicitly treat patterning as a covariate).

Also, how is the "neural rosette area" defined for quantification differences in RGC number?

Gene expression analysis:

It is difficult to evaluate the statistical significance of interpretations from the scRNA-seq data, including the elevated Fzd8 level in Fig. 3r and Extended data 5b and the increased G2M fraction. Perhaps these trends are consistent with the more fine scale quantifications presented earlier? Also, why does the effect on mitotic RGC look so much stronger in 3f vs 3s?

-Extended data Fig 5 could also clarify the 2 control vs 2 HARE5-HS/HS design.

-For the bulk RNA seq, were there 3 biological replicates for each genotype at each developmental stage - the supplemental figure could better explain the design. Similarly, the supplemental figure could better illustrate the design to compare E14.5

and E12.5 within genotypes (rather than compare gene expression between genotypes at each timepoint).

-Some enrichments are discussed related to genes that may have specific temporal changes only in HARE5-HS/HS but not control lines. But it could be that these genes have similar temporal dynamics in both genotypes and just reached the significance threshold in one, which the authors could check for or illustrate with additional plots.

-Did the authors analyze differential expression between HARE5-HS/HS and control lines with the bulk or the scRNA-seq data and were there any insights into radial glia genes with altered regulation?

Other comments

-the authors could add a reference for the definition of ANC516 (PMID 17578567?)

-Page 5 - "profound" is a strong word for a 5-10% reduction

Figure 1:

-Legend a, ends with ? instead of .

-Add y-axis on Figure 1C

Figure 2:

-Clarify in 2b,e,f if colors correspond to litter

-The trend for HS alleles increasing cortical area looks more subtle at P0 than the significant result in adult in Extendedata Fig 2b. Are there marginal p-values associated with this result? Also, should the dots be colored by litter?

Figure 3:

-3g legend (or panel) should state BrdU treatment timing

Figure 5

It is an interesting finding that all 4 human-specific mutations individually act with the same direction of effect (that might be consistent with independent directional selection on all 4 events) and could be noted

Referee #3

(Remarks to the Author)

This manuscript by Liu and colleagues uses a mouse model with a human accelerated region (HAR) knock-in and modified human and nonhuman primate cell models to study the function of a specific HAR. The authors previously showed that this HAR, HARE5, can alter Fzd8 expression and Fzd8 can alter development of the neocortex. Here, the authors find more specifics about the role of this HAR showing that Hs-HARE5 can affect cortical development by modifying progenitor proliferation and neuronal differentiation. Furthermore, the authors pinpoint specific human variants that underlie enhancer function to drive progenitor proliferation.

In general, the results were mostly clear and the conclusions were supported by the data. The inclusion of the cKO mouse was a nice contrast to the knock-in mouse for many comparisons. The inclusion of the chimpanzee cell lines and the chimp variants were also improved comparisons over simply using a mouse context and was much appreciated.

I think the major question I have remaining is whether altered WNT signaling is the main driver here and if all of these phenotypes could simply be recapitulated by manipulating WNT and Fzd8 in the absence of modifying HARE5? I'm not asking the authors to repeat all of their findings to investigate this as some WNT signaling manipulation is already done in the manuscript. In addition, the authors already demonstrated that Fzd8 overexpression can result in some of these phenotypes. So the remaining question really is whether the purpose of HARE5 is to only modify Fzd8, and if so, we have learned a lot about the mechanism of Fzd8 function in cortical development but the purpose of the HARE5 was already solved, so to speak. Also, if this is the case, how does this modification of WNT-Fzd8 signaling work in 2D culture? All of the necessary cell types for the signaling are in place? The organoid data using the chimp-modified human cells is compelling in this regard. Would manipulation of WNT signaling in these organoids "rescue" a human phenotype? Again, I am asking the authors to speculate.

Specific comments:

1) The absolute value of cortical area should be normalized to body length or weight. This claim about changes in cortical area is especially important given that cortical thickness doesn't look different in figures 1c/d.

- 2) In figure 1d, the authors could use publicly available data to also add mouse data (e.g. from Di Bella et al., 2021). I realize the authors later compare Fzd8 expression in control vs knock-in mice but this is only at one timepoint. There should also be a note about how developmental stages were matched for both species.
- 3) Extended Data Fig.2b, it appears there only two samples from cKO—do these mice have survival issues?
- 4) Fig. 2c: NeuN may not be the best antibody to address the question at P0 since the deep layer neurons are mostly not stained with NeuN. At this time point perhaps other neuronal markers (such as TBR1 for deep layer neurons and SATB2 for upper layers) could be used. But the result appears to be consistent with P21—it just might be overestimating a finding due to the low labeling. Moreover, for this quantification, the appropriate quantification should be NeuN+DAPI+/area for neuronal density. A similar comment about quantification is for Extended Data Fig 4.
- 5) Fig. 3h,k,l,m: 1) Where is the region for quantification? Based on the data, it should not be the whole column. Please specify. 2) the representative image does not appear to show the difference in the quantification. Given the increasing RG, IP, and new IP proportion in Hs/Hs, the double negative DAPI cell should be doubled in Mm/Mm. It is hard to tell from Fig. 3h.
- 6) Fig.3o and corresponding result: 1) the proportion of cycling RGCs should be BrdU+PAX6+/PAX6+ not BrdU+PAX6+/BrdU+. 2) fewer BrdU+ RGCs would not suggest a higher proportion of RGCs exit the cell cycle, since BrdU-RGCs could also be the RGCs during the non-S phase of the cell cycle. Instead, exit cycling RGCs should at least be BrdU+SOX2-TBR2- DAPI. It seems challenging to determine the exit cycling RGCs here, because how can one determine if those exit cycling cells are from RGCs or IPs?
- 7) The single genomics data results do not have any statistics applied to the various comparisons that are made about proportion differences or even Fzd8 expression. It is impossible to tell if any of the claims are statistically relevant and what the variance is among biological replicates. The feature plots are not informative to make claims about proportions. Also, why did the authors chose to include the damaged cells within the UMAP presented? The authors should present only the filtered/cleaned data for presentation and analysis and deposit all data for public use to be filtered as needed.
- 8) Did the authors compare the scRNA-seq at E14.5 with the bulk RNA-seq at E14.5? The bulk RNA-seq results do not provide much additional functional information about the role of HARE5 in development beyond what is presented in other ways. Importantly, WNT signaling does not appear to be affected which is in contrast to the functional study presented with the WNT reporter and WNT inhibitor. Could this be due to loss of cellular resolution. Is WNT signaling altered in the E14.5 scRNA-seq data?
- 9) In figure 4, why do the authors analyze Hs/Mm rather than Hs/Hs? Also by carrying out IUE at E14.5 how can the authors specifically label the RGCs and not other cell types? This was not clear.
- 10) The authors state: "Rhesus macaque is a closely related species to humans and a commonly studied nonhuman primate." Closely related is subjective, and yes, compared to mouse, more closely, but this could be reframed.
- 11) The authors state Regarding figure 3a: "HARE5 enhancer activity was remarkably high in the developing neocortex at E11.5 and E14.5, mainly within the germinal regions." Remarkably high compared with what? No other regions or timepoints are shown. This is subjective without a point of comparison.

Version 1:

Reviewer comments:

Referee #1

(Remarks to the Author)

The authors have performed a number of additional experiments, analyses and significant changes in the manuscript in response to all my previous criticisms and points of concern. I am fully satisfied with the revised manuscript, and I congratulate the authors for great work and a groundbreaking discovery.

Referee #2

(Remarks to the Author)

The authors have improved the manuscript and addressed my major concern about the impact of the enhancer mutations on Wnt signaling with extensive additional experiments and a clearer model.

Note, the minor Y-axis point in Fig 1c was for the H3K27ac and DHS plots, not the HiC map

Referee #3

(Remarks to the Author)

The authors have been very responsive to the prior critiques and the manuscript is substantially improved.

I have two suggestions for clarifications:

- 1) The data to support Hs-HARE5 regulating non-canonical WNT signals at later developmental stages for differentiation of neurons is not strong. I suggest toning down this interpretation.
- 2) The calculation of RGCs and IPs exiting the cell cycle still seems open to other interpretations. SOX2 or TBR2 could only be detected in postmitotic cells for a short period of time. Thus, only a portion cycling RGCs and IPs exiting can be captured. The authors do now discuss this in their rebuttal. But the question is about the proportion of those cells and whether it represents the whole population of exiting progenitors. Based on the authors' protocol, the interval between BrdU injection and dissection is 24 hours. Some BrdU labelled postmitotic cells might experience two cell cycles. It is known that the

number of BrdU+ cells is negatively correlated with cell cycle length. However, we do not know the cell cycle length between knock in and WT mice. If the authors could further discuss or disentangle these issues, that would be great.

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Response to Reviewers

A human-specific enhancer fine-tunes radial glia potency and corticogenesis

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We thank the editors and reviewers for their careful evaluation of our manuscript. We are pleased the reviewers found our study of potential interest and significance for the field. Their insightful comments and suggestions have significantly improved our manuscript. Our study shows for the first time that human-specific accelerated regions have a functional impact on the developing brain. To address reviewer concerns we have pursued extensive new experiments and new analyses, which are described in the response.

Major new experiments we include in this revision:

- **Additional evidence that *Hs-HARE5* is a transcriptional enhancer controlling *FZD8* expression:**
 - **Fig. 1e-g, Extended data Fig. 1c,d:** New experiments showing *Hs-HARE5* is a direct and specific transcriptional enhancer of *FZD8* using a) H3K27me3 CHIP-qPCR and b) qPCR for *FZD8* and 2 additional *HARE5* targets identified by Hi-C.
 - **Fig. 1d and Extended data Fig. 1b:** Graphs of *FZD8* expression across comparable prenatal and postnatal stages from human, macaque and mice cortices. Integrating expression data from mouse neocortex shows *Fzd8* levels are highest prenatally.
 - **Fig. 3a:** Images showing *Hs-HARE5* enhancer is active in the germinal zone of E11.5, E14.5 and 16.5 cortices (the latter stage is new).
 - **Fig. 3m-o:** Revised analyses of scRNA seq of control and *Mm-HARE5^{Hs/Hs}* cortex showing *Fzd8* is significantly upregulated in RGCs in E14.5 but with similar spatial patterns with control.
- **Detailed mechanisms by which *Hs-HARE5* influences progenitors and neural cell fate:**
 - **Fig. 3j-l, Extended data Fig. 5k-m:** New quantifications of E14.5 brains showing overall higher density (and proportion) of RGCs, IPs, and newborn IPs in *Mm-HARE5^{Hs/Mm}* and *Mm-HARE5^{Hs/Hs}*.
 - **Fig. 4a-c, Extended data Fig. 7a-h:** New quantifications of cell cycle exit from E13.5 to E14.5 and from E14.5 to E15.5, indicating that *Hs-HARE5* increases cell cycle exit from E14.5 to E15.5.
 - **Fig. 5n:** New quantification of D30 human *Hs-HARE5^{Hs/Hs}* and *Hs-HARE5^{Pt/Pt}* cortical organoids showing that *Pt-HARE5* causes decreased mitosis of RGCs.
 - **Extended data Fig. 5j-l:** New quantifications showing more mitotic IPs in E14.5 *Mm-HARE5^{Hs/Hs}* cortices.
 - **Extended data Fig. 6a-f, Supplementary table 1:** Revised scRNA seq analysis of E14.5 *Mm-HARE5^{Hs/Hs}* cortices showing alterations in composition and transcriptomes of RGCs and IPs.
 - **Extended data Fig. 8a-g, Supplementary table 2:** Revised bulk RNA seq analysis showing scatter plot of all transcripts and different GO categories of altered transcripts in control versus *Mm-HARE5^{Hs/Hs}* cortex from E12.5 to E14.5.
 - **Extended data Fig. 9a, b:** Summary of ASD variants in human *HARE5* locus and known impact on enhancer activity.
 - **Extended data Fig. 10f-u:** New graphs showing that *FZD8* expression across genotypes is not due to differentiation quality, and examples of rosette regions.
- **Mechanisms by which *Hs-HARE5* impacts neural number, neural connectivity, and brain size:**
 - **Fig. 2e,f:** Quantification of NeuN number at P21 showing *Hs-HARE5* induces higher density of mature neurons.
 - **Fig. 2g-k, Extended data Fig. 2f:** New quantification of laminar-specific excitatory neurons in P21 cortices showing *Hs-HARE5* induces increased ratio of upper to deep layer neurons.
 - **Fig. 4p-r:** New lineage analysis experiment *in vivo* showing that embryonic Tbr2+ expressing progenitors in *Mm-HARE5^{Hs/Hs}* ultimately generate more neurons in P30 cortex.
 - **Extended data Fig. 2b:** Graph showing *Hs-HARE5* causes larger cortical area after normalization to body weight in adult mice.
 - **Extended data Fig. 4a-c:** New quantifications showing no significant change in synapse number in the somatosensory cortex (S1bf) of *Mm-HARE5^{Hs/Hs}* and *Mm-HARE5^{Hs/Mm}* mice.

- **Extended data Fig. 4d-g:** New quantifications showing no significant alteration in glia number in P21 *Mm-HARE5^{Hs/Hs}* cortices.
- **New evidence that *Hs-HARE5* controls canonical WNT signaling to regulate progenitor proliferation:**
 - **Fig. 6n:** We include a revised model supported by new data summarized below. *Hs-HARE5* increases *FZD8* expression, which triggers higher canonical WNT signaling in early-stage radial glia. Data from our lab and others show that higher canonical WNT signaling promotes radial glia proliferation and self-renewal. At later neurogenic stages, *Hs-HARE5* does not impact canonical WNT signaling in radial glia. This shift in neurogenesis and WNT activity may be due to exposure to different neurogenic cues, including WNT ligands.
 - **Fig. 6a-c:** New experiment with a canonical WNT reporter in Day12 human knock-in NPCs showing *Pt-HARE5* decreases canonical WNT signaling.
Fig. 6d-i: New experiment with a canonical WNT reporter in E11.5 primary mouse NPCs and *in vivo* tissue sections, showing *Hs-HARE5* increases canonical WNT signaling.
 - **Fig. 6j-l; Extended data Fig. 11g,h:** New experiment with overexpression of *mFzd8* in E10.5 primary mouse NPCs, showing it activates canonical WNT signaling activity evidenced by reporter and qPCR of WNT targets.
 - **Fig. 6m; Extended data Fig. 11i,j:** New experiment using a canonical WNT agonist, CHIR 99021, showing it increases mitosis of D12 human NPCs.
 - **Extended data Fig. 11c-e:** New analyses of WNT canonical reporter focused only on RGCs, showing that canonical WNT signaling at E15.5 is not altered.
 - **Extended data Fig. 11f:** Volcano plots depicting WNT ligand expression changes from E12.5 to 14.5 in control and *Mm-HARE5^{Hs/Hs}* brains.
- **Additional data for reviewers**
 - Reviewer Fig 1: Data showing P0 *Mm-HARE5^{Hs/Hs}* brain trends to have prolonged neurogenesis compared to control.
 - Reviewer Fig 2: Data showing WNT agonist in *Hs-HARE5^{Pt/Pt}* NPCs modifies NPC mitosis defect.

Referee #1 (Remarks to the Author):

The authors of this manuscript study HARE5 and its functional contribution to species-specific cerebral cortex development. HARE5 is an evolutionarily accelerated genomic region that this lab previously showed has gene enhancer activity during brain development, including the cerebral cortex. They also previously showed that this enhancer activity occurs in patterns that are different for the human, non-human primate and mouse sequence of HARE5, suggesting the possibility of having a different influence on brain development. So here they study the effect of HARE5 activity on cerebral cortex development, with a focus on differences between the human version (Hs-HARE5) and the mouse (Mm) and chimpanzee (Pt) versions.

The authors report that Hs-HARE5 fine-tunes early event of cortical development by regulating the proliferation and neurogenic balance of neural progenitor cells, with long term consequences on neuron number and brain size. They first show that HARE5 is particularly active in NPCs compared to neurons, where it regulates the levels of *Fzd8* transcription, and via genetic shuffling of human and mouse HARE5 they study species-specific effects on multiple parameters of cortical NPCs dynamics and function. They conclude that Hs-HARE5 specific enhancer activity promotes NPC proliferation and amplification early on, and later in development it drives neurogenesis, finally leading to increased brain size.

The study is extremely interesting and timely, nailing down how specific modifications of non-coding gene regulatory elements of the genome affect particular aspects of brain development, and contributed to brain expansion and complexity during evolution. The authors use a large variety of experimental and analytical tools, including several transgenic mouse lines and genetically-edited iPSC lines, cell and tissue culture, single cell and bulk transcriptomic and epigenomic analysis, in a remarkable effort to elucidate the specific innovative aspects of Hs-HARE5 on brain development. In my opinion, the story merits publication in Nature, but

unfortunately the manuscript in its present form has a number of caveats and issues that need to be addressed before it is ready for publication.

Overall, the manuscript is quite difficult to read and with many points of confusion, which only upon the second or third read become vaguely clear. Description of results is frequently confusing and needs significant improvement. Insertion of comments about data from previous studies into the description of new results, plus spreading the new results between main and supplementary figures, makes it very confusing and difficult to follow.

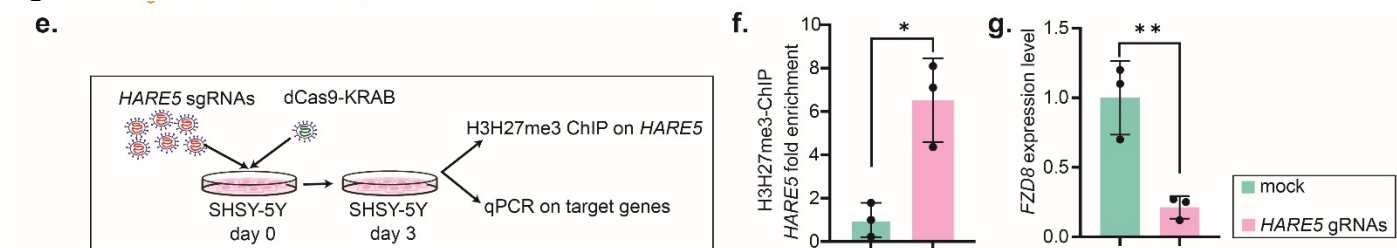
We thank this reviewer for their enthusiasm and excellent summary highlighting the key advances of our manuscript, as well as for their insightful and helpful comments which have improved our paper. We have carefully edited the manuscript to improve clarity and added substantial additional experiments in response to specific concerns.

Specific points:

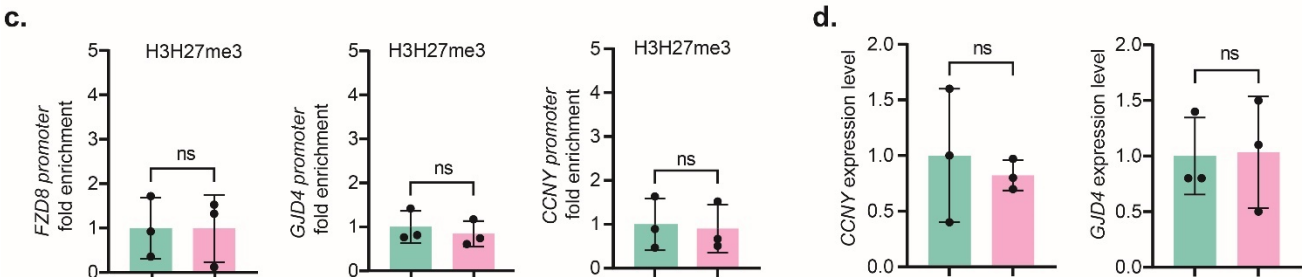
1- The authors use CRISPRi to target and manipulate HARE5. How were these sgRNAs validated? Were off-target effects tested? If FZD8 had robust expression after iCRISPR, how does this indicate that HARE5 is an active enhancer?

We appreciate the reviewer's question. To address this concern, we performed several new experiments. First, to further validate the sgRNAs, we performed H3K27me3 CHIP-qPCR on human SH-SY5Y cells following transduction of dCas9-KRAB and *HARE5* sgRNAs. Compared to mock control, H3K27me3 levels were significantly elevated at *HARE5*, indicating repression of the enhancer (Fig. 1e,f). In contrast, the promoters for *FZD8* and nearby Hi-C targets, *GJD4* and *CCNY*, were unaffected (Extended data Fig. 1c). To further assess specificity, we measured the expression of all three genes. Only *FZD8* levels, but not *GJD4* and *CCNY*, were significantly reduced following CRISPRi treatment (Fig. 1g, Extended data Fig. 1d). These data indicate that 1) the sgRNAs specifically inhibit the *HARE5* enhancer and 2) reinforce that *HARE5* is a bona fide active enhancer controlling *FZD8* expression.

Fig. 1



Extended data Fig. 1



2- “These data indicate human-specific expression patterns of FZD8 in the developing neocortex.” - This is inaccurate, as the patterns were not specific, only the relative expression levels.

We agree and have modified the text for accuracy.

3- Panels Fig. 1e-1g really belong to Fig. 2, and should be moved there for coherence with the presentation of results in the main text.

This is a good suggestion, and we have moved the mouse model to **Fig. 2a**.

4- “These data demonstrate that the ancestral mouse HARE5 enhancer is essential for cortical size, whereas human HARE5, due to stronger activity, has the opposite impact to increase cortical size.” - This is confusing: it seems that both human and mouse HARE5 promote brain size, so why they have an opposite impact?

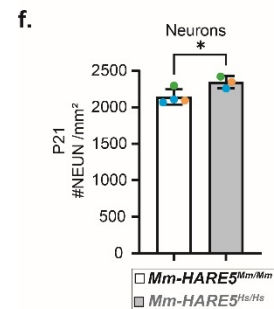
We agree this was confusing as written and have modified the text to clarify.

5- The authors show an increase in brain size of mice with Hs-HARE5 compared to controls, carrying the endogenous Ms-HARE5. Quantitatively this is an extremely mild defect of about 2-3%. They then compare this to mutant mice with one or both copies of endogenous Ms-HARE5 missing, finding a brain size reduction of 5-10%, which they define as profound microcephaly. Clearly, given the very small extent of these changes, the authors must tone-down their remarks.

We agree with the reviewer’s suggestion and have modified the text accordingly.

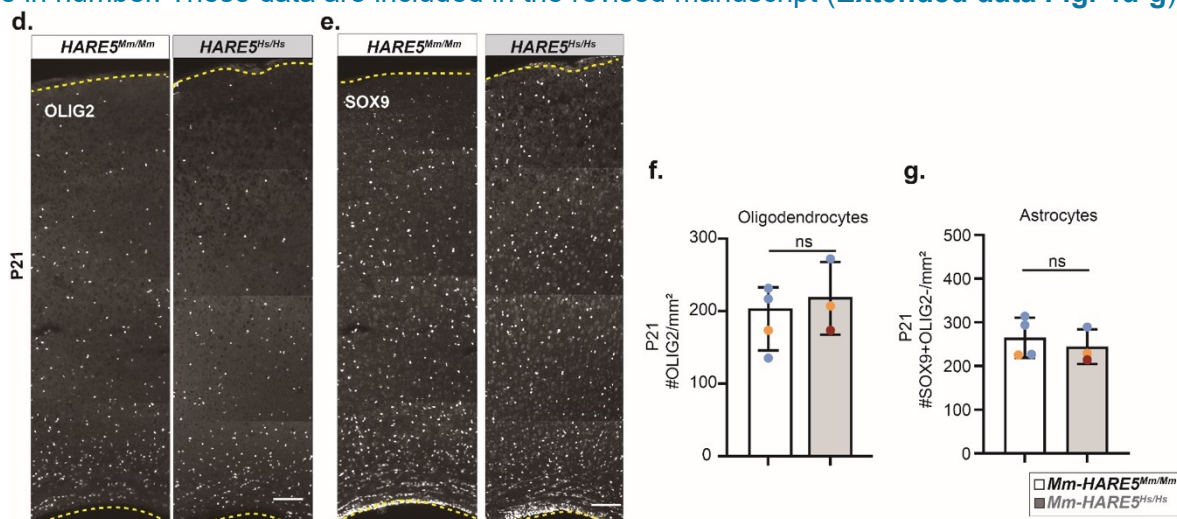
6- “Quantification of NeuN positive cells revealed significantly more mature neurons in the Mm-HARE5Hs/Hs mouse cortex compared to controls at both stages (Fig. 2e,f)” – This is strictly not true, as their quantification is of proportion of all cells that are neurons, not absolute amount of neurons, nor neuron density per piece of cortex. Rather, they measure % of DAPI cells that are neurons. Greater % of DAPI cells that are neurons correspondingly indicates lower % of cells that are not neurons.

This is a great suggestion. We re-quantified NeuN density per area (mm²) in P21 brains (**Fig. 2e,f**). These data support the same conclusion that there are significantly more mature neurons in the *Mm-HARE5^{Hs/Hs}* mouse cortex.



Is there a loss of astrocytes? Other glia types? If so, given that these are generated after neurons, does it mean a premature exhaustion of progenitor cells?

To address this reviewer concern we quantified the density of oligodendrocytes and astrocytes in P21 control and *Mm-HARE5^{Hs/Hs}* somatosensory cortex, using OLIG2 and SOX9 markers. We did not observe any significant difference in number. These data are included in the revised manuscript (**Extended data Fig. 4d-g**).



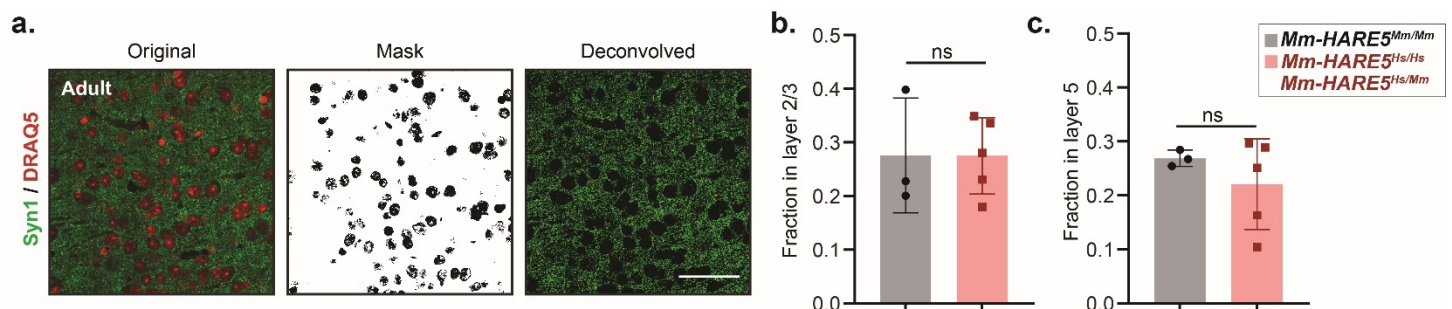
To assess the possibility of premature exhaustion of neural progenitors, we quantified the ventricular zone (VZ) as well as progenitor proliferation in the VZ in P0 brains using SOX2/Ki67 staining (**Rev Fig. 1**). Interestingly there was a slight but insignificant trend towards a thicker VZ and more proliferative cells in the VZ. These data indicate there is no premature exhaustion of progenitors, rather that neurogenesis may be slightly prolonged. However, given this is not significant it is unlikely to be a driving factor in the phenotype.

FIGURE REDACTED

Rev Fig. 1. **a.** Representative sections of P0 control and *Mm-HARE5^{Hs/Hs}* brains stained with SOX2 (green), Ki67 (red) and DAPI (blue). **b.** Quantification of VZ thickness in P0 control and *Mm-HARE5^{Hs/Hs}* cortices. n=5-8 animals; 4 litters per genotype. **c.** Quantification of SOX2+Ki67+/SOX2+ in P0 control and *Mm-HARE5^{Hs/Hs}* VZ regions. n=5-8 animals; 4 litters per genotype. Scale bars: 20µm (a top), 50µm (a bottom). Graphs and bar plot, means ± S.D. ns, not significant. Student's unpaired, two-tailed t-test (b, c).

Related to the phenotype of functional connectivity, it would be very informative to measure if there is a difference in tissue density of neurons because that would be indicative of changes in neuropile, hence maybe in dendritic or axonal arborizations and connectivity. The analysis of functional connectivity reveals decreased interareal activity, suggesting loss of connectivity... perhaps due to neuropile defects?

We thank the reviewer for this insightful point. Indeed, a loss of connectivity may contribute to the decrease in interareal correlated activity in *Mm-HARE5^{Hs/Hs}* mice. In the cortical neuropil, such a reduction in connectivity may be evident by altered synapse number. To investigate this, we quantified synapse number in control and *HARE5^{Hs/Hs}* brains that we had measured functional connectivity, using the general synaptic marker Syn1¹. Our results show no change in the global synapse number in both deep and upper layers (**Extended Fig. 4a-c**). It should be noted however that because *Hs-HARE5* also leads to an increase in neuron number, these results do not preclude the possibility that synaptic connectivity per neuron is decreased. Alternatively, our functional connectivity data may be explained by a change in how specific circuit motifs are formed. Answering these questions will require full mapping of the cellular and circuit-level changes in the cortical connectivity of *Mm-HARE5^{Hs/Hs}* mice, which will be highly intriguing for future work.

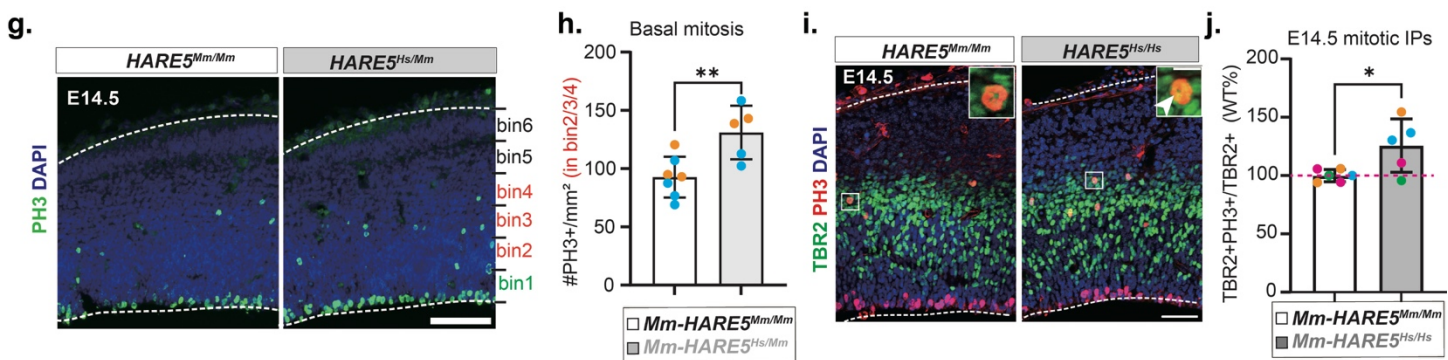


7- "In *Mm-HARE5^{Hs/Hs}* there were significantly more NeuN cells in upper layer bins at P21 (bins 6, 8-10) while P0 showed no significant change (Extended Data Fig.2c,d)." - Why are there no differences at P0 when the analysis is binned, while altogether there is an even greater difference (22%) than at P21?

These are very good points. As noted in point 6 response, we re-quantified NeuN density using methods suggested by this reviewer. While NeuN positive cells were significantly increased at P21, the data were variable and no longer significant at P0. As Reviewer 3 also points out, NeuN mainly marks mature neurons. However at P0, neurogenesis has just finished. Thus, this lack of significant phenotype at P0 may result from labeling mainly mature but not immature neurons. Importantly, the increase in neuron number at P21 is reinforced by new lamination data, as described in response to concern 10 below. Because NeuN is biased to mature neurons, and because we have quantified laminar markers at P21, we have removed the P0 data.

8- “However, we observed a striking increase in mitotic RGCs in *Mm-HARE5^{Hs/Hs}* cortices (Fig.3d,f).” - Were these excess mitoses apical? or located elsewhere?

The reviewer refers to analyses at E12.5 and we did not observe any shift in mitoses more basally at this stage. However, we addressed this question at E14.5 in our initial submission and showed there are more basal divisions. These data are regraphed in **Extended data Fig. 5g,h**. Additionally using PH3 and TBR2 staining we include new quantifications showing there are more mitotic Tbr2+ cells at E14.5 (**Extended data Fig. 5i, j**).

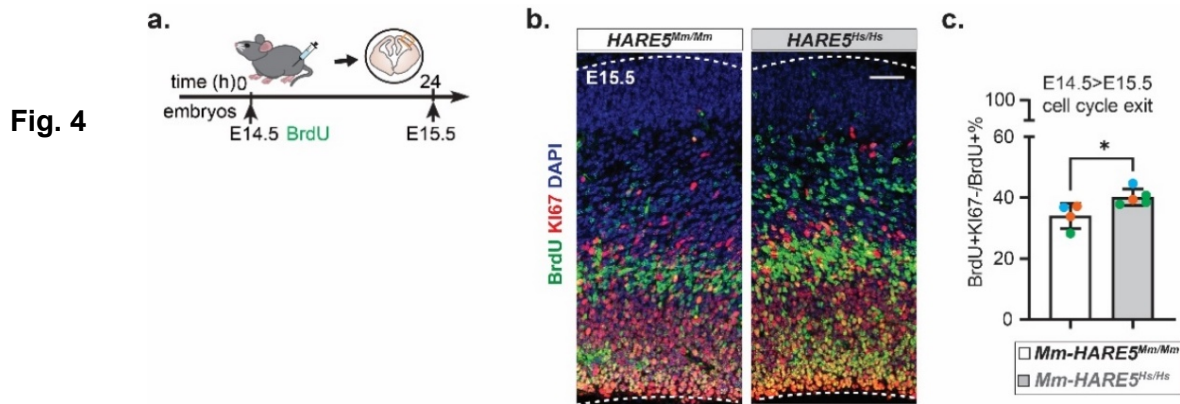


9- For clarity, in Figure 3 panel f should go next to d, and panel g should go next to current e.

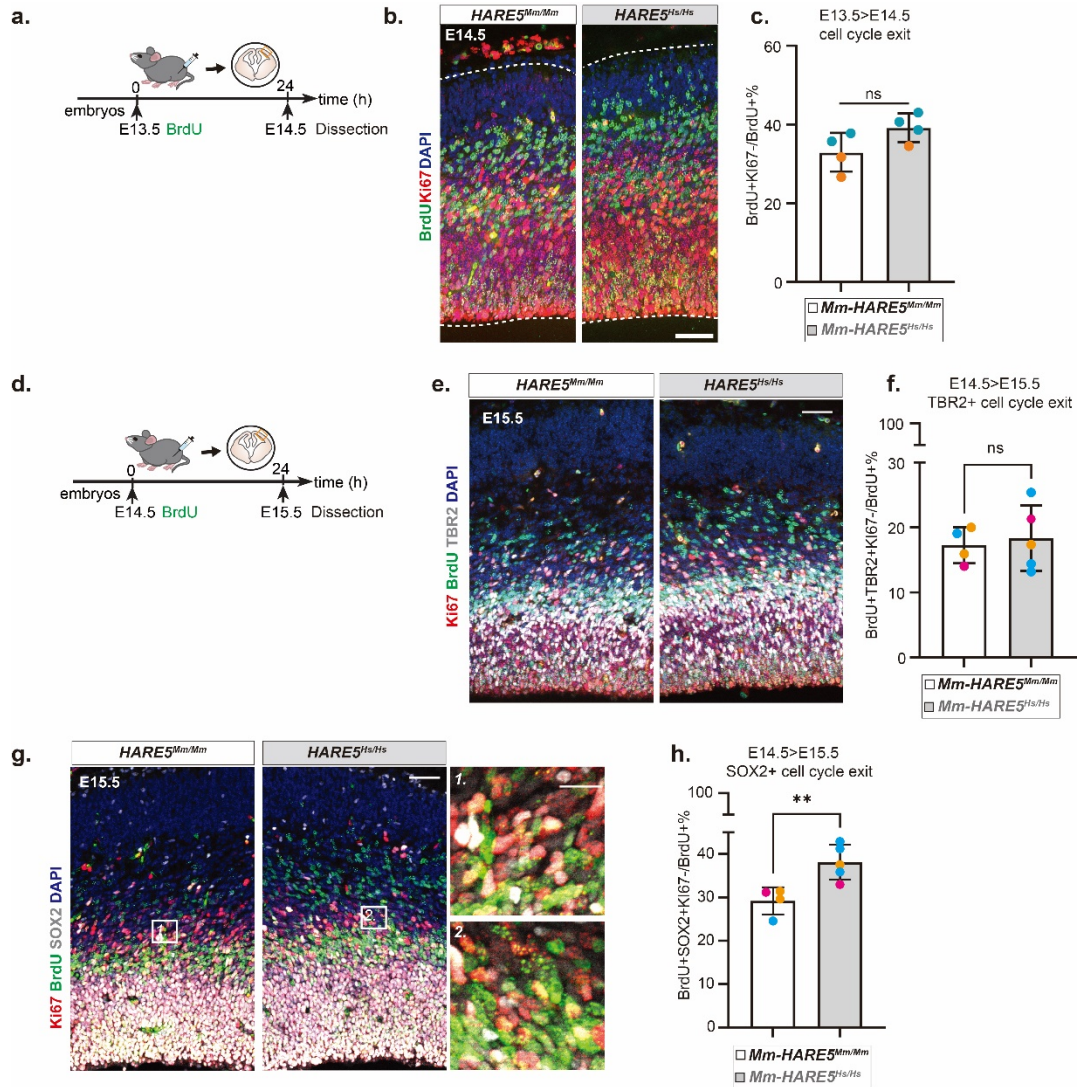
Thank you for this suggestion and we have modified the order accordingly to put graphs beside images.

10- “these data demonstrate that *Hs-HARE5* promotes proliferative capacity and self-renewal of early stage RGCs” - What about late stage? Given the increased number of neurons at P21, the authors should investigate what happens later on to this excess of Sox2+ cells. Was there increased cell cycle exit later? When?

The reviewer refers to analyses at E12.5. Indeed, we agree it is important to assess cell cycle exit at later stages. To address this concern, we assessed cell cycle exit at two later stages (measuring BrdU+Ki67-/BrdU+ cells). We observed a slight but insignificant increase in cell cycle exit from E13.5 to E14.5 (**Extended data Fig. 7a-c**). This result is consistent with the increased numbers of RGCs and IPs at E14.5. In contrast, cell cycle exit from E14.5 to E15.5 was significantly increased in *Mm-HARE5^{Hs/Hs}* brains (**Fig. 4a-c**). We also quantified the fraction of BrdU+ that were Ki67-TBR2+ (**Extended data Fig. 7d-f**). This should measure neurons produced from TBR2+ cells (although we note it is an underestimate as it will not include newborn neurons which have lost TBR2 expression). Cell cycle exit was similar across genotypes in this population. We also quantified the fraction of BrdU+ that were Ki67-SOX2+ (**Extended data Fig. 7g-h**). SOX2 is inherited in newborn IPs (about half of all TBR2+ cells), however IP generating neurons typically no longer express SOX2^{2,3}. Thus, we reasoned this population should report newborn neurons generated primarily from SOX2+ RGCs. As above, this is also a slight underestimate and does not include newborn neurons which have lost SOX2 expression. Our data show that SOX2+ cell cycle exit is significantly increased in *Mm-HARE5^{Hs/Hs}* brains. Our new figures include clearer schematics describing the experimental process including BrdU pulse chase and the analyzed timepoints. Taken together, use of both markers suggests that at E15.5 *Hs-HARE5* promotes increased differentiation by RGCs.



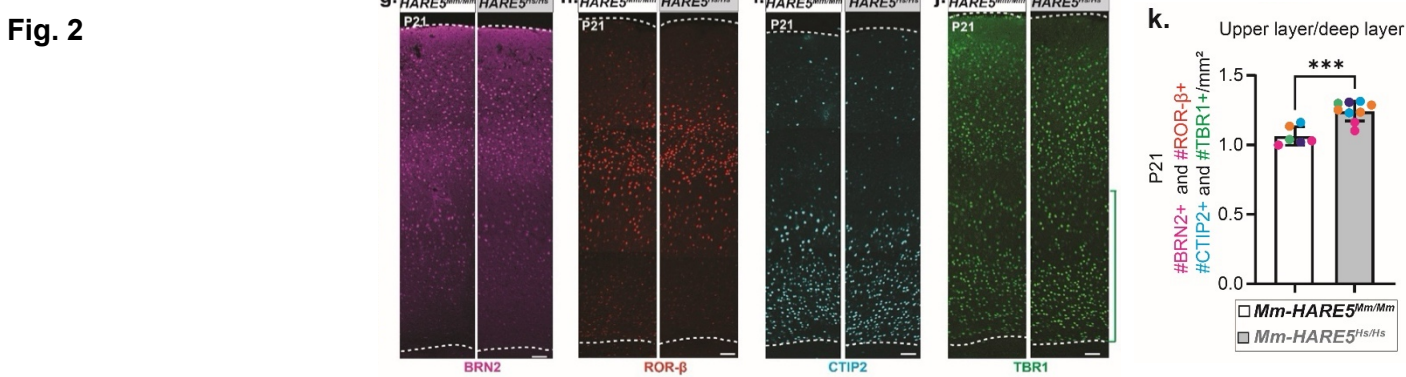
Extended Fig. 7



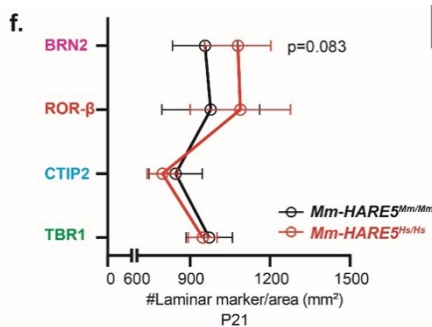
Which cortical layer/types of neurons are being generated?

To resolve which types of excitatory neurons are increased, we performed new analyses of P21 brains, using laminar specific markers to label layer II/III by BRN2, layer IV by ROR- β , layer V by CTIP2 and layer VI by TBR1. There were overall subtle changes in each marker (**Extended data Fig. 2f**). These data show that the ratio of upper layer to deep layer neurons is increased in *Mm-HARE5^{Hs/Hs}* brains (**Fig. 2g-j,k**). This biased increase in upper layer neurons is interesting in light of comparative data showing that upper layers are particularly expanded

in primates⁴. We theorize this could reflect cell cycle exit data showing the most pronounced impact on cell cycle exit at later stages (E15.5) when upper layer neurogenesis is more prominent.



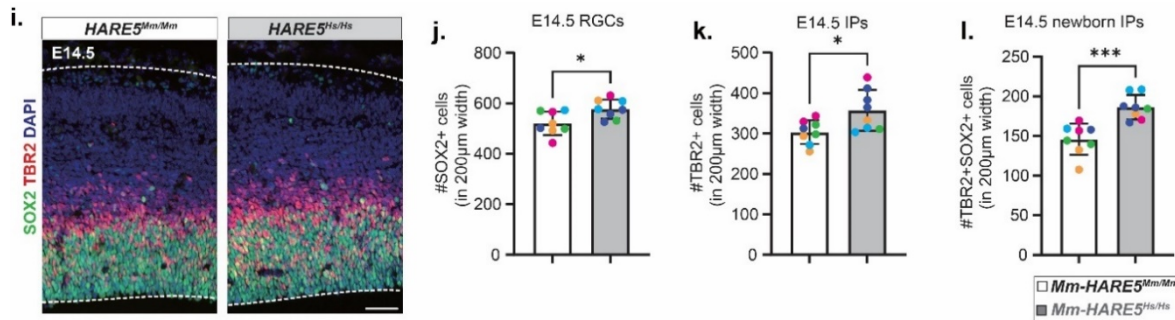
Extended Fig. 2



11- “we assessed whether Hs- HARE5 impacts progenitor composition at peak stages of neurogenesis. We quantified RGCs and IPs by staining for SOX2 and TBR2, respectively, in E14.5 cortices (Fig.3h,i).” – This quantification, relative to DAPI+ cells, is ill defined and seems rather wrong. According to the result, Sox2+ cells (presumptive RGCs) account for only 35-40%, which most likely means the quantification was done over the entire cortical thickness. If so, this is wrong because many confounding factors may change this abundance, such as change in neuron abundance (excitatory or inhibitory), etc. Instead, the authors must quantify exclusively within germinal layers.

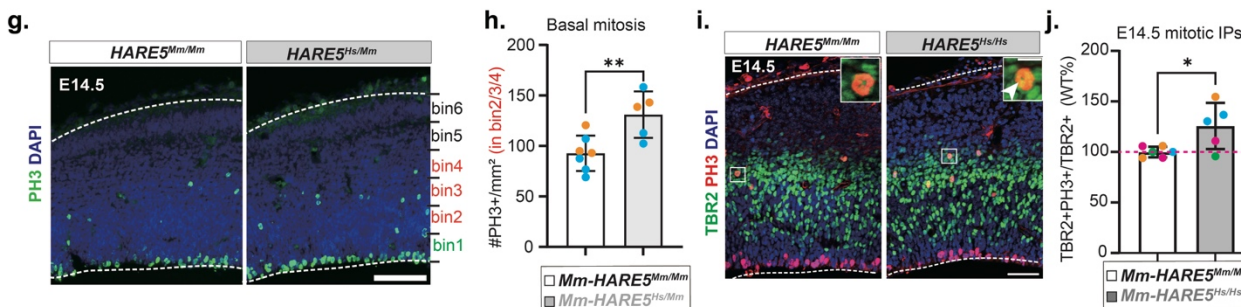
Alternatively, the analysis would greatly benefit from quantification of the density of Sox2+ and Tbr2+ cells per unit length of ventricular surface. This will reveal differences in absolute density, independent from amount of DAPI cells outside of germinal layers, and will take into account potential changes in density of cells within the germinal layers, which goes unnoticed when calculating % to DAPI cells. Moreover, IPs are here considered as all Tbr2+ cells, which is wrong as newborn neurons still are Tbr2+. Tbr2+Ki67+ cells should be counted instead, or else refer to these only as Tbr2+ cells.

We appreciate the reviewer’s point and have repeated the analysis as suggested by measuring the number of SOX2 and TBR2 positive cells per 200 μm of ventricular surface. To further assess IPs, we quantified both TBR2 alone as well as TBR2+SOX2+ which should reflect newborn IPs and not newborn neurons. These data show the density of RGCs, IPs, and newborn IPs, are significantly increased in E14.5 $HARE5^{Hs/Hs}$ brains. We include graphs for density (Fig. 3 j-l) and the proportion data are now moved to **Extended data Fig. 5k-m**.



12- Given the problematic use of Tbr2 to selectively mark IPs, the authors should combine this with quantification of basal PH3+Tbr2+ mitoses, a defining feature of IPs in mouse.

We thank the reviewer for this suggestion. We previously showed there were more basal mitosis in *Mm-HARE5^{Hs/Hs}* brains. We have now re-graphed these data (**Extended data Fig. 5g,h**). To perform the suggested experiment, we quantified mitotic IPs (PH3+TBR2+) relative to total TBR2+ cells. Notably, mitotic IPs significantly increase in the E14.5 *Mm-HARE5^{Hs/Hs}* brains (**Extended data Fig. 5i,j**).



13- The authors use PhVim stains to study oRG abundance. The example shown for oRG in Fig 3i is definitely non-convincing. While a basal mitosis positive for Pax6 is a good indicator of oRG, the definite evidence is the presence of a basal process, which cannot be really seen in Fig. 3i. I am not sure what the arrowheads point at in the high mag, but if this is their best example, I really worry about how they quantified this.

We appreciate this reviewers' concern. We quantified only basally located Sox2+ cells which contain a clear basal process. We feel confident in these blind quantifications, however we acknowledge it is difficult with Phospho-Vimentin (combined Ser55 and Ser82) to visualize the entire basal process. Given this, together with the very mild contribution of oRGs to our phenotype (only 0.4 % of cells to 0.7% of cells show an increase with large variability) we have opted to remove these data.

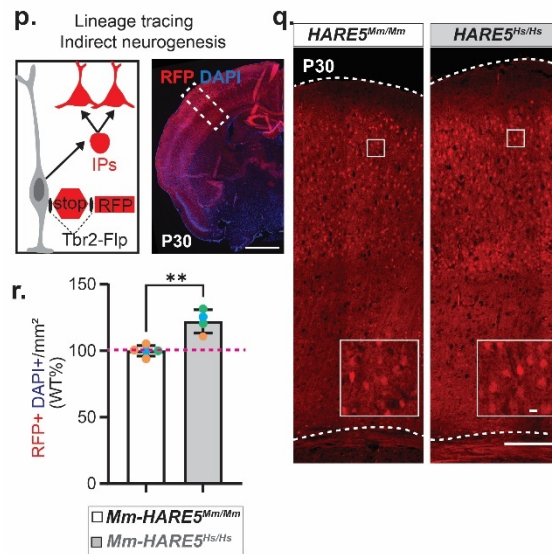
14- "brains contained significantly fewer BrdU+PAX6+ cells, indicating a higher proportion of RGCs exit the cell cycle (Fig.3o)." - This is incorrect, as RGCs incorporating BrdU at E13.5 may have become Tbr2+ IPs at E14.5, without exiting cell cycle.

We appreciate this reviewers' point and agree this was an inaccurate calculation. As noted in response 10 we have carried out additional cell cycle exit from E14.5 to E15.5 in which we include Ki67 to monitor cycling cells.

15- "In comparison, there was no notable change in cell cycle exit of IPs(Extended Data Fig.4i)." - This statement is definitely wrong. According to the y-axis legend, they measured proportion of BrdU+ cells at E14.5 that are Tbr2+, and this does not indicate cell cycle exit of IPs. BrdU+ cells at E14.5 include neurons born at E13.5 after BrdU labeling, plus cycling RGCs and cycling IPs. This analysis is invalid for this purpose. The same is true for the results in Fig. 3o.

We appreciate this reviewers' point and agree this was an inaccurate calculation. As noted in response 10 we have carried out additional cell cycle exit from E14.5 to E15.5 in which we include Ki67 to monitor cycling cells.

To further measure the output of IPs in *Mm-HARE5^{Hs/Hs}* brains, we took advantage of the genetic tool: Tbr2-flp driven Ai65-frt reporter mice lines⁵. In this model, cells derived from TBR2+ expressing cells are labeled by Td-tomato. At D30, we detected significantly more Tdtomato+ cells in *Mm-HARE5^{Hs/Hs}* brains (**Fig. 4p-r**). This data support the conclusion that increased number of IPs ultimately exit the cell cycle and contribute to increased neuron number.



16- “These data demonstrate that Hs-HARE5 promotes RGC cell cycle exit at E14.5, resulting in production of more excitatory neurons.” - This conclusion requires the analyses indicated above to have a solid basis. Also, for greater clarity, the authors must clearly define the timeline of cell cycle exit experiments at early and later stages. Was the histological analysis done at E12.5 and E14.5? Or at E13.5 and then E14.5. There is a big difference in timing, and this is important for the conclusions on the underlying mechanism.

As noted above, the cell cycle exit analyses have been better defined with cartoon timelines. Our previous data show that cell cycle exit from E11.5 to E12.5 is reduced (Fig. 3f-h). Our new data (referenced in response 10) show that cell cycle exit is unaffected from E13.5 to E14.5 (Extended data Fig 7a-c) and increased from E14.5 to E15.5 (Fig 4a-c). We appreciate this suggestion as it has helped us to better understand how *HARE5* influences neurogenesis.

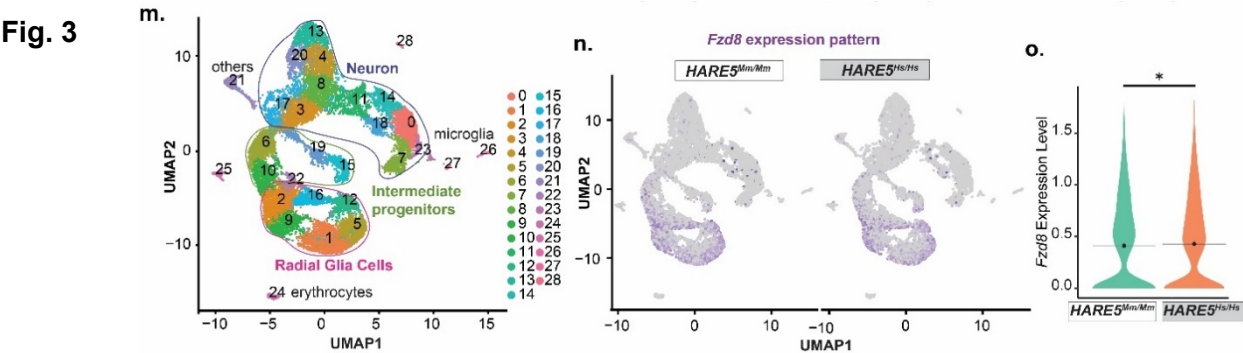
17- “Hs-HARE5 promotes proliferation and self-renewal of RGCs at the onset of neurogenesis and continues to expand the precursor pool while amplifying neuronal and progenitor generation as development proceeds.” - This statement is not accurate, because it is in the same developmental stage (E14.5) that they observe (if confirmed with the analyses proposed in my above comments) both progenitor amplification and increased neurogenesis / cell cycle exit. Instead, the authors should be able to explain how these two aspects can occur simultaneously.

Our live cell imaging, in utero lineage analysis, and cell cycle exit measurements from E14.5 to E15.5 indicate that at E15.5, *Mm-HARE5^{Hs/Hs}* RGCs produce more neurons and more IPs (revised Fig. 4). At E14.5, RGCs are modestly increased by 11% while IPs have an even higher fold increase (Fig. 3j). We interpret this is due to an earlier expansion of the RGC population. Thus, we don’t think events of RGC amplification and increased neurogenesis are happening simultaneously. We have clarified this in the text.

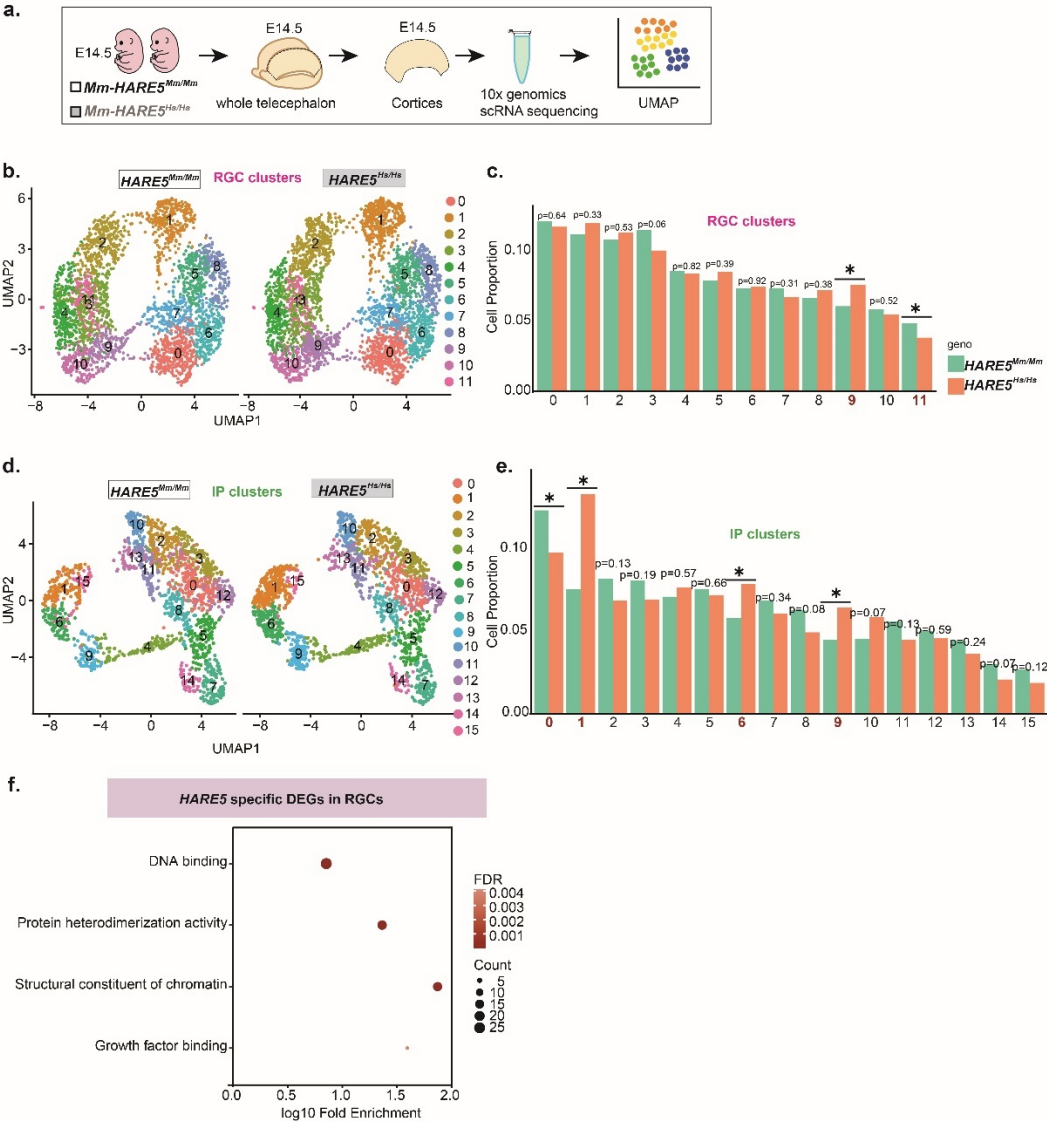
18- “the proportion of G2/M RGCs was increased relative to other cell cycle phases in the *Mm-HARE5^{Hs/Hs}* cortex (Fig.3s).” – This increase is really very small (1-2% difference), much smaller than the above histological analyses. Is it statistically significant?

We thank the reviewer for their comments on the scRNA analysis. In the new manuscript, in response to these and other reviewer concerns, we reassessed these data. 1) We filtered out all the dead/low quality cells and redid the UMAP; 2) we updated the *Fzd8* expression with statistics to show it is enriched in *Hs-HARE5* RGCs and this spatial pattern is maintained in *Mm-HARE5^{Hs/Hs}* brains; 3) we graphed the cell composition of RGCs and IPs with statistics; and 4) we include new tables showing differentially expressed transcripts and their gene ontology enrichment. These data are included in Fig. 3m-o; Extended Data Fig. 6; Supplementary Table 1.

We also performed new cell cycle analysis using a different Seurat tool: https://satijalab.org/seurat/articles/cell_cycle_vignette. These data indicated a slight trend towards more G2M cells for both RGCs and IPs, however these were not statistically significant. While the quantitative data in situ carefully measures cell cycle states, the sc-seq is based upon transcript expression of some genes. Because of this and the lack of significance, we have removed the cell cycle predictions of scRNA seq data from the study.



Extended Fig. 6



19- “these scRNA-seq data provide orthogonal evidence that Hs-HARE5 promotes expression of Fzd8 in RGCs, with changes in cell cycle composition of both RGCs and IPs.” - This analysis is puzzling: Hs-HARE5 mice had many more cluster 8 cells and fewer cluster 0 cells, but both are in G1. In fact, cluster 8 contains cells in G1, S and G2M, so it is confusing what are they, and what does it mean an increase in the G2M population. IP cluster 5 is importantly increased in Hs, and is a G2M cluster. It is also strange the dramatically smaller proportion of cells in S compared to G2M, considering that in mouse S is much longer than G2M. The conclusion refers to RGCs and IPs, but changes in cell cycle are only shown for IPs.

As described above in response 18, we reassessed cell composition for both RGCs and IPs for significant differences. Gene expression in these clusters is included in **Supplementary Table 1**. These did not show any clear bias to cell cycle state.

20- “These data demonstrate that Hs-HARE5 causes RGCs to directly produce more IPs, which is also consistent with more newborn IPs (Fig.3m).” - This analysis is really nice and smart, but progenitor cells in this experiment are completely out of cellular context, without lateral inhibition by Notch signaling, for example. Therefore, conclusions should be toned down at this point. The next set of experiments in slice are just fabulous and conclusions are much stronger then. This result contradicts the increased cell cycle exit in vivo at E14.5.

We appreciate the reviewer’s point on this. We were surprised to not see increased production of neurons in this assay. However, as noted by the reviewer, our cell culture conditions may not completely recapitulate progenitor potential. We have added this caveat to the manuscript. Importantly we draw our conclusions regarding RGC progeny collectively from live cell imaging, in utero lineage analysis, and cell cycle exit measurements which were all performed at identical stages, from E14.5 to E15.5.

21- The result shown in Fig. 3o-p seems perfectly parallel to above analyses (Fig. 3k) and should be better presented together, before the single cell transcriptomics.

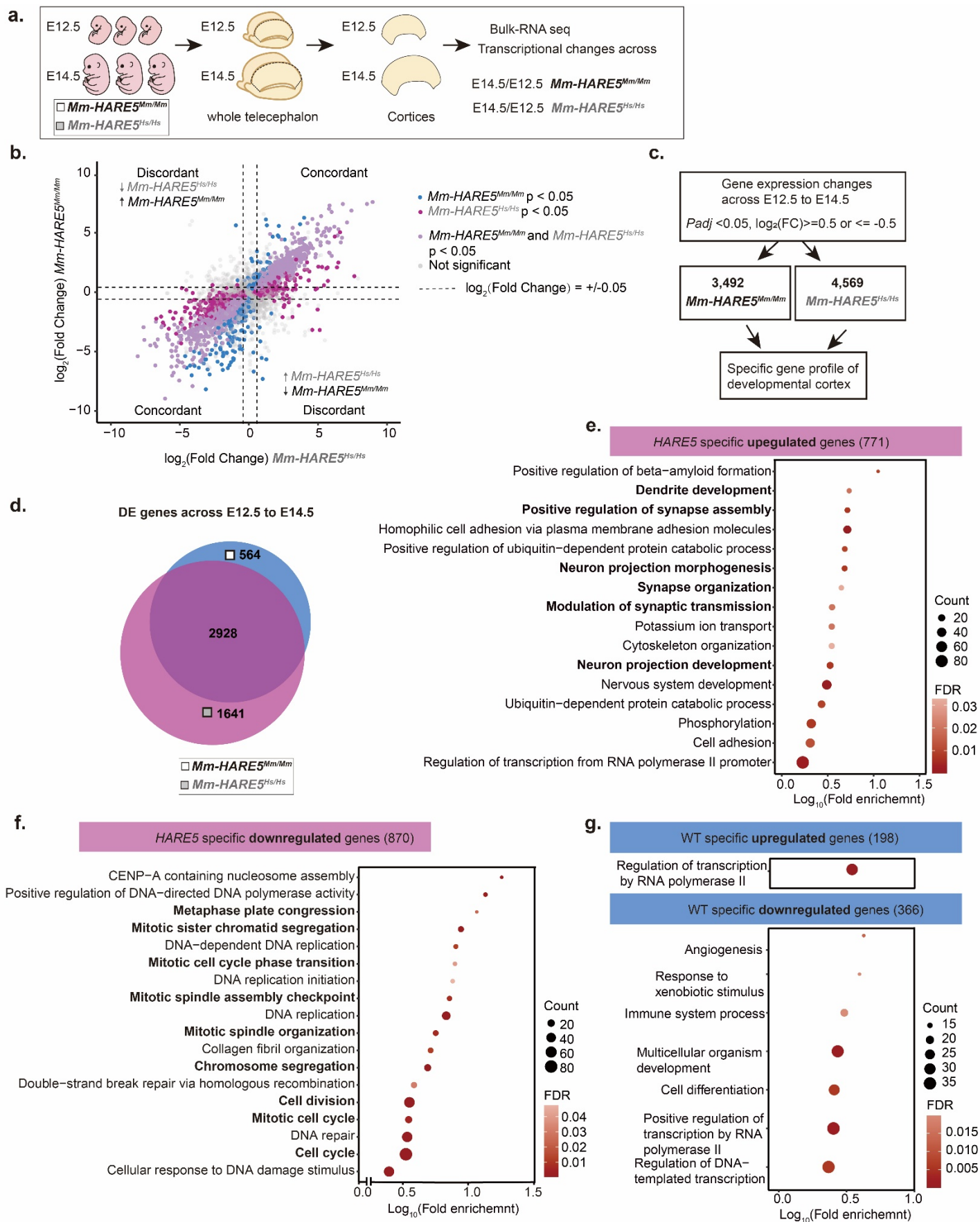
Thank you for the suggestion. We agree and have moved all the cell cycle exit data to **Fig. 4a-c** and **Extended Data Fig 7** in the revised manuscript.

22- “Expression of 1645 transcripts were specifically changed in the HARE5Hs/Hs cortex from E12.5 to E14.5,” - Figure S6c indicates 1641 genes. This disparity should be corrected.

We apologize for this mistake and have modified this sentence.

23- “These molecular changes are consistent with observations of the developmental impact of Hs-HARE5 upon RGCs, with reduced cell cycle exit at E12.5 and increased neural differentiation at E14.5.” - This conclusion seems wrong, as downregulated genes involved in cell cycle and mitosis are downregulated at E14.5 compared to E12.5; thus, cortex at E12.5 expresses more cell cycle genes than at E14.5, just like in wild-type brains. To reach the conclusion here stated, the authors must show that GO of these changes specific to HsHs is different from GO of changes specific to MmMm.

We thank the reviewer for this suggestion. We examined the 564 significantly altered control (*Mm-HARE5^{Mm/Mm}*)-specific transcripts using GO analysis. There was only 1 significant category of up-regulated genes in WT, related to regulation of transcription by RNA polymerase II (FDR=3.8X10⁻⁶). For down-regulated genes in WT, we observed distinct categories than those for *Mm-HARE5^{Hs/Hs}* (**Extended data Fig. 8g; Supplementary Table 2**). Moreover, following a suggestion from reviewer 2, we compared the log2Fold Change values for all transcripts differentially expressed between E12.5 to E14.5 cortices for both *Mm-HARE5^{Mm/Mm}* and *Mm-HARE5^{Hs/Hs}* (**Extended data Fig. 8b**). Most of these transcripts show concordant trends, either increasing or decreasing in both genotypes. Indicating that most genes have similar temporal dynamics across genotypes but show a fine-tuning of expression levels. A subset of genes are discordant, showing opposing changes in gene expression for the evaluated genotypes. These data suggest that *Hs-HARE5* has mild qualitative and quantitative impacts on gene expression, aligning with its role as an enhancer.



Extended Data Fig.8

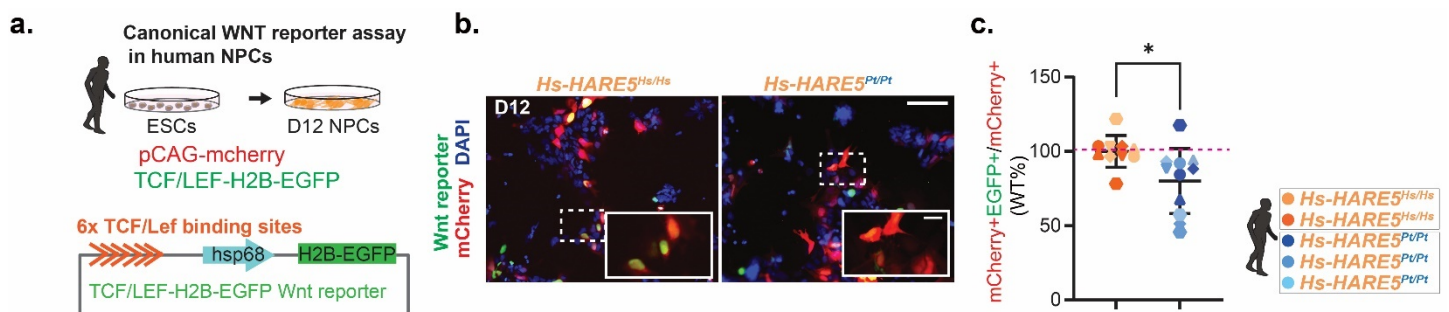
24- “This suggests that increased neural differentiation by Hs-HARE5 is associated with reduced canonical WNT signaling.” - This is again very confusing. If HARE5 increases Fzd8 expression, shouldn't Hs-HARE5 increase Wnt signaling, and this block neural differentiation? These results suggest that Hs-HARE5 acts on different pathways at early and late stages, to produce its different effects.

We thank the reviewer for this question which was also raised by other reviewers. We have pursued extensive new experiments to understand how *HARE5* influences WNT signaling during neurogenesis. A completely **new Fig. 6, and Extended data Fig. 11** with a revised model clarifying how *Hs-HARE5* influences WNT signaling.

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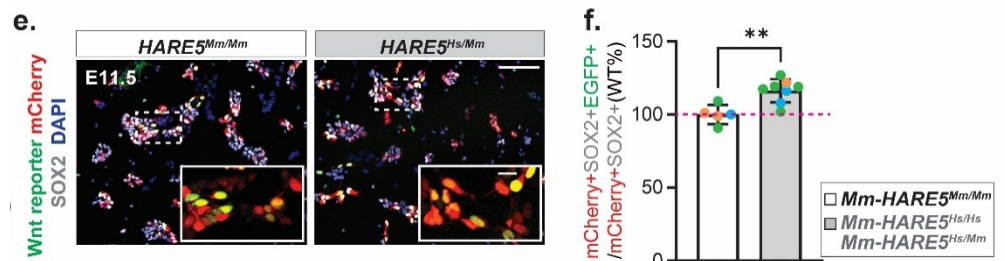
FIGURE REDACTED

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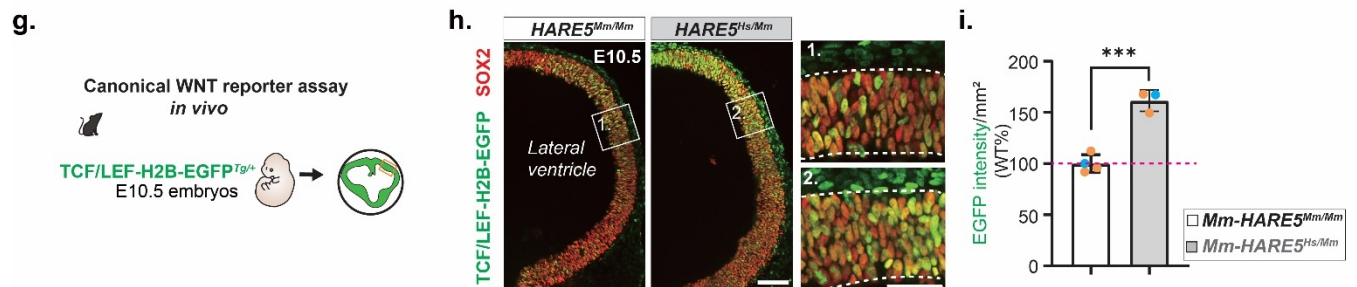


- 3) **Fig.6d-f:** We measured canonical WNT signaling at early mouse stages when RGCs are primarily self-renewing. For this, we dissociated E10.5 mouse controls, *Mm-HARE5*^{Mm/Hs} and *Mm-HARE5*^{Mm/Hs} cortices and performed AMAXA electroporation on the primary NPCs with WNT-reporter and pCAG-mcherry. Analysis was then performed at E11.5, using SOX2 staining to quantify only RGCs. These results indicate that *Hs-HARE5* significantly increases canonical WNT signaling in early stage mouse RGCs.

FIGURE REDACTED



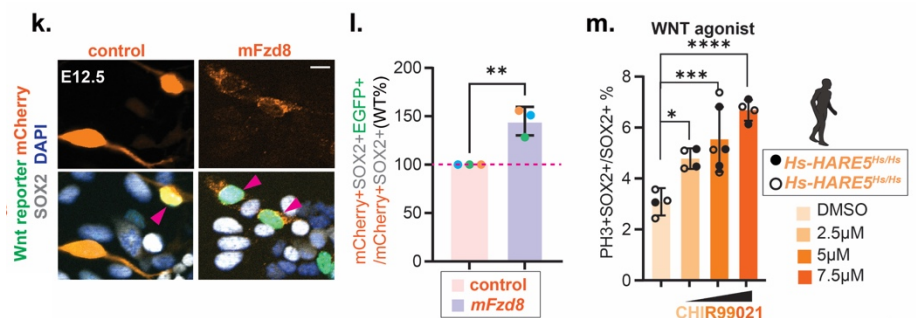
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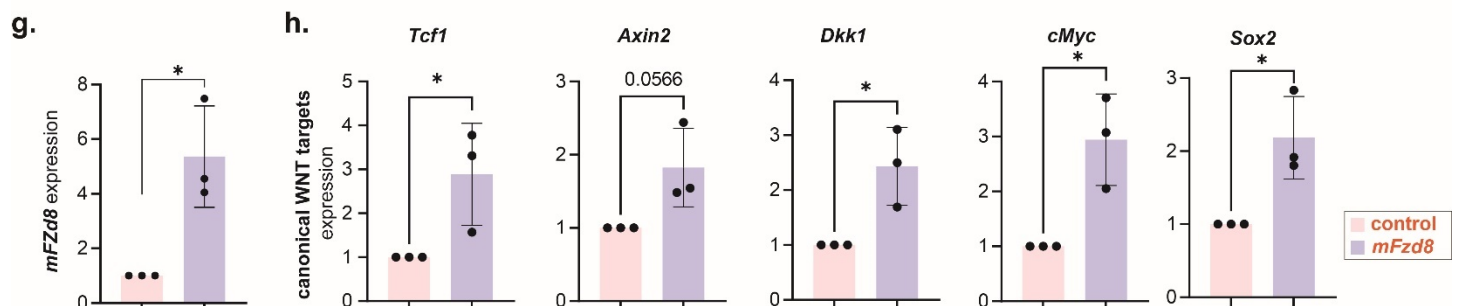
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Fig.6

FIGURE REDACTED

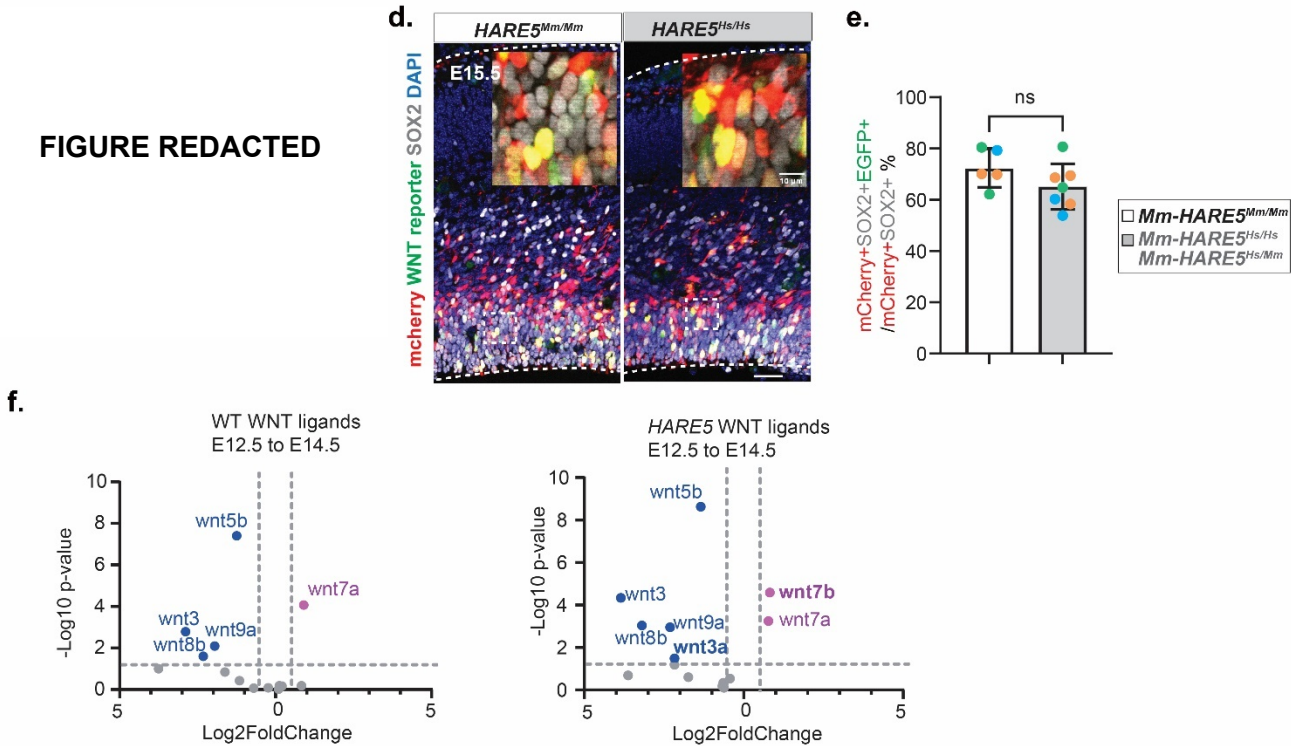


Extended Data Fig. 11

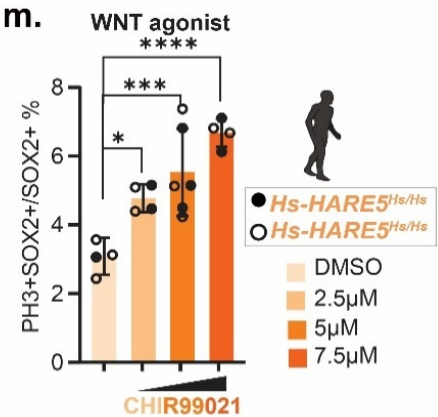


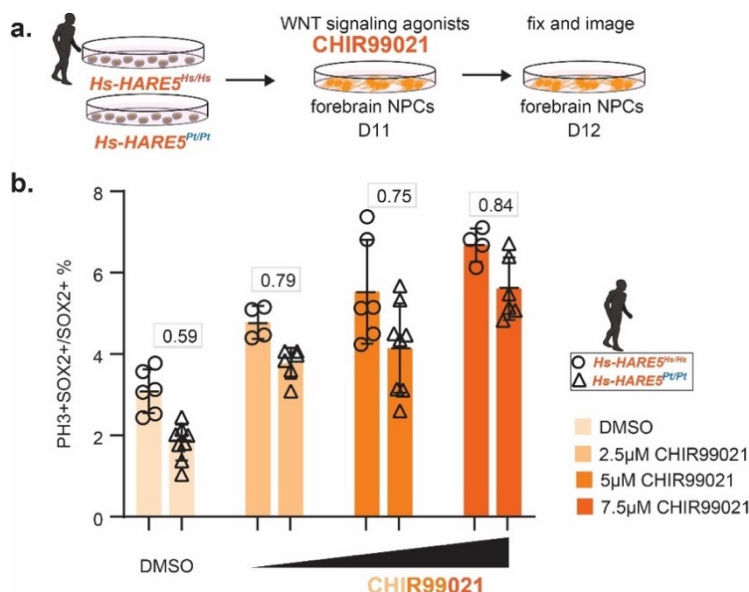
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neural progeny, and not only RGCs. To measure canonical signaling only in RGCs, we re-quantified the images focusing on WNT signaling only in SOX2+ RGCs (**Extended data Fig. 11c-e**). We detected no change in canonical WNT signaling at E15.5. Canonical WNT signaling is strongly associated with self-renewing divisions but not differentiation^{3,7-13}, and thus our data at E12.5 (above) and E15.5 are consistent with this. We posit that at later stages, the cortex contains more neurogenic cues (WNT ligands) which favor progenitors to differentiate. Consistent with this we observed a shift in WNT ligands between stages, including downregulation of ligands associated with canonical WNT signaling (**Extended data Fig. 11f**). It is also possible that *HARE5* could influence differentiation via non-canonical WNT signaling¹⁴, which is not tested with this reporter. We have added these points to the manuscript and modified our model as well.



7) We assessed if increased WNT activation influences proliferation of early-stage wild-type human NPCs. NPCs were treated with a WNT agonist in a dose dependent fashion. This caused NPCs to increase their proliferation (**Fig.6m**), consistent with the conclusion that canonical WNT signaling activation promotes progenitor proliferation⁷⁻⁹. We also attempted to use this agonist to rescue mitosis defect phenotypes of the *Hs-HARE5*^{Pt/Pt} hNPCs. The agonist also increased mitosis of the *Hs-HARE5*^{Pt/Pt} hNPCs. However, given the baseline impact upon control hNPCs this is difficult to interpret as a rescue. We include these data for the reviewer but not in the manuscript (see **Rev Fig. 2**).





Rev Fig. 2 Overview of experiment for rescuing the mitotic defect of *Hs-HARE5*^{Pt/Pt} hNPCs with canonical WNT signal agonist-CHIR99021. **b**, Quantification of mitotic D12 control and *Hs-HARE5*^{Pt/Pt} human NPCs. n=2-3 cell lines of each condition; 2-3 individual differentiations; each dot represents the average value of the cell line from each differentiation. Value above the graph bars represent the fold change of mutant/controls.

25- “Our data using mouse models indicate that Hs-HARE5 activates RGC expansion and neuron production.”
- How HARE5 increases RGC expansion if it decreases canonical Wnt-bCat signaling, consistent with neuron differentiation?

The temporal dynamics of HARE5 must be presented much more clearly, or else it is very difficult to follow the multiple functions that the authors attribute to it.

With additional experiments suggested by this and other reviewers we have refined our understanding of the temporal dynamics of *HARE5* and WNT signaling. Importantly we have modified our text to reflect this as well as the summary model in the manuscript (**Fig. 6n**), as described in response 24.

26- The results related to ASD are very nice, but it is totally unclear what is the genetic relationship between ASD and HARE5 driving gene expression. For example, is ASD also associated with decreased expression of genes under HARE5 regulation? I don't see this clearly explained, and its relevance is very confusing.

We agree that the relevance could have been clearer. We included these data to support the notion in the field that with evolutionary modifications also can come increased propensity for neurological disease¹⁵. In line with this, 5 ASD variants are enriched in *Hs-HARE5*. We include a small table to show what is known about these variants (**Extended data Fig. 9a,b**). Our results align with high throughput screens in which another ASD variant in *HARE5* was shown to affect enhancer activity¹⁶. To further understand how ASD variants influence *HARE5* function, we would need to perform CRISPR on *Hs-HARE5* by replacing them with the ASD mutations, followed by in depth phenotyping. This work will be valuable for future studies.

a. ASD mutations in human *HARE5* sequence

Coordinate(hg38)	Alternative	Patients numbers	Clinical phenotype	Affect <i>HARE5</i> enhancer activity?
chr10:35949522	T> -	44 homo+100 het	Both affected and unaffected	unknown
chr10:35949547	T> C	1 het	Affected	yes(This study)
chr10:35949716	-> T	10 homo+12 het	Both affected and unaffected	no (Doan et al,2016)
chr10:35949743	G> -	2 homo+10 het	Both affected and unaffected	yes(Doan et al,2016)
chr10:35949985	T> G	1 het	Both affected and unaffected	unknown

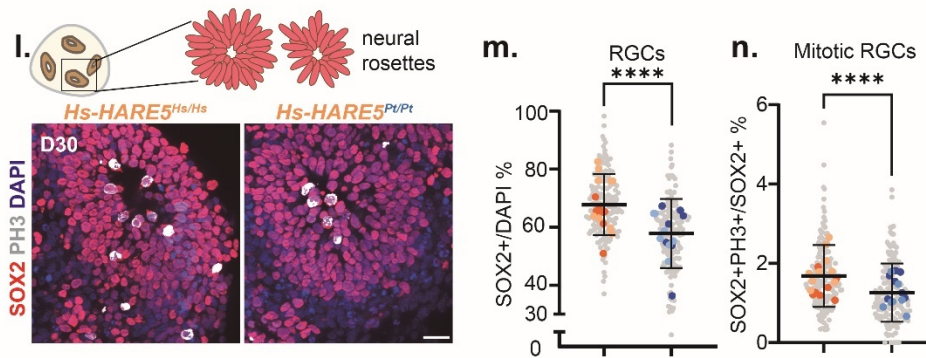
b. TCTCAACTGTTTGATTCAATGATTGTTAATAAGCAGTAACCCACACCAACCAATGCAGTCATTGTGCTTCTGAAAAATGTAATGTCACCTTTTGGTCTTTAAAAATTATTAAGCAGAGAGAAAGATAGCAA
TTTCCAGTGTTTGCTCGGATGGTAAGATGCAGGAGAATTGCACTTACTCCTGAGAGCTGCCATTG
GTTAGCTGGAGGGAAAAAGGATATCAATTATACATAATCTAAGAAGCCTCTGCATAATAATCAGG
CCCAGTAGGGGCTTTGGGGCAGCTTCGTTATTCCTTGTAACACAAGAGCCAGATAGACATGT
CAGTCAGGAGTAGCGGCAGCAATTCCTCTTTTAACACCTTCACATTCTATTGCTTCAATATGCA
AACAAGGCTGTTTAGCTGAGGGTAGAGCGGTGCTAATGAGCTCCCTGCACTAACCCCTGAAGTAA
AAAGTTCTGTTTCACAGCTGCTCAGAGACAGCTCCTCTGGGCATGCTCAGGTATCGGAAAAATAA
TGCCCTTTAAACAGAGAAATGCCAATCAGGATTTTGTGCATATTTCCCTTTTCTATGTGCTAAT
GCAATTACCTCACAACTGTGAACCTCCA

27- “This finding demonstrates that Hs-HARE5 is essential for promoting proliferation of NPCs, consistent with observations in mice.” - Maybe the authors could also test the late pro-neurogenic effect of HARE5 in this system. It is not essential to the story, but it would nicely clarify whether this effect reported here is specific or rather a general effect of HARE5 no matter what.

We thank the reviewer for this interesting suggestion. Indeed, we have an ongoing project to look at the later neurogenic effect of *Hs-HARE5* in human organoids. While we agree this is super interesting this will require a much longer timeline to assess older cortical organoids in a comprehensive fashion.

28- “just 4 chimpanzee specific variants within human cells reduces proliferative capacity of progenitors.” - This is not accurate, as SOX2 stains do not reveal proliferation, but only cortical progenitor cell identity. Pt/Pt cells may proliferate more but produce neurons, hence reducing the overall % of cells that are Sox2+. Proliferative activity should be revealed by PH3 frequency and/or cell cycle re-entry rate, which these authors actually do in the following experiments.

As suggested, we quantified mitotic RGCs by PH3 and SOX2 staining of D30 organoids. We quantified a significant reduction in the fraction of mitotic RGCs in D30 *HARE5*^{Pt/Pt} cortical organoids (**Fig. 5n**). This is consistent with the 2D analysis in which *Pt-HARE5* led to a proliferative defect in human NPCs.



29- “These findings are consistent with studies showing that during in vitro neural differentiation of human NPCs and in vivo mouse cortical neurogenesis, canonical WNT signaling decreases.” - If HARE5 increases Fzd8 expression, shouldn't this increase Wt signaling?

We thank the reviewer for the question. As discussed above, we have further clarified our model with extensive new experiments. Please refer to our response 24.

Referee #2 (Remarks to the Author):

Liu et al., perform functional characterization of HARE5, a conserved non-coding sequence with an accelerated nucleotide substitution rate in humans. The non-coding element had previously been shown to interact with the Fzd8 promoter and drive reporter gene expression during cortical development. Compared to the chimpanzee element, the human sequence when driving to Fzd8 expression more strongly increased proliferation. However, previous studies had not directly evaluated the function of the human sequence at the endogenous locus. In the present study, Liu et al., demonstrate that HARE5 regulates FZD8 expression in human, chimpanzee, and mouse cells and can increase cortex size (~6%) and neuron number (12.5-21.9%) by increasing the radial glia progenitor pool initially and then by increasing intermediate progenitor producing divisions and neurogenesis later in development, with the effects likely driven by 4 nucleotide substitution that each act to increase enhancer activity.

The study represents an important step for modeling human-specific changes affecting brain development. Previous studies have modulated the expression of candidate genes by ectopic overexpression, but direct engineering of the mutations at the endogenous locus to identify a functional consequence has been lacking. The authors make excellent use of orthogonal models to test the effects of gain and loss of function. Orthogonal evidence includes quantitative differences in cortical area and neuron number and adult neural calcium dynamics in adult mice, analysis of underlying developmental mechanisms by histology, scRNA and bulk RNA-seq and timelapse imaging, and analysis of genetic changes by luciferase assays and by measuring developmental cell behavior in engineered human and chimpanzee organoids. I applaud the authors for combining so many techniques to evaluate the consequences of HARE5.

We thank this reviewer for their enthusiasm and excellent summary, as well as for their insightful and helpful comments which have improved our paper.

My remaining comments that I hope will be constructive are listed below:

Major comments

I want to make sure the proposed developmental mechanism is clearly articulated and that there are not any missing experiments.

Specifically, why would increased Fzd8 expression switch from promoting proliferation at E12.5 (consistent with previous HARE5 Fzd8 study and other studies of Wnt activation) to promoting more cell cycle exit and neuronal differentiation at E14.5? The argument seems to be that HS-HARE5 causes more IP generating divisions and possibly that HS-HARE5 decreases canonical Wnt activation later in development.

This result of decreased Wnt activation at E14.5 was a surprise to me - the authors suggest that this reduction may reflect the increased neural differentiation driven by HS-HARE5, citing other studies showing decreased Wnt signaling during differentiation. Can the authors clarify the argument:

-Is the suggestion that increased Fzd8 expression directly reduces Wnt signaling to drive differentiation (which I did not expect) as mentioned in the discussion (“...Hs-HARE5 inhibits canonical signaling at E14.5 correlates with the developmental impact of this enhancer from promoting RGC self-renewal to differentiation.”)

-Or is the suggestion that increased Fzd8 expression first increases canonical Wnt signaling at E12.5 (which could/should be tested? and would be consistent with the effect of Wnt on radial glia), which first increases RGC proliferation and then increases IP-producing divisions and neural differentiation later, with reduced Wnt signaling as a secondary/indirect consequence?

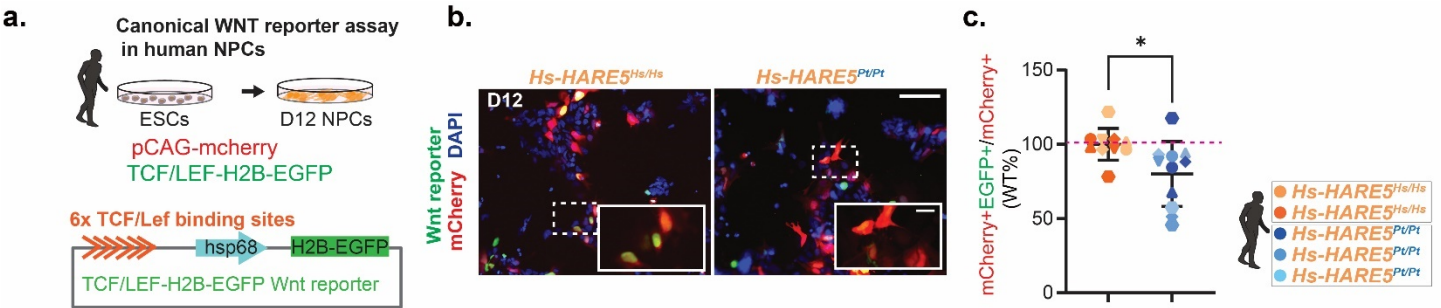
We thank the reviewer for this question which was also raised by other reviewers. We have pursued extensive new experiments to understand how *HARE5* influences WNT signaling during neurogenesis. A completely **new Fig. 6, and Extended data Fig. 11** with a revised model clarifying how *Hs-HARE5* influences WNT signaling.

Our new data support the second possibility noted by the reviewer and further clarify WNT signaling at both early and late stages.

- 1) **Fig. 6n:** We include a revised model supported by new data summarized below. *Hs-HARE5* increases *FZD8* expression, which triggers higher canonical WNT signaling in early-stage radial glia. Data from our lab and others show that higher canonical WNT signaling promotes radial glia proliferation and self-renewal. At later stages, *Hs-HARE5* does not impact canonical WNT signaling in radial glia. Accordingly, at these stages, *Hs-HARE5* increases neuron number via direct neurogenesis and production of neurogenic IPs. We posit that at later stages, the cortex contains more neurogenic cues which favor progenitors to differentiate. This may include WNT ligands which contribute to shifts in WNT activity.

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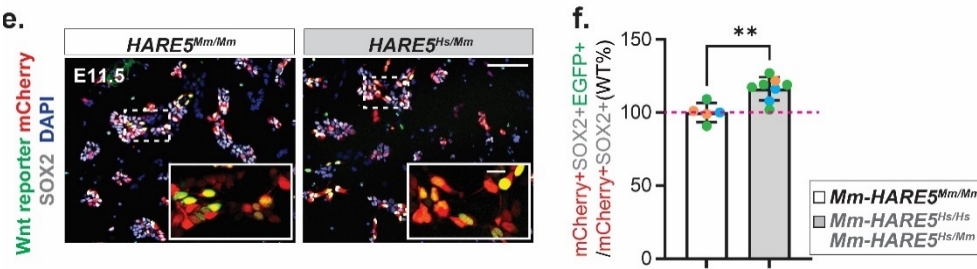
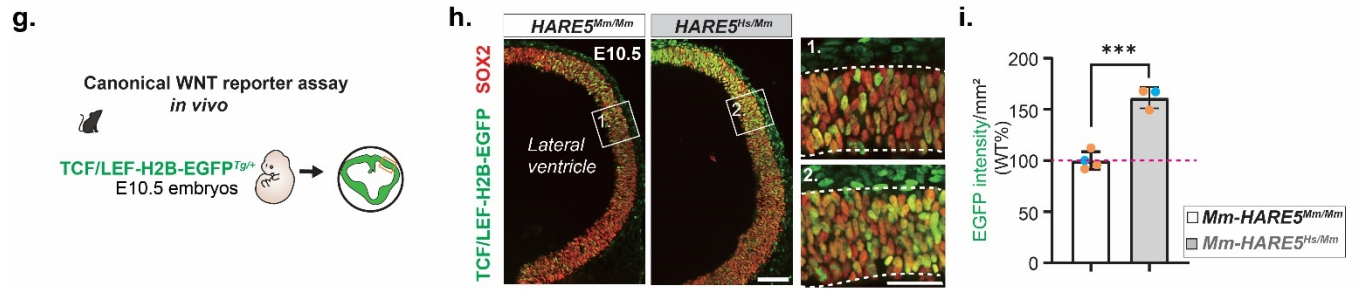


FIGURE REDACTED

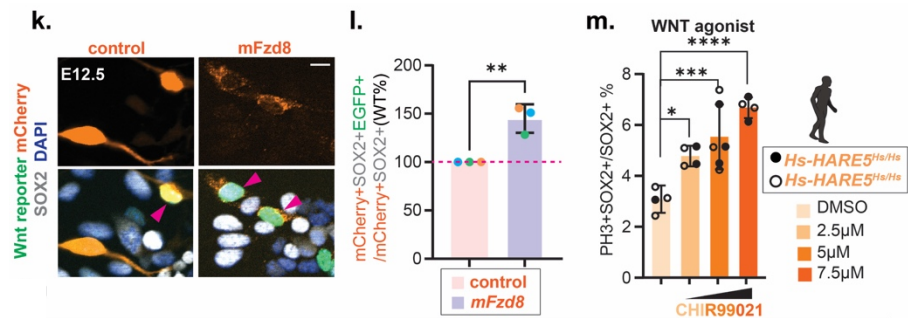
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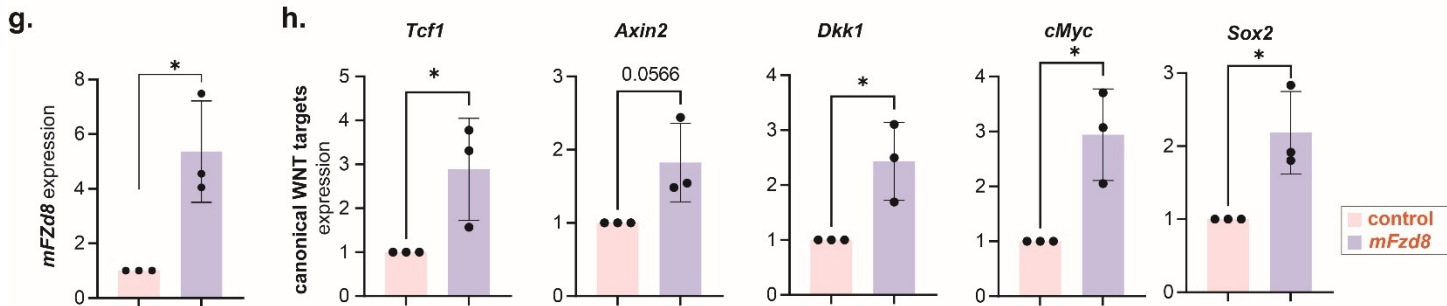
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Fig. 6

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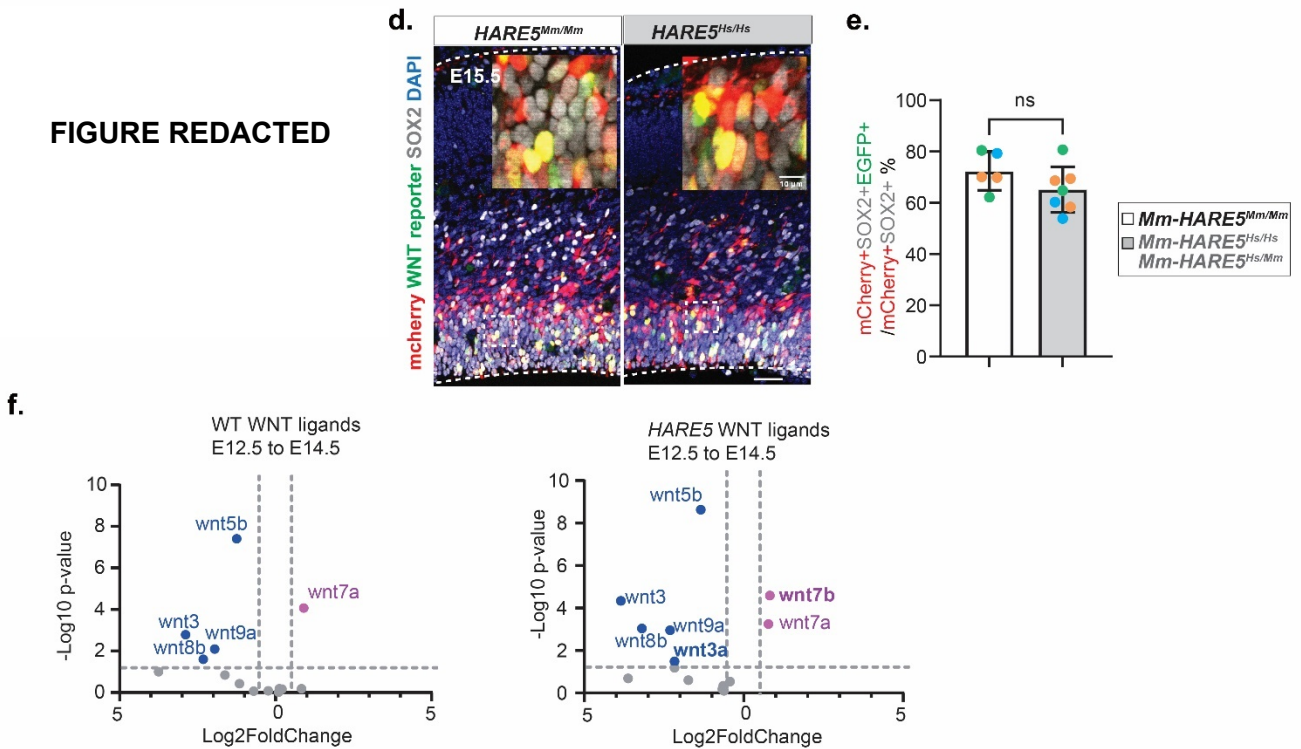


Extended Data Fig. 11

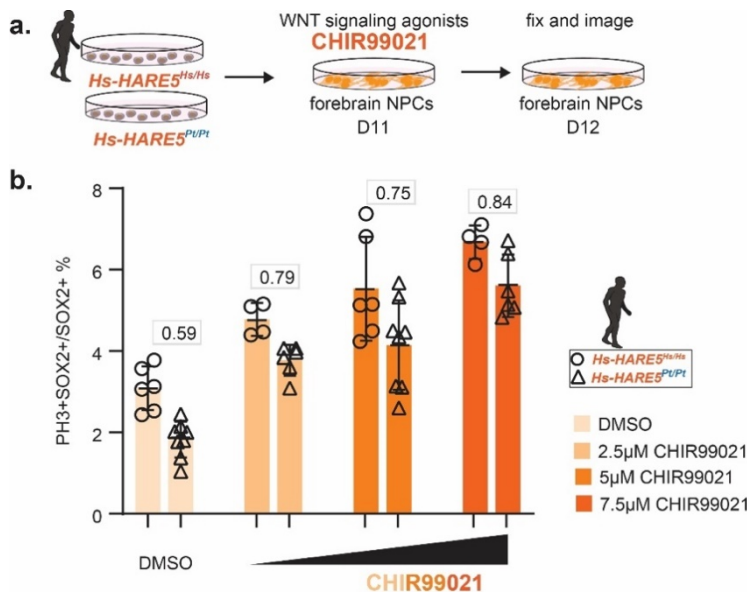
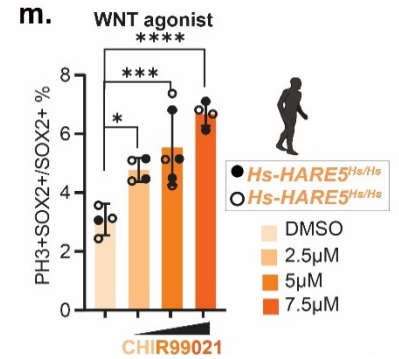


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5) We assessed if increased WNT activation influences proliferation of early-stage wild-type human NPCs. NPCs were treated with a WNT agonist in a dose dependent fashion. This caused NPCs to increase their proliferation (**Fig.6m**), consistent with the conclusion that canonical WNT signaling activation promotes progenitor proliferation⁷⁻⁹. We also attempted to use this agonist to rescue mitosis defect phenotypes of the *Hs-HARE5^{Pt/Pt}* hNPCs. The agonist also increased mitosis of the *Hs-HARE5^{Pt/Pt}* hNPCs. However, given the baseline impact upon control hNPCs this is difficult to interpret as a rescue. We include these data for the reviewer but not in the manuscript (see **Rev Fig. 2**).



Rev Fig. 2. Overview of experiment for rescuing the mitotic defect of *Hs-HARE5^{Pt/Pt}* hNPCs with canonical WNT signal agonist-CHIR99021. **b.** Quantification of mitotic D12 control and *Hs-HARE5^{Pt/Pt}* human NPCs. n=2-3 cell lines of each condition; 2-3 individual differentiations; each dot represents the average value of the cell line from each differentiation. Value above the graph bars represent the fold change of mutant/controls.

Minor comments

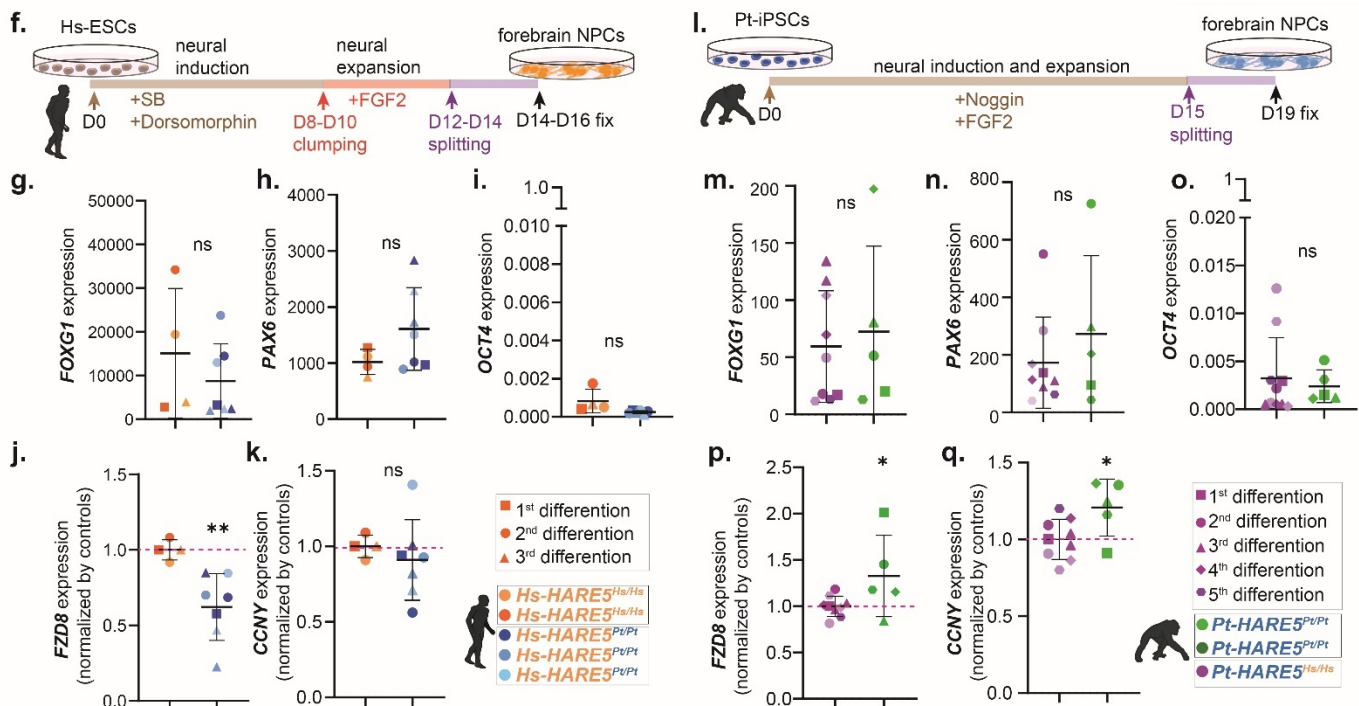
-Although the chimpanzee allele was not also engineered into the mouse models for direct in vivo comparisons, the figure 5 data sufficiently address this issue by indicating that mouse and chimp alleles drive similar levels of reporter gene expression and also by examining the chimp and human alleles in the human and chimp organoid context. In addition, the mouse CKO data also support the claims.

We thank the reviewer for their summary and appreciating our approaches.

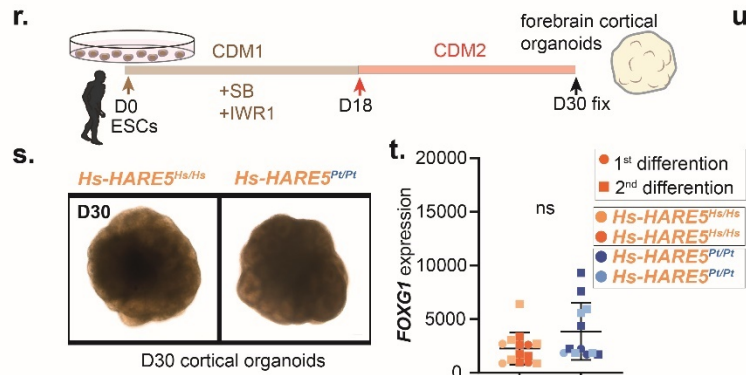
-What was the *FOXG1* threshold for excluding organoids - the text states “samples were excluded based on the extremely not comparable *FOXG1* level from each differentiation,” and Extended Data Fig 8 shows some variation by RT-qPCR. The authors could examine whether variation in patterning efficiency and *FOXG1*/*PAX6* levels correlates with some measured phenotypes like *FZD8* expression (or explicitly treat patterning as a covariate).

We appreciate the reviewer's question. We only included organoids in which *FOXG1* levels were 1000-fold higher than day 0 ESCs (almost all organoids fit this threshold). We clarified this in the methods of the manuscript.

We agree that it is valuable to show that the phenotype is not related to differentiation status. To further clarify how differentiation status correlates with gene expression, in our 2D and 3D data, we have shape-coded each NPC differentiation to the RT-PCR of *FOXG1*, *PAX6* and *FZD8*. Importantly, in 2D, we did not observe any significant difference in patterning efficiency across cell lines/genotypes, detected by levels of *FOXG1*, *PAX6* and *OCT4* (we have added “ns” to these graphs) (**Extended data Fig. 10g-q**). The chimpanzee differentiation was based on a different protocol which has comparable, albeit lower *FOXG1* and *PAX6* expression across genotypes^{17,18}. In 3D, we added in analysis of another differentiation, and show *FOXG1* is not significantly different between genotypes (**Extended data Fig. 10r-t**). Together these data indicate that *FZD8* levels are different between genotypes and this phenotype was not due to variable differentiation.

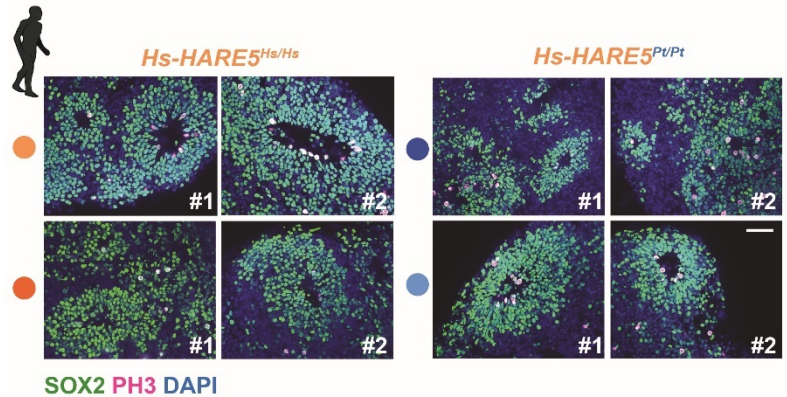


Extended Fig. 10



Also, how is the “neural rosette area” defined for quantification differences in RGC number?

We thank the reviewer for this question. We didn’t analyze by rosette, as rosettes had variably intact borders. We used 40x objective to image fields which include the rosettes based on the SOX2+ clusters, and then quantified the entire field. At D30, the control lines have an average of 4-5 rosettes, while the *Hs-HARE5^{Hs/Hs}* lines have an average of 3-4 from each section. We now include representative images (**Extended data Fig. 10u**) and clarify this in the methods.



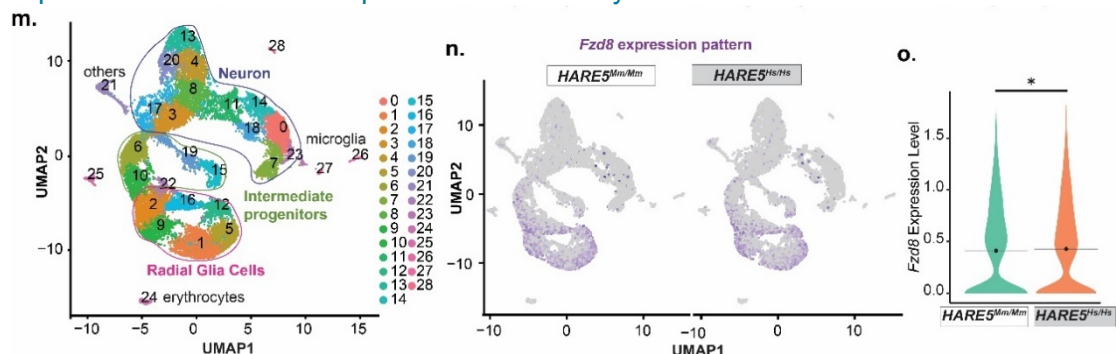
Gene expression analysis:

It is difficult to evaluate the statistical significance of interpretations from the scRNA-seq data, including the elevated *Fzd8* level in Fig. 3r and Extended data 5b and the increased G2M fraction. Perhaps these trends are consistent with the more fine scale quantifications presented earlier? Also, why does the effect on mitotic RGC look so much stronger in 3f vs 3s?

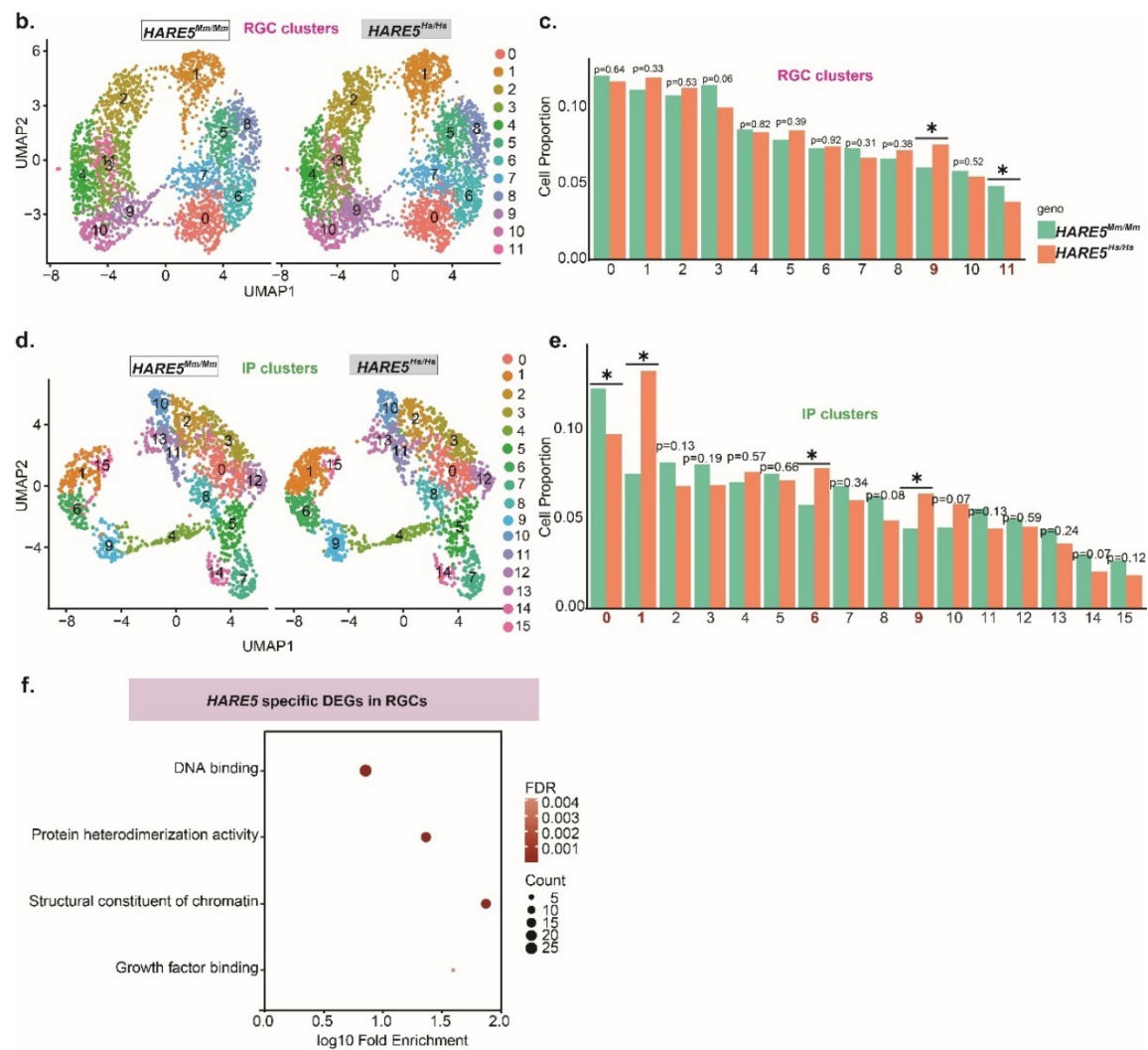
We thank the reviewer for their comments on the scRNA analysis. In the new manuscript, in response to these and other reviewer concerns, we reassessed these data. 1) We filtered out all the dead/low quality cells and redid the UMAP; 2) we updated the *Fzd8* expression with statistics to show it is enriched in *Hs-HARE5* RGCs and this spatial pattern is maintained in *Mm-HARE5^{Hs/Hs}* brains; 3) we graphed the cell composition of RGCs and IPs with statistics; and 4) we include new tables showing differentially expressed transcripts and their gene ontology enrichment. These data are included in **Fig. 3m-o**; **Extended Data Fig. 6**; **Supplementary Table 1**.

We also performed new cell cycle analysis using a different Seurat tool: https://satijalab.org/seurat/articles/cell_cycle_vignette. These data indicated a slight trend towards more G2M cells for both RGCs and IPs, however these were not statistically significant. While the quantitative data in situ carefully measures cell cycle states, the sc-seq is based upon transcript expression of some genes. Because of this and the lack of significance, we have removed the cell cycle predictions of scRNA seq data from the study.

Fig. 3

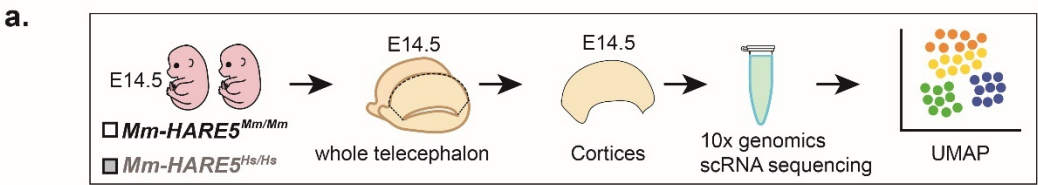


Extended Fig. 6



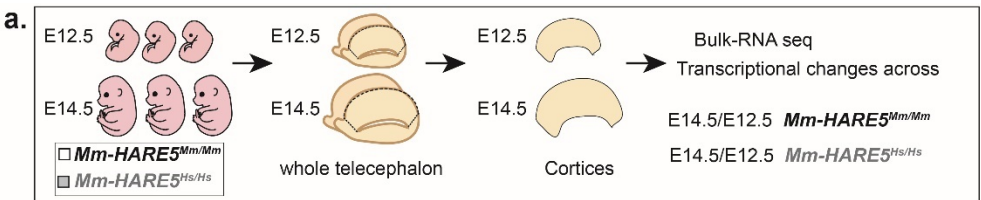
-Extended data Fig 5 could also clarify the 2 control vs 2 HARE5-HS/HS design.

We have modified the cartoon to clarify the experimental design (Extended Data Fig. 6a).



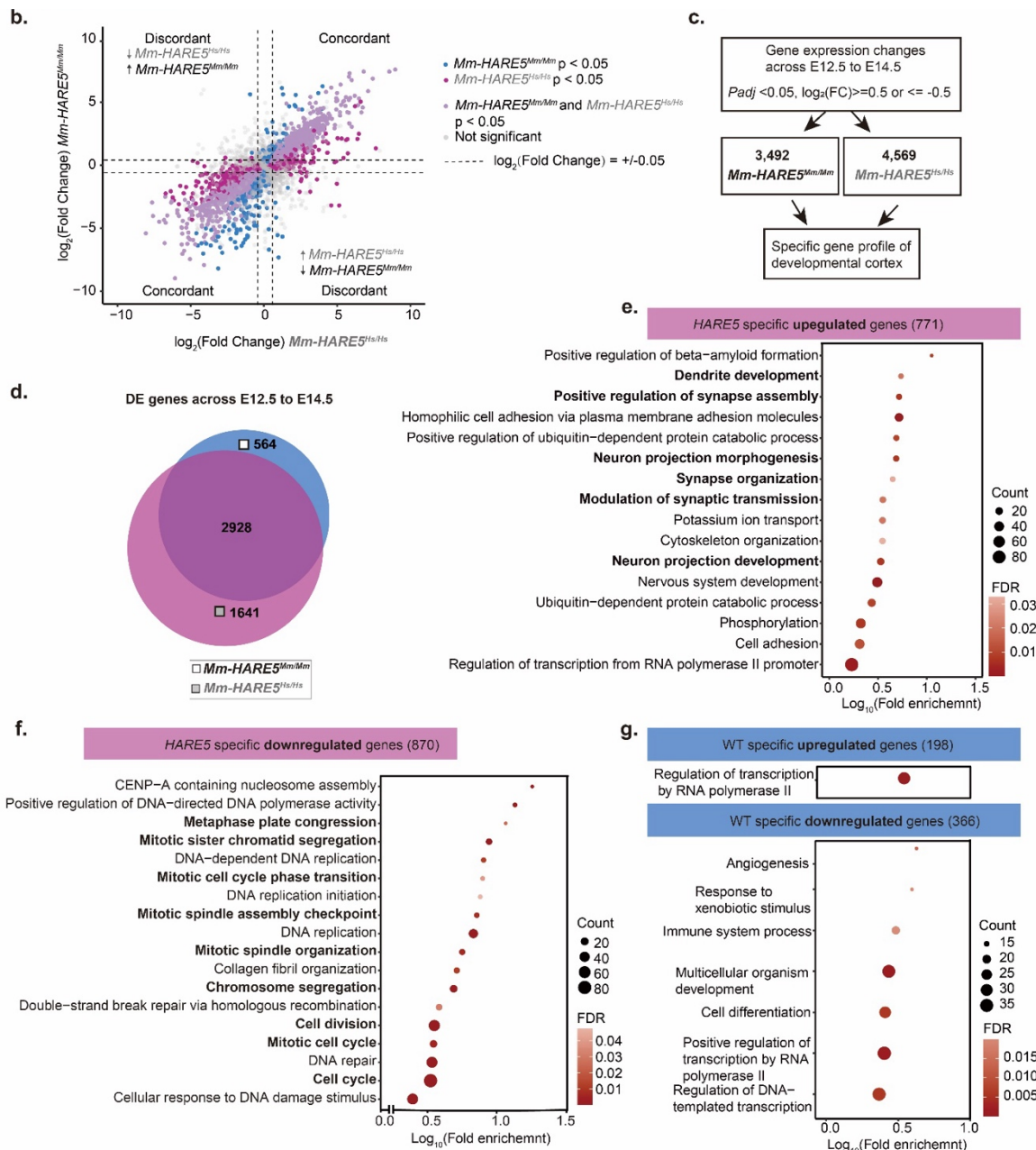
-For the bulk RNA seq, were there 3 biological replicates for each genotype at each developmental stage - the supplemental figure could better explain the design. Similarly, the supplemental figure could better illustrate the design to compare E14.5 and E12.5 within genotypes (rather than compare gene expression between genotypes at each timepoint).

There were 3 biological replicates for each genotype. We modified the cartoon to clarify the experimental design (Extended Data Fig. 8a).



-Some enrichments are discussed related to genes that may have specific temporal changes only in HARE5-HS/HS but not control lines. But it could be that these genes have similar temporal dynamics in both genotypes and just reached the significance threshold in one, which the authors could check for or illustrate with additional plots.

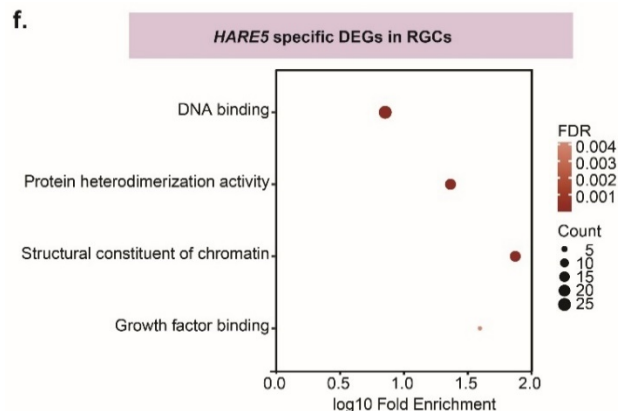
We thank the reviewer for this suggestion. We examined the 564 significantly altered control (*Mm-HARE5*^{Mm/Mm})-specific transcripts using GO analysis. There was only 1 significant category of up-regulated genes in WT, related to regulation of transcription by RNA polymerase II (FDR=3.8X10⁻⁶). For down-regulated genes in WT, we observed distinct categories than those for *Mm-HARE5*^{Hs/Hs} (**Extended data Fig. 8g; Supplementary Table 2**). Moreover, following a suggestion from reviewer 2, we compared the log2Fold Change values for all transcripts differentially expressed between E12.5 to E14.5 cortices for both *Mm-HARE5*^{Mm/Mm} and *Mm-HARE5*^{Hs/Hs} (**Extended data Fig. 8b**). Most of these transcripts show concordant trends, either increasing or decreasing in both genotypes. Indicating that most genes have similar temporal dynamics across genotypes but show a fine-tuning of expression levels. A subset of genes are discordant, showing opposing changes in gene expression for the evaluated genotypes. These data suggest that *Hs-HARE5* has mild qualitative and quantitative impacts on gene expression, aligning with its role as an enhancer.



Extended Data Fig.8

-Did the authors analyze differential expression between HARE5-HS/HS and control lines with the bulk or the scRNA-seq data and were there any insights into radial glia genes with altered regulation?

Because *Hs-HARE5* is active in a subpopulation of cortical cells, mainly RGCs, the bulk RNA seq did not have sufficient resolution to detect expression changes. As noted above, we observed some differences in gene expression developmentally. To gain insight into cell specificity with scRNA-seq, we include transcript tables and GO analyses of RGC clusters (Extended Data Fig.6f, Supplementary Table 1). DEGs were identified in both IPs and RGCs. In RGCs these included DNA binding factors. Functional studies will be valuable in the future to follow up on these downstream targets.



Other comments

- the authors could add a reference for the definition of ANC516 (PMID 17578567?)
- Page 5 - "profound" is a strong word for a 5-10% reduction

We agree with the reviewer's suggestion and have modified both points in the revised manuscript.

Figure 1:

- Legend a, ends with ? instead of .
- Add y-axis on Figure 1C

We have fixed this typo in the Figure 1 legend. We are not sure how to add a Y-axis to Figure 1c. These TAD representations show chromosomal contacts between *Hs-HARE5* and target genes and do not typically include a y axis.

Figure 2:

- Clarify in 2b,e,f if colors correspond to litter

These are littermates and we have clarified this in the figure legend (and in other figure legends).

- The trend for HS alleles increasing cortical area looks more subtle at P0 than the significant result in adult in Extended Data Fig 2b. Are there marginal p-values associated with this result? Also, should the dots be colored by litter?

We have added 2 more *Mm-HARE5*^{KO} mice to the P0 graph, which showed a significant reduction by 1 way ANOVA (**Extended data Fig 2d**). We also re-analyzed cortical area differences between P0 control and *Mm-HARE5*^{Hs/Hs} using an unpaired t-test. The p-value is 0.0934 with a trend to increase but not significant (**Extended data Fig 2e**). We didn't color-code this graph because all the mutant brains are already normalized to their WT littermates. We discuss these data in the manuscript.

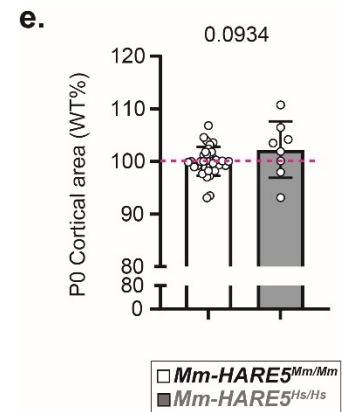


Figure 3:

- 3g legend (or panel) should state BrdU treatment timing

We apologize for this omission and have added a new carton in the new manuscript (as well as for other BrdU related panels).

Figure 5

It is an interesting finding that all 4 human-specific mutations individually act with the same direction of effect (that might be consistent with independent directional selection on all 4 events) and could be noted

We thank the reviewer for this suggestion, and we added this interesting point to the results.

Referee #3 (Remarks to the Author):

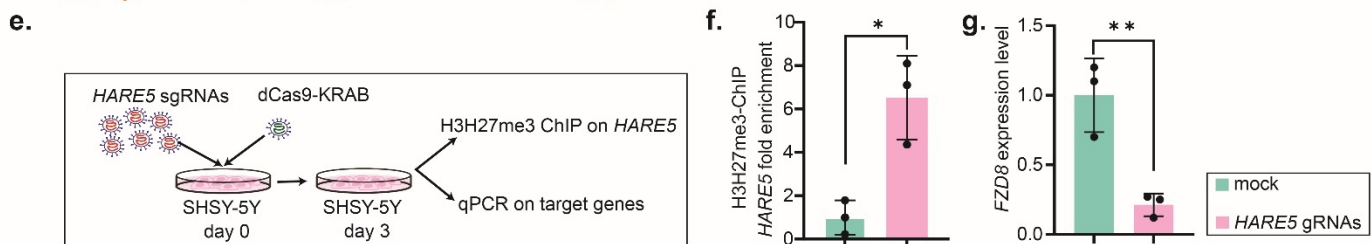
This manuscript by Liu and colleagues uses a mouse model with a human accelerated region (HAR) knock-in and modified human and nonhuman primate cell models to study the function of a specific HAR. The authors previously showed that this HAR, HARE5, can alter *Fzd8* expression and *Fzd8* can alter development of the neocortex. Here, the authors find more specifics about the role of this HAR showing that Hs-HARE5 can affect cortical development by modifying progenitor proliferation and neuronal differentiation. Furthermore, the authors pinpoint specific human variants that underlie enhancer function to drive progenitor proliferation.

In general, the results were mostly clear and the conclusions were supported by the data. The inclusion of the cKO mouse was a nice contrast to the knock-in mouse for many comparisons. The inclusion of the chimpanzee cell lines and the chimp variants were also improved comparisons over simply using a mouse context and was much appreciated.

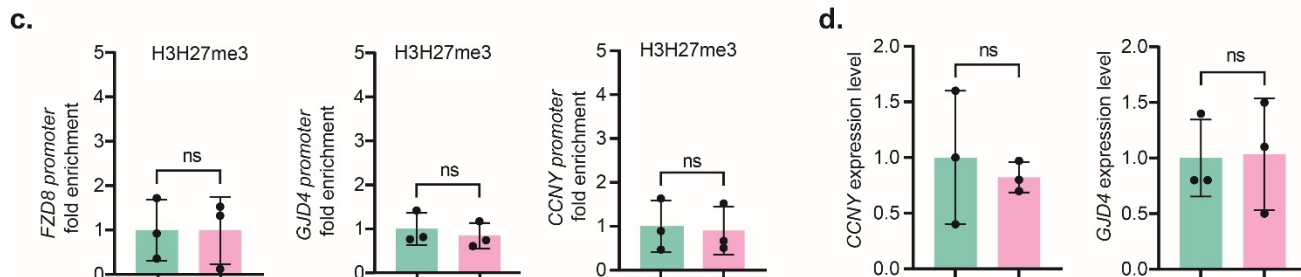
I think the major question I have remaining is whether altered WNT signaling is the main driver here and if all of these phenotypes could simply be recapitulated by manipulating WNT and *Fzd8* in the absence of modifying HARE5? I'm not asking the authors to repeat all of their findings to investigate this as some WNT signaling manipulation is already done in the manuscript. In addition, the authors already demonstrated that *Fzd8* overexpression can result in some of these phenotypes. So the remaining question really is whether the purpose of HARE5 is to only modify *Fzd8*, and if so, we have learned a lot about the mechanism of *Fzd8* function in cortical development but the purpose of the HARE5 was already solved, so to speak. Also, if this is the case, how does this modification of WNT-*Fzd8* signaling work in 2D culture? All of the necessary cell types for the signaling are in place? The organoid data using the chimp-modified human cells is compelling in this regard. Would manipulation of WNT signaling in these organoids "rescue" a human phenotype? Again, I am asking the authors to speculate.

We thank the reviewer for appreciating the work and our use of diverse approaches (mouse, human and chimpanzee knock-in) to draw our conclusions. We also appreciate the questions regarding WNT signaling, which we discuss below. Indeed we can draw important conclusions from our prior study of *FZD8* overexpression, in which we showed increased brain size and neuron number and reduced E12.5 cell cycle exit. Importantly, Hi-C data (**Extended data Fig. 1a**), our new epigenetic data (**Fig.1e-g; Extended data Fig.1c,d**), as well as expression analysis of knock-in mice (**Fig. 2b, Extended data Fig. 2a**) and cell lines (**Fig. 5i,q, Extended data Fig. 10k,q**) collectively support the notion that *FZD8* is the main target of *Hs-HARE5* in NPCs.

Fig. 1



Extended data Fig. 1

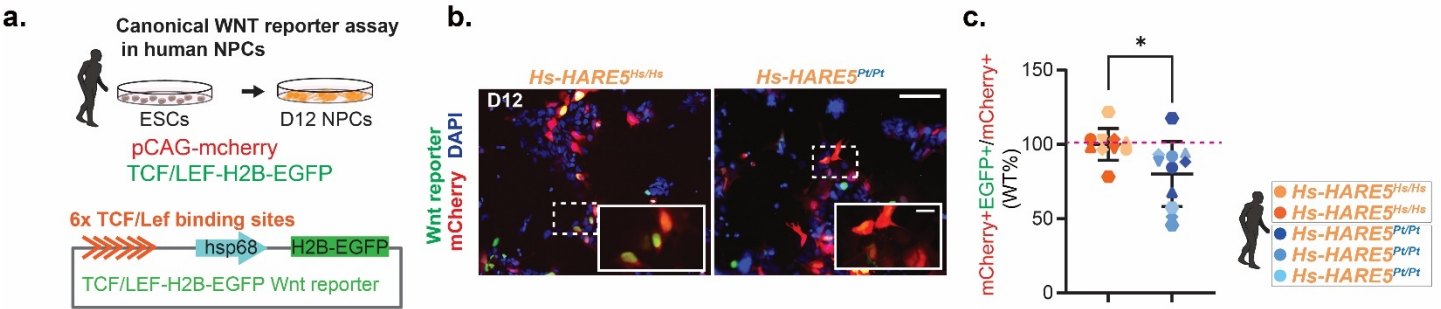


We have pursued extensive new experiments to understand how *HARE5* influences WNT signaling during neurogenesis. A completely new Fig. 6, and Extended data Fig. 11 with a revised model clarifying how *Hs-HARE5* influences WNT signaling.

- 1) **Fig. 6n:** We include a revised model supported by new data summarized below. *Hs-HARE5* increases *FZD8* expression, which triggers higher canonical WNT signaling in early-stage radial glia. Data from our lab and others show that higher canonical WNT signaling promotes radial glia proliferation and self-renewal. At later stages, *Hs-HARE5* does not impact canonical WNT signaling in radial glia. Accordingly, at these stages, *Hs-HARE5* increases neuron number via direct neurogenesis and production of neurogenic IPs. We posit that at later stages, the cortex contains more neurogenic cues which favor progenitors to differentiate. This may include WNT ligands which contribute to shifts in WNT activity.

FIGURE REDACTED

- 1) **Fig.6a-c:** We introduced a canonical WNT reporter into human knock-in NPCs. Compared to control NPCs, *Hs-HARE5^{Pt/Pt}* exhibited reduced canonical WNT signaling. This indicates that lower *HARE5* enhancer activity, mediated by *Pt-HARE5*, reduces canonical WNT signaling in human NPCs.



- 2) **Fig.6d-f:** We measured canonical WNT signaling at early mouse stages when RGCs are primarily self-renewing. For this, we dissociated E10.5 mouse controls, *Mm-HARE5^{Mm/Hs}* and *Mm-HARE5^{Mm/Hs}* cortices and performed AMAXA electroporation on the primary NPCs with WNT-reporter and pCAG-mcherry. Analysis was then performed at E11.5, using SOX2 staining to quantify only RGCs. These results indicate that *Hs-HARE5* significantly increases canonical WNT signaling in early stage mouse RGCs.

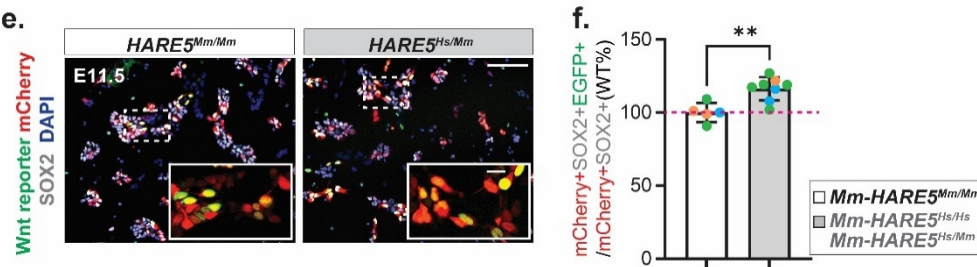
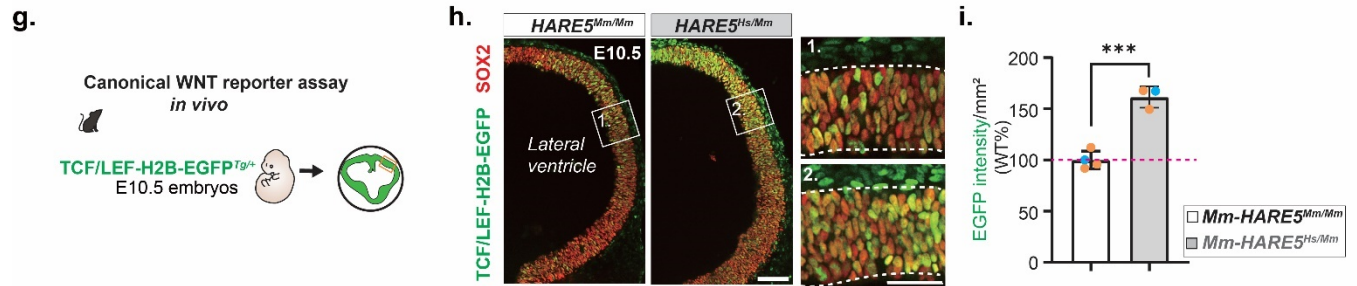
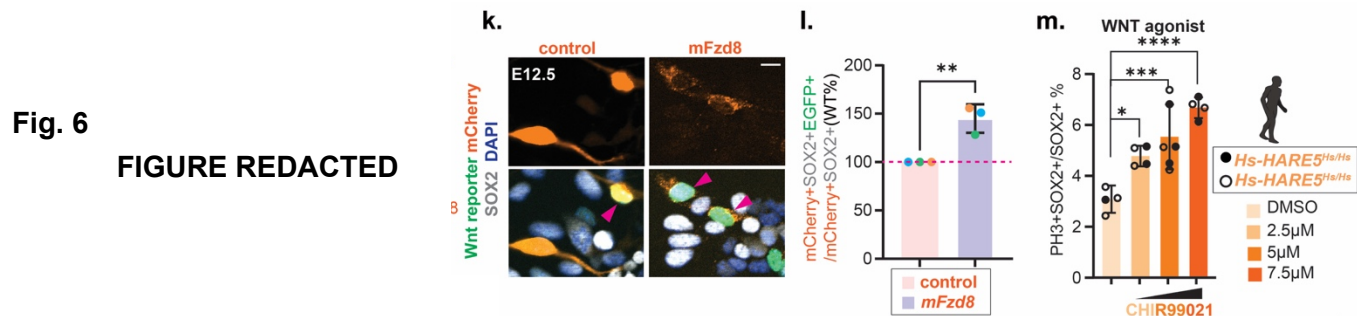


FIGURE REDACTED

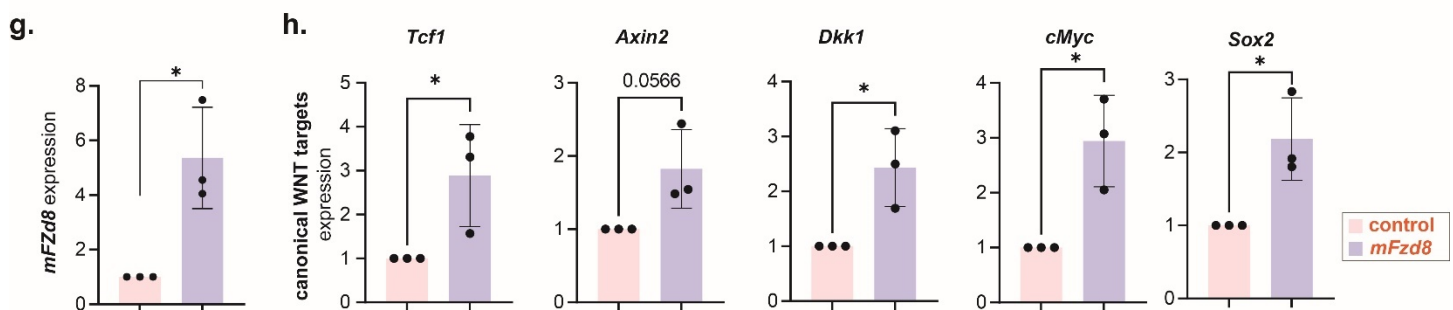
- 3) **Fig.6g-i:** We crossed *Mm-HARE5*^{Mm/Hs} mice with a canonical WNT reporter mouse line-TCF/LEF:H2B-EGFP^{Tg/+6}. Quantification of EGFP intensity in the E10.5 cortical germinal zone indicated a higher activity of canonical of WNT signaling in early-stage *Mm-HARE5*^{Mm/Hs} progenitors *in vivo*. This further supports findings *in vitro* in mouse cells.



- 4) **Fig.6j-l; Extended data Fig. 11g,h:** We assessed if increased *FZD8* expression affects canonical WNT signaling in E10.5 mouse wild type primary NPCs. We analyzed canonical signaling after 48 hours by WNT reporter, as well as qPCR on WNT signaling target genes. Both measurements revealed that increased *FZD8* expression promotes higher canonical WNT signaling in early stage mouse RGCs.

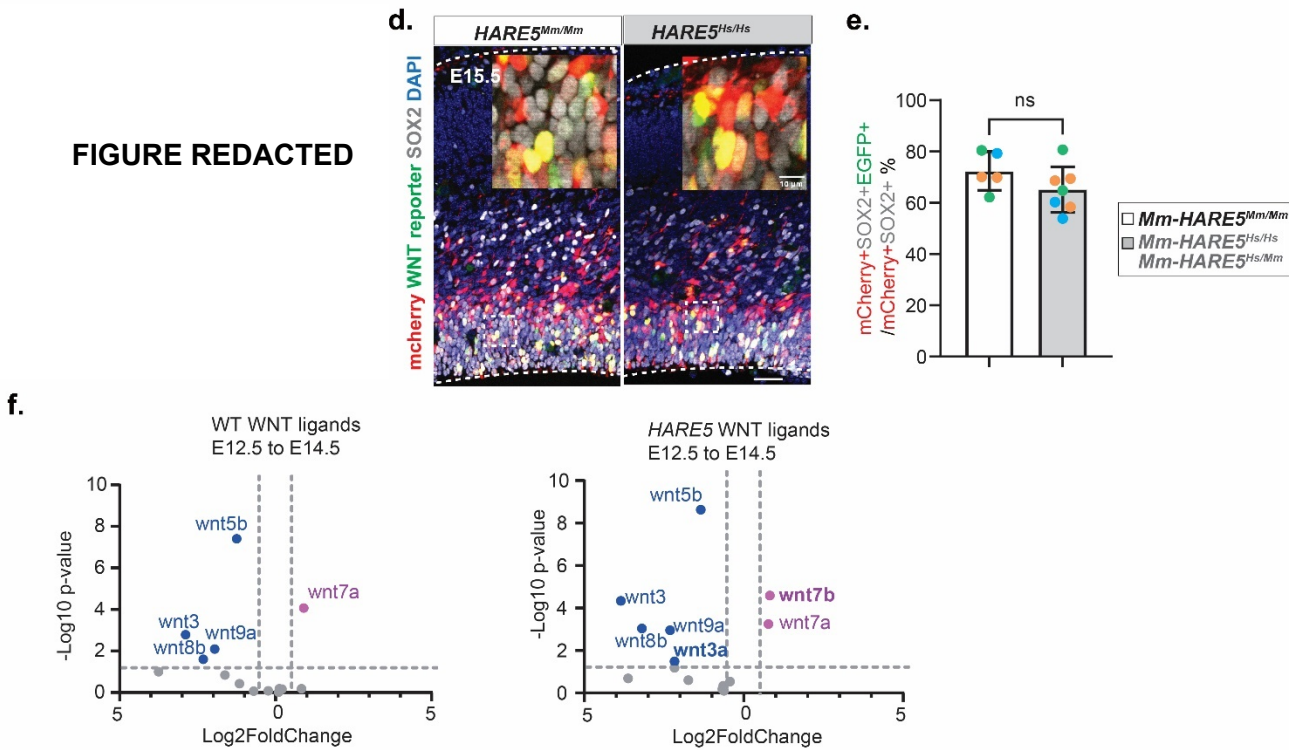


Extended Data Fig. 11

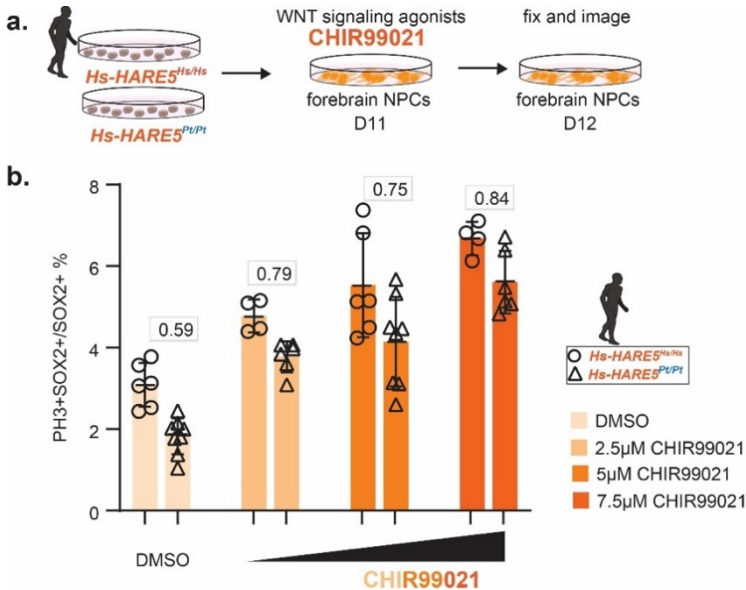
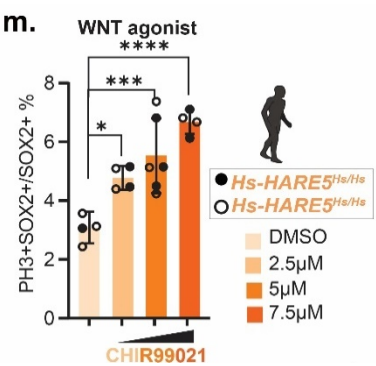


- 3) Our data in the initial submission showed that canonical WNT signaling is reduced in E15.5 brains. However, as we reflected on these data, we realized this quantification included all mCherry positive neural progeny, and not only RGCs. To measure canonical signaling only in RGCs, we re-quantified the images focusing on WNT signaling only in SOX2+ RGCs (**Extended data Fig. 11c-e**). We detected no change in canonical WNT signaling at E15.5. Canonical WNT signaling is strongly associated with self-renewing divisions but not differentiation^{3,7-13}, and thus our data at E12.5 (above) and E15.5 are consistent with this. We posit that at later stages, the cortex contains more neurogenic cues (WNT ligands) which favor progenitors to differentiate. Consistent with this we observed a shift in WNT ligands between stages, including downregulation of ligands associated with canonical WNT signaling (**Extended data Fig. 11f**). It is also possible that *HARE5* could influence differentiation via non-canonical

WNT signaling¹⁴, which is not tested with this reporter. We have added these points to the manuscript and modified our model as well.



5) We assessed if increased WNT activation influences proliferation of early-stage wild-type human NPCs. NPCs were treated with a WNT agonist in a dose dependent fashion. This caused NPCs to increase their proliferation (**Fig.6m**), consistent with the conclusion that canonical WNT signaling activation promotes progenitor proliferation⁷⁻⁹. We also attempted to use this agonist to rescue mitosis defect phenotypes of the *Hs-HARE5^{Pt/Pt}* hNPCs. The agonist also increased mitosis of the *Hs-HARE5^{Pt/Pt}* hNPCs. However, given the baseline impact upon control hNPCs this is difficult to interpret as a rescue. We include these data for the reviewer but not in the manuscript (see **Rev Figure 2**).

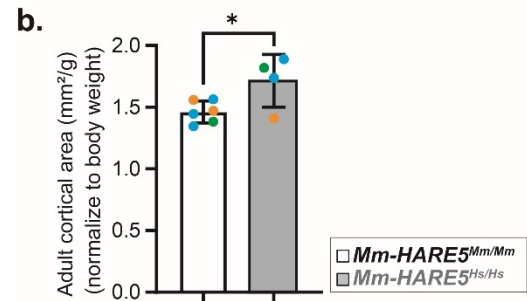


Rev Figure 2. Overview of experiment for rescuing the mitotic defect of *Hs-HARE5^{Pt/Pt}* hNPCs with canonical WNT signal agonist-CHIR99021. **b.** Quantification of mitotic D12 control and *Hs-HARE5^{Pt/Pt}* human NPCs. n=2-3 cell lines of each condition; 2-3 individual differentiations; each dot represents the average value of the cell line from each differentiation. Value above the graph bars represent the fold change of mutant/controls.

Specific comments:

1) The absolute value of cortical area should be normalized to body length or weight. This claim about changes in cortical area is especially important given that cortical thickness doesn't look different in figures 1c/d.

We have normalized the cortical area to body weight. This reveals a similar conclusion, and this is now included in the results (**Extended Data Fig. 2b**).

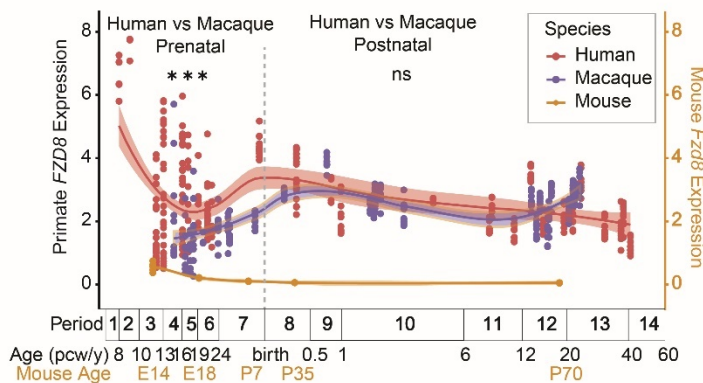


2) In figure 1d, the authors could use publicly available data to also add mouse data (e.g. from Di Bella et al., 2021). I realize the authors later compare *Fzd8* expression in control vs knock-in mice but this is only at one timepoint. There should also be a note about how developmental stages were matched for both species.

We thank the reviewer for the suggestion, and we have added developmental mouse data to the plot. Because the data from human and macaque was bulk RNA seq we looked for datasets that also fulfilled this criteria. The developmental stages between human and macaque were matched using TranscriptomeAge, a published algorithm that matches development across species based on gene expression data¹⁹. These datasets are from the same study¹⁹. For the newly added mouse data²⁰, we have used TranslatingTime (<https://www.translatingtime.org>)²¹ to match mouse and human developmental stages. We have added all the details of this analysis in the revised Methods section. These data are now included in **Fig. 1d**. We also include the mouse data alone (**Extended data Fig. 1b**). This new analysis revealed that mouse *Fzd8* expression is highest during early prenatal neocortical development, similar to human.

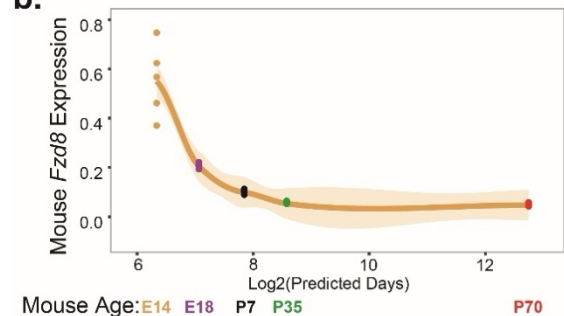
Fig. 1

d.



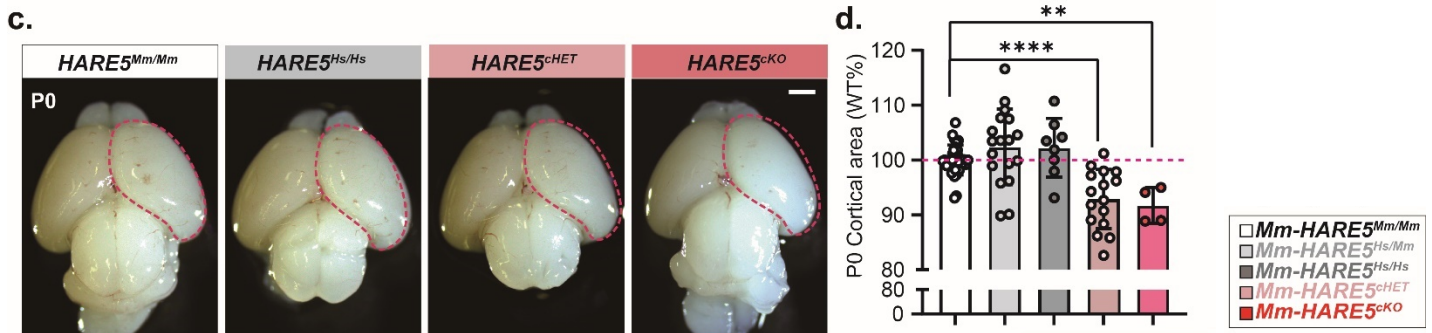
Extended data Fig. 1

b.



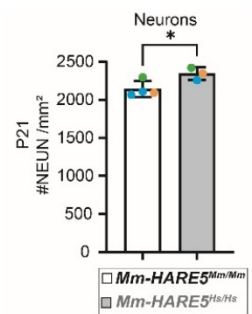
3) Extended Data Fig.2b, it appears there are only two samples from cKO—do these mice have survival issues?

We thank the reviewer for this observation. We didn't observe a notable survival problem of *Mm-HARE5^{CKO}* mice. We have added 2 more *Mm-HARE5^{CKO}* mice to this graph. This shows a significant reduction in cortical size of the cKO mice (**Extended data Fig. 2d**).

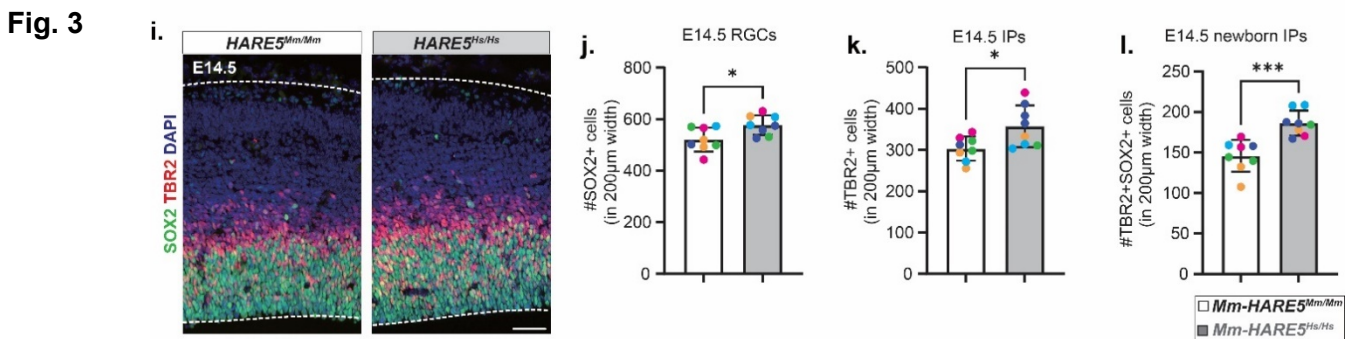


4) Fig. 2c: NeuN may not be the best antibody to address the question at P0 since the deep layer neurons are mostly not stained with NeuN. At this time point perhaps other neuronal markers (such as TBR1 for deep layer neurons and SATB2 for upper layers) could be used. But the result appears to be consistent with P21—it just might be overestimating a finding due to the low labeling. Moreover, for this quantification, the appropriate quantification should be NeuN+DAPI+/area for neuronal density. A similar comment about quantification is for Extended Data Fig 4.

We thank the reviewer for suggesting these important analyses. First, we quantified the absolute NeuN number per area (mm^2) in P21 brains (Fig. 2e,f). These data support the same conclusion that there are significantly more mature neurons in the *Mm-HARE5^{Hs/Hs}* mouse cortex. We agree that NeuN may not be the best marker of neurons at P0. Indeed, when we re-graphed these data by density they were no longer significant at P0. Thus, this lack of significant phenotype at P0 may result from labeling mainly mature but not immature neurons. Because NeuN is biased to mature neurons, and because we have quantified laminar markers at P21, we have removed the P0 data.

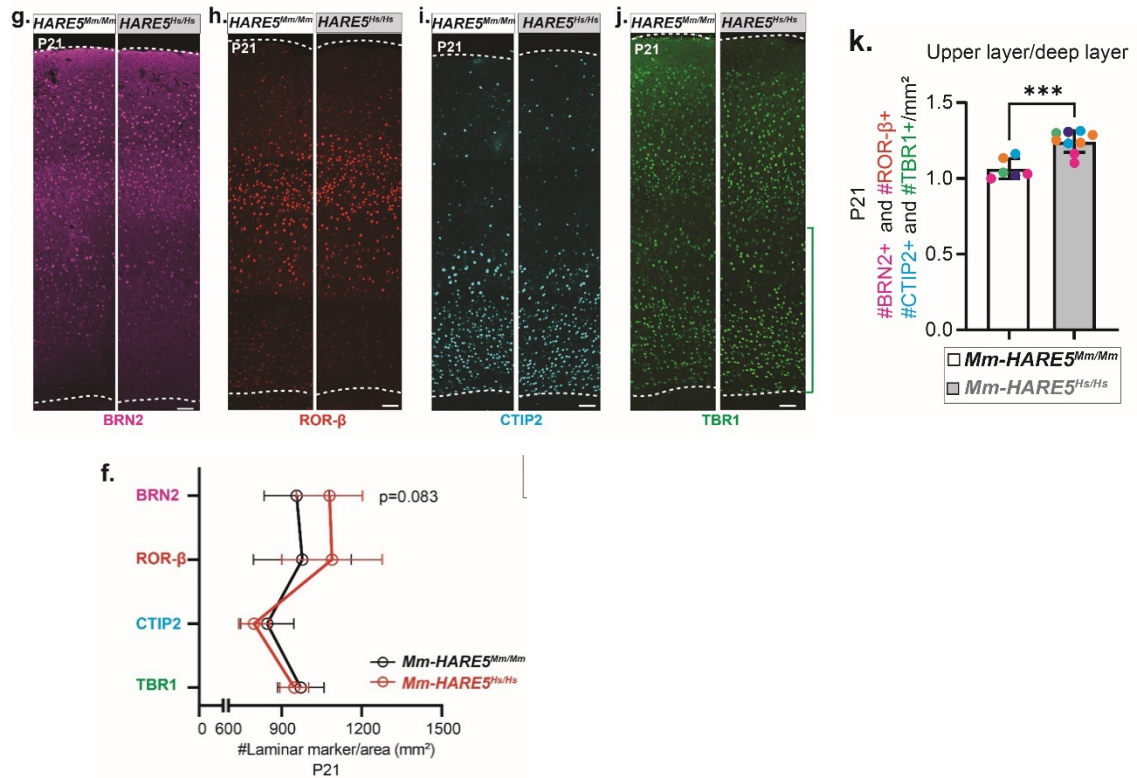


In addition, also as requested by reviewer 1, we have re-quantified graphs for neural progenitors by measuring the density of SOX2 and TBR2 positive cells (Fig. 3j-l). These data show the density of RGCs, IPs, and newborn IPs, are significantly increased in E14.5 *HARE5^{Hs/Hs}* brains. The proportion data are now moved to Extended data Fig. 5k-m.



To resolve which types of excitatory neurons are increased, we performed new analyses of P21 brains, using laminar specific markers to label layer II/III by BRN2, layer IV by ROR- β , layer V by CTIP2 and layer VI by TBR1. There were overall subtle changes in each marker (Extended data Fig. 2f). These data show that the ratio of upper layer to deep layer neurons is increased in *Mm-HARE5^{Hs/Hs}* brains (Fig. 2g-j,k). This biased increase in upper layer neurons is interesting in light of comparative data showing that upper layers are particularly expanded in primates⁴. We theorize this could reflect cell cycle exit data showing the most pronounced impact on cell cycle exit at later stages (E15.5) when upper layer neurogenesis is more prominent.

Fig. 2



Extended Fig. 2

5) Fig. 3h,k,l,m: 1) Where is the region for quantification? Based on the data, it should not be the whole column. Please specify. 2) the representative image does not appear to show the difference in the quantification. Given the increasing RG, IP, and new IP proportion in Hs/Hs, the double negative DAPI cell should be doubled in Mm/Mm. It is hard to tell from Fig. 3h.

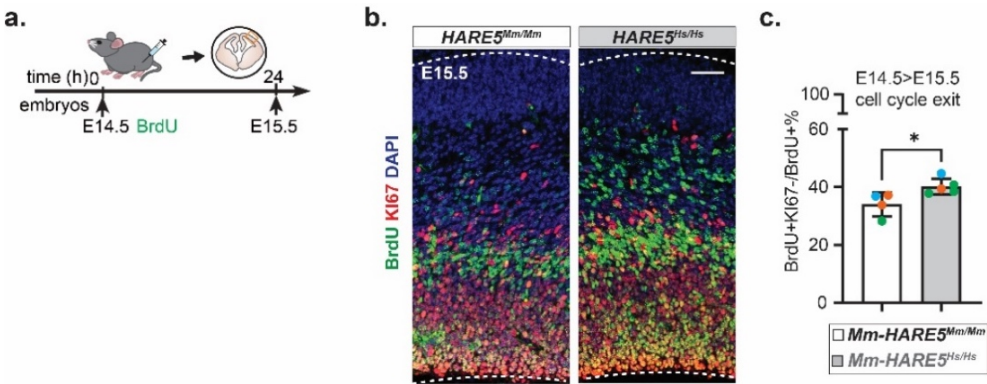
The region quantified was the radial column cropped from pia to ventricle. The proportion of the 3 progenitor subtypes per DAPI in the controls is comparable to the literature. To further address the concern, as noted in response 4, we have re-quantified progenitor density per 200μm of ventricular surface, using methods suggested by this reviewer and reviewer 1 (Fig. 3 j-l). We note that these are significant but subtle differences which may be difficult to observe by eye. Importantly all quantifications were done blind to genotype.

6) Fig.3o and corresponding result: 1) the proportion of cycling RGCs should be BrdU+PAX6+/PAX6+ not BrdU+PAX6+/BrdU+. 2) fewer BrdU+ RGCs would not suggest a higher proportion of RGCs exit the cell cycle, since BrdU- RGCs could also be the RGCs during the non-S phase of the cell cycle. Instead, exit cycling RGCs should at least be BrdU+SOX2-TBR2- DAPI. It seems challenging to determine the exit cycling RGCs here, because how can one determine if those exit cycling cells are from RGCs or IPs?

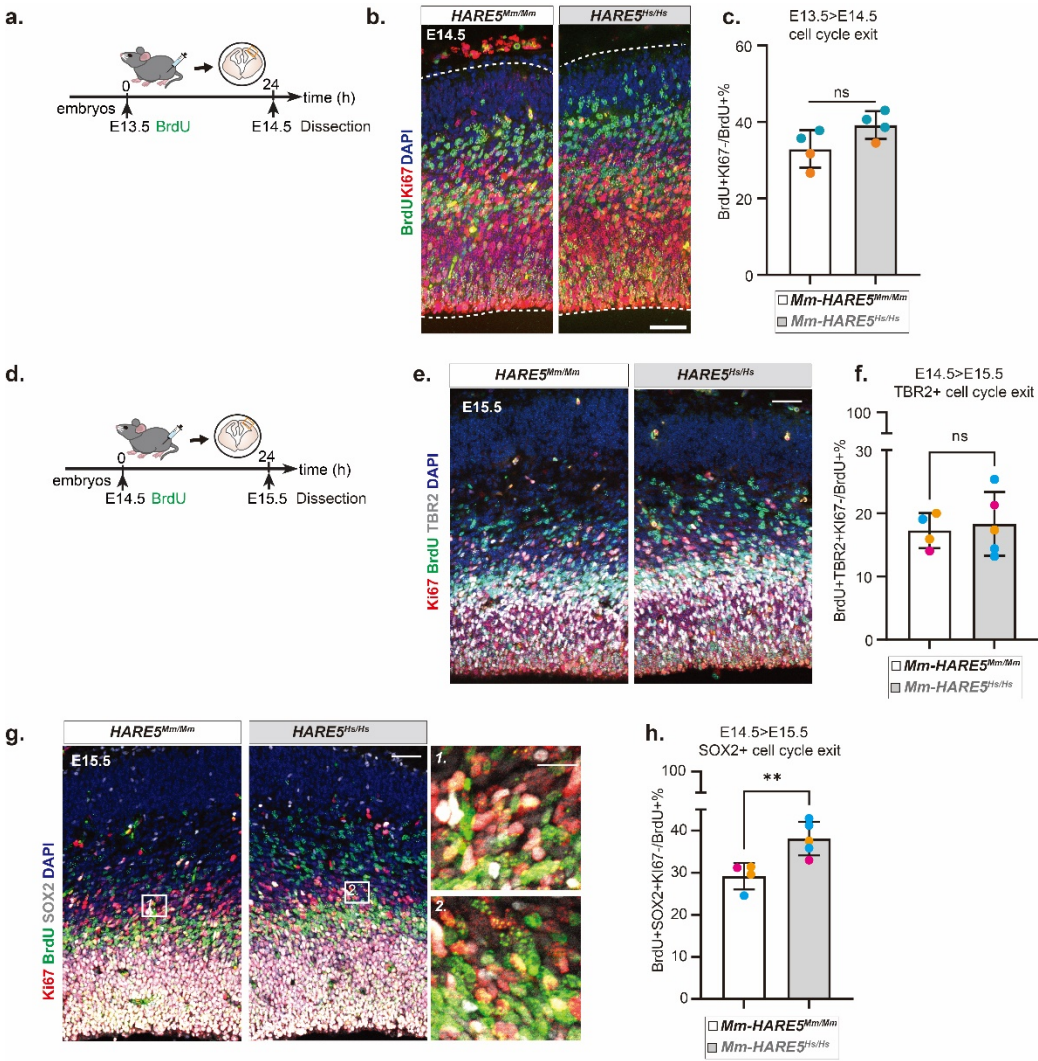
We appreciate this reviewers' point and agree this was an inaccurate calculation. We have now carried out more comprehensive cell cycle analysis. First we observed no significant increase in cell cycle exit from E13.5 to E14.5 (Extended data Fig. 7a-c). This result is consistent with the increased numbers of both RGCs and IPs at E14.5. In contrast, cell cycle exit from E14.5 to E15.5 was significantly increased in Mm-HARE5^{Hs/Hs} brains (Fig. 4a-c). We also quantified the fraction of BrdU+ that were Ki67-TBR2+ (Extended data Fig. 7d-f). This should measure neurons produced from TBR2+ cells (although we note it is an underestimate as it will not include any neurons which have lost TBR2 expression). Cell cycle exit was similar across genotypes in this population. We also quantified the fraction of BrdU+ that were Ki67-SOX2+ (Extended data Fig. 7g-h). SOX2 is inherited in newborn IPs (about half of all TBR2+ cells), however IP generating neurons typically no longer express SOX2^{2,3}. Thus, we reasoned this population should report newborn neurons generated primarily from SOX2+ RGCs. As above this is also a slight underestimate and does not include any neurons which have lost SOX2 expression. Our data show that SOX2+ cell cycle exit is significantly increased in Mm-HARE5^{Hs/Hs} brains. Our new figures include clearer schematics describing the experimental process including BrdU pulse chase and the analyzed timepoints.

Taken together use of both markers suggests that at E15.5 *Hs-HARE5* promotes increased differentiation by RGCs.

Fig. 4



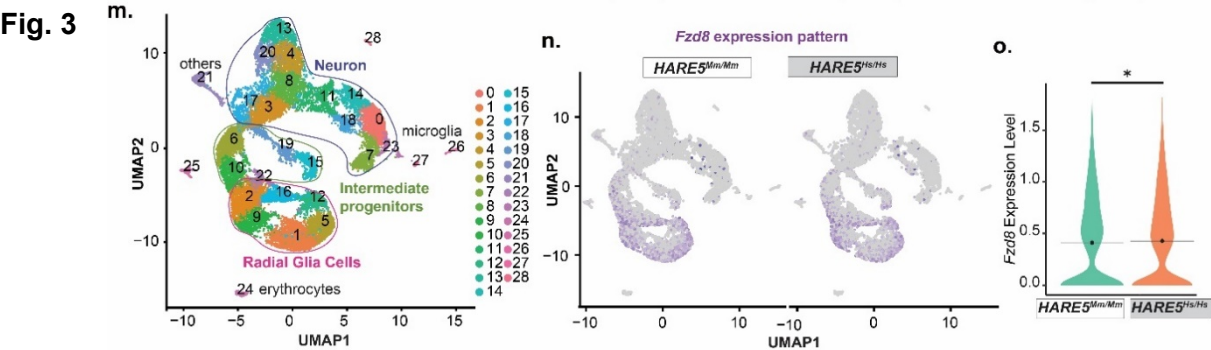
Extended Fig. 7



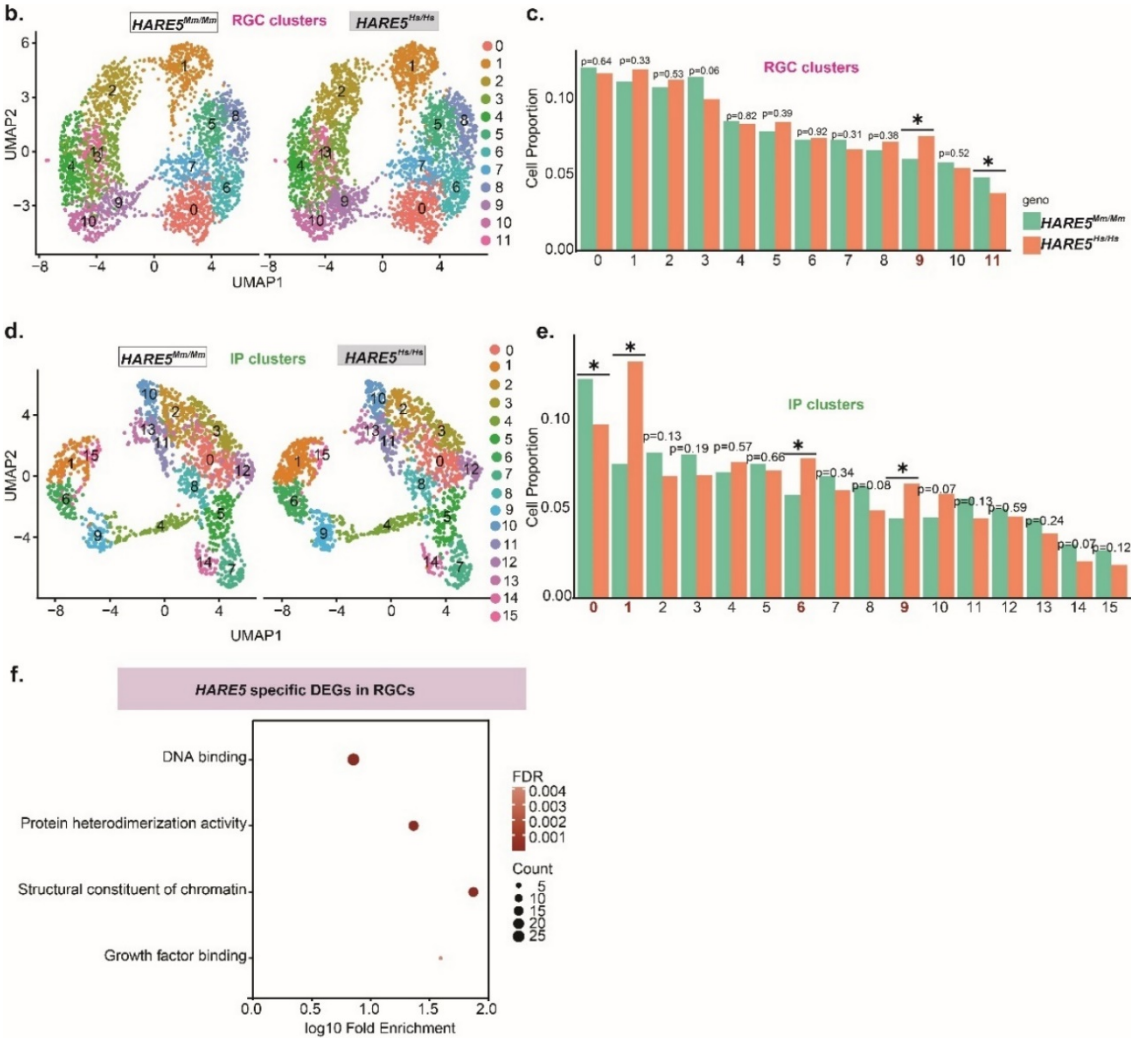
7) The single genomics data results do not have any statistics applied to the various comparisons that are made about proportion differences or even *Fzd8* expression. It is impossible to tell if any of the claims are statistically relevant and what the variance is among biological replicates. The feature plots are not informative to make claims about proportions. Also, why did the authors chose to include the damaged cells within the UMAP presented? The authors should present only the filtered/cleaned data for presentation and analysis and deposit all data for public use to be filtered as needed.

We thank the reviewer for their comments on the scRNA analysis. In the new manuscript, in response to these and other reviewer concerns, we reassessed these data. 1) We filtered out all the dead/low quality cells and redid the UMAP; 2) we updated the *Fzd8* expression with statistics to show it is enriched in *Hs-HARE5* RGCs and this spatial pattern is maintained in *Mm-HARE5^{Hs/Hs}* brains; 3) we graphed the cell composition of RGCs and IPs with statistics; and 4) we include new tables showing differentially expressed transcripts and their gene ontology enrichment. These data are included in **Fig. 3m-o**; **Extended Data Fig. 6**; **Supplementary Table 1**.

We also performed new cell cycle analysis using a different Seurat tool: https://satijalab.org/seurat/articles/cell_cycle_vignette. These data indicated a slight trend towards more G2M cells for both RGCs and IPs, however these were not statistically significant. While the quantitative data in situ carefully measures cell cycle states, the sc-seq is based upon transcript expression of some genes. Because of this and the lack of significance, we have removed the cell cycle predictions of scRNA seq data from the study.



Extended Fig. 6

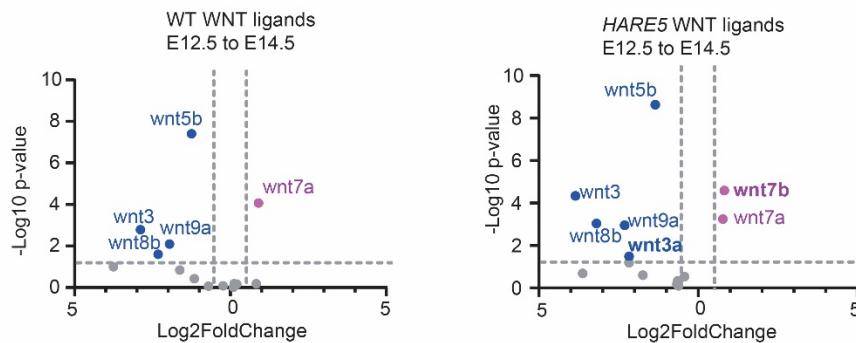


8) Did the authors compare the scRNA-seq at E14.5 with the bulk RNA-seq at E14.5? The bulk RNA-seq results do not provide much additional functional information about the role of HARE5 in development beyond what is presented in other ways. Importantly, WNT signaling does not appear to be affected which is in contrast to the functional study presented with the WNT reporter and WNT inhibitor. Could this be due to loss of cellular resolution. Is WNT signaling altered in the E14.5 scRNA-seq data?

We thank the reviewer for these important questions. We did not directly compare these datasets. *Hs-HARE5* is active only in a subpopulation of cortical cells, mainly RGCs. Thus, bulk RNA seq had less resolution to detect transcript changes. As noted above, we observed some differences in gene expression developmentally. In the scRNA-seq some DEGs were identified in both RGCs and IPs. In RGCs these included DNA binding factors (**Extended Data Fig.6f, Supplementary Table 1**). Functional studies will be valuable in the future to follow up on these downstream targets.

We agree that understanding how *Hs-HARE5* alters WNT signaling is fascinating. While we did not observe many significant changes in WNT associated transcripts in the scRNA-seq data, there were a few significantly changed in IPs or RGCs, including *Nkd1*, *Igfbp2*, and *Ccnd1* (**Supplementary table 1**). To further address this question, from the bulk RNA seq, we plotted those WNT ligands expressed at detectable levels from E12.5 to E14.5. Overall, the general change was consistent between genotypes, however, *Hs-HARE5* specifically affected temporal expression of some ligands, such as downregulation of WNT3a and upregulation of WNT7b (**Extended data Fig. 11f**). These data support the notion that developmental differences in WNT ligand expression may influence shifts to neural differentiation, as well as tempered impact on RGC canonical WNT signaling observed in older *Hs-HARE5* brains.

f.



9) In figure 4, why do the authors analyze Hs/Mm rather than Hs/Hs? Also by carrying out IUE at E14.5 how can the authors specifically label the RGCs and not other cell types? This was not clear.

We thank the reviewer for the opportunity to clarify this. In the live imaging experiments, we needed to incorporate the TBR2-EGFP^{Tg/+} reporter line into the *Hs-HARE5* knock-in background and obtain enough embryos of the right genotype for live imaging across several differentiations. Due to these genetics, we performed these experiments with Hets. Importantly, our data demonstrate that Hets also exhibit progenitor phenotypes (**Extended data Fig. 5k-m**), and Hets exhibit similar connectivity phenotypes in adulthood (**Extended data Fig. 3a**). Further, the live imaging results demonstrate there is a phenotype with the het. For our experimental design of the IUE experiment we aimed to have littermate controls, Hs/Mm and Hs/Hs. Including both the Hs/Hs and Hs/Mm allowed us to have sufficient sample size. Importantly, as above, we observed no difference in phenotypes when doing this experiment in Hets or Homo.

The plasmid (either pCAG-EGFP or pCAG-mcherry) does not selectively label specific cell type²². However, in utero electroporation (injecting plasmids in the lateral ventricle of embryos and using electronic pulse to deliver to cells lining the ventricle) is thought to primarily target dividing RGCs²³. In the results we interpret this population as “primarily RGCs.” As the RGC cell-cycle length at this stage is estimated around 15-18hr²⁴, we collected samples at 17hrs after the IUE before the EGFP protein was passed along to several generations. The IUE

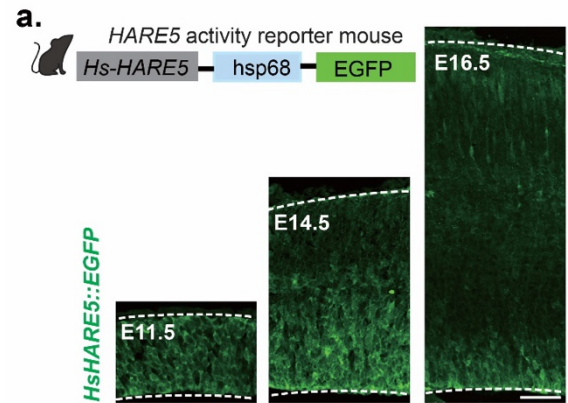
results, together with the cell cycle exit and live imaging data, collectively indicate that *Hs-HARE5* promotes RGC differentiation into IPs and newborn neurons at E15.5.

10) The authors state: “Rhesus macaque is a closely related species to humans and a commonly studied nonhuman primate.” Closely related is subjective, and yes, compared to mouse, more closely, but this could be reframed.

We thank the reviewer for this suggestion and have modified the wording.

11) The authors state Regarding figure 3a: “HARE5 enhancer activity was remarkably high in the developing neocortex at E11.5 and E14.5, mainly within the germinal regions.” Remarkably high compared with what? No other regions or timepoints are shown. This is subjective without a point of comparison.

We agree with the reviewer that this was a subjective statement. We now include an image of the *Hs-HARE5* reporter at E16.5 (Fig. 3a). Together with data at E11.5 and E14.5, this shows *Hs-HARE5* activity is highest in the VZ and SVZ compared with IZ and CP in early to mid-corticogenesis stages. We have modified the text accordingly.



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Response to Reviewers

A human-specific enhancer fine-tunes radial glia potency and corticogenesis

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We thank the editors and reviewers for their careful evaluation and continued feedback on our revised manuscript. We are pleased the reviewers and editors found our study is significant for the field and is accepted in principle for *Nature*. To address reviewer concerns we have slightly modified the manuscript, as described below.

Referee #1 (Remarks to the Author):

The authors have performed a number of additional experiments, analyses and significant changes in the manuscript in response to all my previous criticisms and points of concern. I am fully satisfied with the revised manuscript, and I congratulate the authors for great work and a groundbreaking discovery.

We thank this reviewer for appreciating the key advances in our revised manuscript.

Referee #2 (Remarks to the Author):

The authors have improved the manuscript and addressed my major concern about the impact of the enhancer mutations on Wnt signaling with extensive additional experiments and a clearer model.

We thank this reviewer for appreciating the key advances in our revised manuscript.

Note, the minor Y-axis point in Fig 1c was for the H3K27ac and DHS plots, not the HiC map.

The H3K27ac and DHS plots were generated from the ChIP-seq and DNase-seq dataset from Kundaje, et al. 2015. In this paper, the y-axis indicated mapped peaks. We include this label as “peaks” in the updated **Fig. 1c**.

Referee #3 (Remarks to the Author):

The authors have been very responsive to the prior critiques and the manuscript is substantially improved.

We thank this reviewer for appreciating the key advances in our revised manuscript.

I have two suggestions for clarifications:

- 1) The data to support Hs-HARE5 regulating non-canonical WNT signals at later developmental stages for differentiation of neurons is not strong. I suggest toning down this interpretation.

We appreciate the reviewer’s points and have modified the discussion to state the following: “It remains to be tested if HARE5 promotes differentiation via non-canonical WNT signaling, and/or via exposure to neurogenic cues.”

- 2) The calculation of RGCs and IPs exiting the cell cycle still seems open to other interpretations. SOX2 or TBR2 could only be detected in postmitotic cells for a short period of time. Thus, only a portion cycling RGCs and IPs exiting can be captured. The authors do now

discuss this in their rebuttal. But the question is about the proportion of those cells and whether it represents the whole population of exiting progenitors. Based on the authors' protocol, the interval between BrdU injection and dissection is 24 hours. Some BrdU labelled postmitotic cells might experience two cell cycles. It is known that the number of BrdU+ cells is negatively correlated with cell cycle length. However, we do not know the cell cycle length between knock in and WT mice. If the authors could further discuss or disentangle these issues, that would be great.

We appreciate this reviewers' point and had noted in our initial response that this paradigm is an underestimate of all exiting progenitors. We have added this important point to the results as well as the figure legend for Extended Data Fig. 8. For example the figure legend states: "BrdU+TBR2+Ki67- cells reflect a subset of TBR2+ cells which have exited the cell cycle. Note this is an underestimate of all exiting progenitors due to the labels used and timing of BrdU." In addition, we added a new cartoon schematic to **Extended Data Fig. 8d.** to clarify the specific cell type labeled and analyzed in our assays in bold.

FIGURE REDACTED