Chd8 mediates cortical neurogenesis via transcriptional regulation of cell cycle and Wnt signaling

Omer Durak1,2, Fan Gao1, Yea Jin Kaeser-Woo1, Richard Rueda1, Anthony J Martorell1,2, Alexi Nott1, Carol Y Liu1, L Ashley Watson1 & Li-Huei Tsai1,3

De novo mutations in CHD8 are strongly associated with autism spectrum disorder, but the basic biology of CHD8 remains poorly understood. Here we report that Chd8 knockdown during cortical development results in defective neural progenitor proliferation and differentiation that ultimately manifests in abnormal neuronal morphology and behaviors in adult mice. Transcriptome analysis revealed that while Chd8 stimulates the transcription of cell cycle genes, it also precludes the induction of neural-specific genes by regulating the expression of PRC2 complex components. Furthermore, knockdown of Chd8 disrupts the expression of key transducers of Wnt signaling, and enhancing Wnt signaling rescues the transcriptional and behavioral deficits caused by Chd8 knockdown. We propose that these roles of Chd8 and the dynamics of Chd8 expression during development help negotiate the fine balance between neural progenitor proliferation and differentiation. Together, these observations provide new insights into the neurodevelopmental role of Chd8.

Autism spectrum disorder (ASD) is a complex developmental disorder that manifests in social deficits, communication difficulties, stereotyped behaviors and cognitive delays1,2. Around 120 genes have been linked to ASD, often encoding proteins involved in chromatin remodeling, transcriptional regulation and synapse function3–8. Of these, at least 15 distinct mutations in the coding regions of chromodomain helicase DNA binding protein 8 (CHD8), an ATP-dependent chromatin remodeler, were identified in subjects with ASD from exome sequencing of trio families5,8,9. Most of these mutations are predicted to be loss-of-function9. Subjects with CHD8 mutations often display increased head circumference and cognitive deficits, as well as social interaction and communication difficulties5,9.

Little is known about the biological function of CHD8. It was initially identified as a binding partner and negative regulator of β-catenin signaling and was shown to be enriched in the promoters of transcriptionally active genes10–13. Homozygous deletion of Chd8 in mice results in early embryonic lethality resulting from massive apoptosis13,14. However, no induction of Wnt–β-catenin signaling was found in Chd8 null mice14. CHD8 was also shown to be necessary for E2F1-dependent cell cycle gene activation during the G1/S transition11. Downregulation of CHD8 in cultured cells resulted in impaired cell proliferation15. In contrast, an increase in the number of mitotic cells and head size were observed following suppression of the chd8 ortholog in zebrafish16.

Whereas the genetic and molecular underpinnings of ASD are heterogeneous, an accumulating body of evidence indicates that disrupted embryonic cortical development could be one of numerous defects underlying the etiology of ASD2,17,18. Cortical development is a spatially and temporally regulated process that is defined by an early expansion of proliferative neural progenitor cells (NPCs) that reside in the ventricular zone (VZ) of cortical epithelium19,20. At the onset of neurogenesis, NPCs undergo neurogenic divisions to produce pyramidal neurons that migrate radially to the cortical surface to generate the six layers of the neocortex21. Diverse signaling pathways govern the intricate balance between continued proliferation and cell cycle exit/differentiation. Of these, the role of canonical Wnt signaling in cortical neural progenitor proliferation has been described extensively22–25. For instance, induction of Wnt signaling via overexpression of stabilized β-catenin increases neural progenitor proliferation by negatively regulating cell cycle exit and differentiation26. In addition to signaling mechanisms, epigenetic mechanisms also play a crucial role in the spatial and temporal control of developmental genes21,27. For instance, polycomb group proteins are important for maintaining genes necessary for differentiation in a temporally repressed but poised state, which allows for their activation in response to the appropriate differentiation cues27,28.

Here we examine the in vivo effects of Chd8 disruption on embryonic mammalian brain development. We show that in utero knockdown of Chd8 results in reduced cortical neural progenitor proliferation and altered neurogenesis. Transcriptional profiling of cortical neural progenitors via RNA sequencing (RNA-seq) following Chd8 knockdown showed that downregulated genes were enriched for cell cycle control and chromatin remodeling pathways. Upregulated genes were highly enriched for repressive H3K27me3 chromatin, potentially due to reduced PRC2 complex activity. In contrast to previous in vitro findings in non-neuronal proliferating cells, our results indicate that Chd8 is a positive regulator of Wnt signaling in cells of neural lineage both in vivo and in vitro. Finally, knockdown of Chd8 in developing...
cerebral cortex resulted in behavioral deficits in adult mice, which can be rescued upon overexpression of β-catenin. Together, these observations provide new insights into the roles of Chd8 in brain development and how mutations in CHD8 may contribute to ASD.

RESULTS

Chd8 is highly expressed in the developing brain

To understand whether CHD8 has a role in cerebral cortical development, we first examined the temporal expression pattern of Chd8 in the developing mouse cortex. Quantitative polymerase chain reaction (qPCR) analysis demonstrated that Chd8 is highly expressed in the embryonic day (E) 12 cortex and that its expression decreases with the progression of corticogenesis (Supplementary Fig. 1a). Furthermore, an assessment of CHD8 expression in the developing human dorso-lateral and medial prefrontal cortex (DPC and MFC) showed that CHD8 is highly expressed in both DFC and MFC during early fetal development. CHD8 expression peaks in the early midfetal period in DFC and early fetal period in MFC. The temporal expression pattern of CHD8 in the developing human DFC and MFC was very similar to that of mouse Chd8, with reduction in expression throughout early development (Supplementary Fig. 1b,c). Finally, in situ hybridization experiment revealed that Chd8 is ubiquitously expressed in the developing cortex and that, in agreement with the results from our qPCR data above, Chd8 expression in E12 cortex was higher than in E16 cortex (Supplementary Fig. 1a,d). These initial studies suggest that Chd8 could play an important role in cortical neural progenitor proliferation and differentiation.

Chd8 is required for cortical neural progenitor proliferation

To determine the functions of Chd8 in the developing mouse cortex and because the ASD-associated CHD8 mutations are predicted to lead to the loss of protein function, we used two distinct shRNAs (short hairpin RNAs; sh1 and sh2) to knockdown Chd8. Transfection of N2a cells with these shRNAs led to robust reduction of endogenous Chd8 expression, as well as reduced expression of Ccne2, a previously identified target of Chd8 (Supplementary Fig. 2a,b). Next we performed in utero electroporation to knock down Chd8 in cortical progenitors at E13 and analyzed brains at E16. Either scrambled shRNA (control) or Chd8 shRNAs expression constructs were used, in combination with a GFP expression construct to label electroporated cells. We first examined the distribution of GFP-positive (GFP+) cells in the embryonic mouse cerebral wall. A significant reduction in the number of GFP+ cells was observed in the ventricular zone and subventricular zone (VZ/SVZ) of Chd8-knockdown animals compared to controls, along with a comparable increase in the number of GFP+ cell in the cortical plate (Fig. 1a,b). To examine if the loss of GFP+ cells from the VZ/SVZ was due to reduced neural progenitor proliferation, we pulse-labeled them with BrdU 24 h before analysis. The brains were immunolabeled with antibodies against BrdU, GFP and the proliferative marker Ki67. Chd8 shRNAs caused a significant decrease in BrdU incorporation in the GFP+ cell population (Fig. 1a,c). The reduction in cell proliferation was accompanied by increased cell cycle exit (Fig. 1a,d). We further used phosphorylated histone-H3-S10 (pHH3) to label mitotically active cells and observed a significant decrease in mitotic activity in Chd8-knockdown embryos (Fig. 1e,f). This was accompanied by a concomitant increase in the percentage of cells positive for neuronal marker TuJ1 (Fig. 1e,g). In addition to this, we observed that Chd8 knockdown caused a reduction in Sox2+ neural progenitors (Supplementary Fig. 3a,b) but that apoptosis was unaffected (Supplementary Fig. 3a,c). These results, together with the increased number of GFP+ cells in the cortical plate and the reduced number of BrdU+ cells, indicated that Chd8 knockdown resulted in a premature depletion of the neural progenitor pool in the developing mouse cortex.

We next electroporated an shRNA-resistant human CHD8 (hCHD8) overexpression construct in conjunction with the control and Chd8 shRNAs (Supplementary Fig. 2c). First, we found that overexpression of hCHD8 together with the control shRNA caused increased BrdU incorporation and mitotic activity, as well as reduced cell cycle exit and neuronal differentiation (Fig. 1). Co-expression of hCHD8 with mouse Chd8 shRNAs was able to restore levels of BrdU incorporation, mitotic activity and cell cycle exit, as well as normalize the distribution of GFP+ cells and the percentage of TuJ1+ cells to control levels (Fig. 1). These experiments indicated that neural progenitor pool depletion and increased TuJ1+ neurons in the embryonic brain following Chd8 knockdown could result in a reduction in the total number of neurons produced in postnatal brain.

Chd8 is important for the expression of cell cycle genes

To gain a deeper insight into how Chd8 regulates neural progenitor proliferation, we examined the transcriptome of neural progenitors following Chd8 knockdown. We performed in utero electroporation, targeting NPCs at E13 and using either control or Chd8 shRNAs together with a GFP expression construct. The GFP+ cell population was then isolated through fluorescence-activated cell sorting (FAC-sorting) 48 h after the in utero transfection. This approach ensured...
that our analysis included only transfected cell populations with
the same birthdate. Following total RNA collection, we performed
next-generation sequencing to identify differentially expressed genes
(DEGs) between control and Chd8-knockdown cells (Fig. 2a and
Supplementary Data Set 1).

Our analysis revealed a total of 3,762 genes in the Chd8 sh1 and 5,245
genes in the Chd8 sh2 knockdown samples that were differentially
expressed compared with control samples (P < 0.05, fold change > 1.2;
Supplementary Fig. 4 and Supplementary Data Sets 2 and 3). To
identify biological functions perturbed by Chd8 knockdown, we
subjected the DEGs to gene ontology analysis of biological pro-
cesses, which showed that genes downregulated by Chd8 knockdown are
involved in regulation of cell cycle, chromosome organization, RNA
processing and cytoskeleton organization (Fig. 2b and Supplementary

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Data Sets 4 and 5). Upregulated genes in Chd8-knockdown samples were enriched for processes relating to neuronal development such as neuron and cell differentiation, cell migration and defense response (Fig. 2b and Supplementary Data sets 6 and 7). Supporting the analysis of biological processes, an analysis of the biological pathways enriched in the downregulated genes revealed pathways relating to cell cycle, DNA replication and Wnt signaling (Supplementary Fig. 5 and Supplementary Data Sets 8 and 9). Furthermore, mammalian (mouse) phenotype analysis of downregulated genes revealed that they were enriched for categories such as prenatal lethality and abnormal embryo size, brain size and brain development. Together these analyses suggest that Chd8 has an essential role in regulating pre-natal genes necessary for proper embryonic development. We then validated a subset of downregulated (Akap8, Cdc7, Tacc3, Timeless, Notch2, Kif20b, Dnmt3a and Tial1) and upregulated (Camk1d, Pak6, Fgfl8 and Jakmip1) genes via qPCR and correlated these changes in expression with RNA-seq data, which showed significant Pearson and Spearman correlation coefficients (Fig. 2c).
Our results are consistent with previous studies that have assessed the transcriptional consequences of Chd8 suppression. For instance, Suganthan et al. showed that, in human NPCs (hNPCs), CHD8 controls gene expression during embryonic brain development, we generated a chromatin-states map for the E12 mouse brain based on published chromatin immunoprecipitation-sequencing (ChIP-seq) data for seven chromatin marks (ENCOD, https://www.encodeproject.org/). Genomic regions were classified into different chromatin states defined by combinations of these histone marks, including promoters, enhancers, transcribed, bivalent, repressed and low-signal regions (Supplementary Fig. 7). We then mapped CHD8 peaks from hNPCs, human neural stem cells (hNSCs), human midfetal DFC, ChIP-seq data sets and CHD8 peaks from E17.5 mouse cortex to the chromatin states map (Fig. 3a) Both human and mouse Chd8 binding peaks were highly enriched for chromatin states associated with active promoters (Fig. 3a), suggesting that Chd8 binds to the promoters of actively transcribed genes. We then examined the overlap between CHD8 ChIP-seq peaks in hNSCs with differentially expressed genes in both of our Chd8-knockdown data sets and found that 58.4% (367 of 628) of the differentially expressed genes following CHD8 knockdown contained CHD8 ChIP-seq peaks.

Chd8 is a transcriptional activator of genes necessary for early cortical development.

To gain further insight into the molecular mechanisms by which Chd8 controls gene expression during embryonic brain development, we generated a chromatin-states map for the E12 mouse brain based on published chromatin immunoprecipitation-sequencing (ChIP-seq) data for seven chromatin marks (ENCOD, https://www.encodeproject.org/). Genomic regions were classified into different chromatin states defined by combinations of these histone marks, including promoters, enhancers, transcribed, bivalent, repressed and low-signal regions (Supplementary Fig. 7). We then mapped CHD8 peaks from hNPCs, human neural stem cells (hNSCs), human midfetal DFC, ChIP-seq data sets and CHD8 peaks from E17.5 mouse cortex to the chromatin states map (Fig. 3a) Both human and mouse Chd8 binding peaks were highly enriched for chromatin states associated with active promoters (Fig. 3a), suggesting that Chd8 binds to the promoters of actively transcribed genes. We then examined the overlap between CHD8 ChIP-seq peaks in hNSCs with differentially expressed genes in both of our Chd8-knockdown data sets and found that 58.4% (367 of 628) of the differentially expressed genes following CHD8 knockdown contained CHD8 ChIP-seq peaks.

**Figure 3** Direct and indirect regulation of genes necessary for early cortical development by Chd8. (a) Chromatin state enrichment analysis using CHD8 (hNSC, hNPC and midfetal human brain), Chd8 (E17.5 mouse brain), Ezh2 (hNSC) and Suz12 (hNPC) ChIP-seq peaks using published data. (b) Venn diagram showing the overlap between Chd8 target genes (CHD8 ChIP-seq)12 with differentially expressed genes following Chd8-knockdown (Chd8 RNA-seq). Overlap shows enrichment of genes downregulated following Chd8 knockdown (79.5% downregulated vs. 20.5% upregulated). (c) Aggregate plots of average H3K27ac ChIP-seq intensity signal in E12 mouse brain generated using differentially expressed genes in Chd8 RNA-seq data sets. Aggregate plots were generated at the TSS of genes upregulated (red) and downregulated (blue). TSS, transcriptional start site. (d) Aggregate plots of average H3K27me3 ChIP-seq intensity signal in E12 mouse brain generated using differentially expressed genes in Chd8 RNA-seq data sets. Aggregate plots were generated at the TSS of genes upregulated (red) and downregulated (blue). (e) Confirmation of reduced expression of Ezh2 and Suz12 in independent set of FAC-sorted Chd8-knockdown samples from embryonic brain assessed using qRT-PCR (one-way ANOVA followed by Dunnett’s multiple comparison test; DF = 2, F = 32.93, P < 0.0001 for Ezh2; DF = 2, F = 12.03, P = 0.0008 for Suz12; Control, n = 9; Chd8 sh1, n = 5; Chd8 sh2, n, number of different FAC-sorted samples analyzed. (f) Sample western blots shown for Chd8, Ezh2 and α-tubulin (loading control, left panel). Quantification of protein expression levels normalized to α-tubulin (right panel). Knockdown of Chd8 in mouse NPCs results in reduced expression of Ezh2, a major component of polycomb repressive complex 2 (n = 4 for all samples; two-tailed student’s t-test; P = 0.0002 for Chd8, P = 0.0006 for Ezh2). n, number of cultured cell samples analyzed. Full-length western blots are presented in Supplementary Figure 14. (g) Overlap between upregulated genes following Chd8 knockdown that are not Chd8 targets and Ezh2 or Suz12 targets34,35, Ezh2 targets, 3,390 genes; Suz12 targets, 2,290 genes; upregulated genes (non-Chd8 targets) in sh1 data set, 789 genes; upregulated genes (non-Chd8 targets) in sh2 data set, 1,622 genes. P-values calculated using hypergeometric distribution test. *P < 0.05; **P < 0.01; ***P < 0.001. Results are presented as mean ± s.e.m.
in their promoters (Fig. 3b). A significant majority of these 367 differentially expressed genes (291; 79.5%) were downregulated following Chd8 knockdown. These results suggest that Chd8 could function as a transcriptional activator in mouse cortical progenitors (Fig. 3b).

Consistent with this possibility, we found that genes downregulated by Chd8 knockdown are normally enriched for H3K27ac, a chromatin mark associated with active gene promoters (Fig. 3c). Thus our data indicate that Chd8 functions primarily as a transcriptional activator in neural progenitors. Cotney et al. previously published similar findings wherein active histone marks were enriched at transcription starting sites of CHD8 target genes.

In contrast to the scenario with downregulated genes, we observe that upregulated genes following Chd8 knockdown are normally enriched for H3K27me3, a chromatin mark associated with repressed gene promoters (Fig. 3d). The levels of H3K27me3 are regulated through the activities of the PRC2 complex. Our RNA-seq data revealed that the expression of two main components of the PRC2 complex, Ezh2 and Suz12, were significantly attenuated upon Chd8 knockdown. The effects of Chd8 knockdown on these genes were further verified by qPCR (Fig. 3e). Additionally, injection of cultured mouse NPCs with Chd8 shRNA also caused a significant reduction in Ezh2, which is the functional enzymatic component of the PRC2 complex (Fig. 3f).

Furthermore, published CHD8 ChIP-seq data sets show that CHD8 binds to the promoters EZH2 and SUZ12 (refs. 12, 16). These results suggest that Chd8 knockdown could upregulate genes directly targeted by PRC2 complex. In fact, from analysis of publicly available CHD8 (ref. 12), Ezh2 (ref. 34) and Suz12 (ref. 35) ChIP-seq data sets, we found that a significant proportion (approximately 25%) of the upregulated genes that lack CHD8 binding within their promoters are normally bound by Ezh2 and Suz12 (Fig. 3g). These results suggest that downregulation of PRC2 complex components could constitute an additional important mechanism by which Chd8 regulates gene expression.

Overall, our data provide key mechanistic insights into the dual function of Chd8 as promoter of cell cycle progression and a repressor of neural genes: Chd8 binds the promoters of cell cycle genes and serves as a transcriptional activator. At the same time, it also promotes PRC2 expression, which allows for the repression of neural genes during this developmental period.

**Chd8 maintains Wnt signaling in neural progenitors**

While Chd8 knockdown caused the downregulation of a number of genes related to cell cycle progression, gene ontology of biological pathways analysis indicated that genes encoding the Wnt signaling pathway were enriched among the genes downregulated in Chd8 knockdown samples (Supplementary Fig. 5). These include several of the primary transducers and effectors of the Wnt signaling pathway, such as Fzd1, Fzd2, Dvl2, Dvl3 and Ctnnb1 (Supplementary Data Sets 2 and 3). Transcriptional dysregulation of Wnt–β-catenin signaling by CHD8 in neural stem cells (NSCs) has also been reported previously by Cotney et al.

To validate the findings from RNA-seq experiments, we processed FAC-sorted cells for qPCR analysis and found that Chd8 knockdown resulted in reduced expression of several Wnt signaling genes as well as a Wnt target gene (Ccmd1; Fig. 4a). To understand whether Chd8 directly targets these genes, we performed Chd8 ChIP-qPCR experiments using mouse embryonic cortical tissue (E12) and found that Chd8 binds to the promoter regions of Fzd1, Dvl3 and Ctnnb1 genes (Supplementary Fig. 8b). Consistent with these results, analysis of publicly available human and mouse CHD8/Chd8 ChIP-seq data sets revealed the enrichment of Chd8 at the promoters of Fzd1/Fzd1, Fzd2/Fzd2, Dvl2/Dvl2, Dvl3/Dvl3 and Ctnnb1/Ctnnb1 (Supplementary Fig. 8a). Together, these findings suggest that Chd8 is required for maintaining Wnt signaling in embryonic mouse cortical neural progenitors.

To further test this hypothesis, we used a luciferase reporter assay to measure Wnt-mediated transcriptional activity. First, we in utero transfected mouse embryonic NPCs with the luciferase construct along with either control or Chd8 shRNA. Cerebral cortical tissues were collected to measure luciferase reporter activity 72 h later. We found that, following knockdown of Chd8, T-cell factor and lymphoid enhancer factor (TCF/LEF)-reporter activity was markedly reduced compared to controls (Fig. 4b). In contrast, overexpression of hCHD8 resulted in a significant increase in TCF/LEF-reporter activity (Fig. 4b).

Furthermore, co-expression of Chd8 shRNA along with hCHD8 cDNA restored the Wnt signaling activity (Fig. 4b). Similarly, in N2a cells, both Chd8 shRNAs markedly reduced Wnt signaling activity, and this effect was normalized by co-expressing hCHD8 cDNA alongside Chd8 shRNAs (Fig. 4c).

Contrary to our findings, previously published reports identified Chd8 as a negative regulator of canonical Wnt–β-catenin signaling. It is worth noting that these earlier studies were conducted in non-neuronal cells, suggesting that Chd8 could play cell-type-specific roles in regulating Wnt signaling. We therefore examined Wnt signaling in a non-neuronal cell line to determine if the regulation of Wnt signaling by Chd8 is cell-type specific. We conducted the TCF/LEF luciferase assay using human embryonic kidney 293T (HEK293T) cells as used in Nishiyama et al. We observed a significant upregulation in Wnt signaling activity following CHD8 knockdown (Fig. 4d), consistent with previous reports using non-neuronal cells.

Unexpectedly, CHD8 knockdown did not affect the expression of Wnt signaling genes in HEK293T cells (Supplementary Fig. 9). Furthermore, we also examined the consequences of Chd8 loss-of-function on TCF/LEF luciferase activity in both mouse and human cultured NPCs. We found that knockdown of Chd8 caused reduced Wnt signaling activity in both mouse and human NPCs (Fig. 4e.f). These results strongly suggest that the influence of Chd8 on Wnt signaling is cell-type specific and that Chd8 is a positive regulator of Wnt signaling in cells of neural progenitors.

**Increased β-catenin expression can counteract Chd8-knockdown-associated phenotypes**

Our findings suggest that Chd8 could stimulate Wnt signaling activity in proliferating neural cell lines, in part through transcriptional regulation of multiple Wnt pathway components. β-catenin is the critical downstream component of the Wnt signaling pathway. Upon activation of canonical Wnt signaling, β-catenin is stabilized in the cytoplasm and subsequently enters the nucleus, where it binds to TCF/LEF family transcription factors to activate expression of Wnt target genes such as Ccnd1. To determine if Chd8 regulates cortical progenitor proliferation via Wnt signaling, we expressed a degradation-resistant β-catenin mutant construct (S37A) alongside Chd8 sh2. Expression of stabilized β-catenin rescued TCF/LEF luciferase activity following Chd8 knockdown in embryonic mouse brain (E13–E16, Fig. 5a) and in N2a cells (Fig. 5b).

We next evaluated if increased Wnt signaling activity could also rescue reduced progenitor proliferation downstream of Chd8 knockdown. We performed in utero electroporation at E13 with control or Chd8 shRNA and co-expressed either control empty vector or stabilized β-catenin constructs. The pregnant dams were injected with BrdU 48 h later at E15, and the embryos were collected at E16. As shown earlier, we observed a significant shift of GFP + cell from the VZ/SVZ to the cortical plate in Chd8 knockdown samples (Fig. 5c.d). The altered distribution of GFP + cells was normalized upon stabilized β-catenin co-expression (Fig. 5c.d). Furthermore, stabilized β-catenin also rescued
the cell proliferation (Fig. 5c,e), cell cycle exit (Fig. 5c,f), increased TuJ1 cell (Fig. 5g,i) and normalized mitotic index (Fig. 5g,h) phenotypes that occurred in response to Chd8 knockdown.

Finally, we assessed whether induction of Wnt signaling could also ameliorate the transcriptional dysregulation caused by Chd8 knockdown. To this end, we performed in utero electroporation at E13 using

**Fig. 4** Chd8 is a positive regulator of canonical Wnt signaling in neuronal cells. (a) Confirmation of reduced expression of Wnt signaling genes bound by Chd8 (Fzd1, Fzd2, Dvl2, Dvl3, Ctnnb1) and a Wnt response gene (Ccnd1) in independent set of FAC-sorted Chd8-knockdown samples from embryonic brain assessed using qRT-PCR (one-way ANOVA followed by Dunnett’s multiple comparison test; DF = 2, F = 12.94, P = 0.0005 for Fzd1; DF = 2, F = 16.97, P = 0.0006 for Fzd2; DF = 2, F = 27.08, P = 0.0002 for Dvl2; DF = 2, F = 10.21, P = 0.0016 for Dvl3; DF = 2, F = 16.39, P = 0.0001 for Ctnnb1; DF = 2, F = 10.38, P = 0.0015 for Ccnd1; Control, n = 9; Chd8 sh1, n = 4; Chd8 sh2, n = 5 for Fzd1, Dvl3 and Ctnnb1; Control, n = 7; Chd8 sh1, n = 3; Chd8 sh2, n = 3 for Fzd2 and Dvl2; Control, n = 10; Chd8 sh1, n = 5; Chd8 sh2, n = 5 for Ctnnb1. n, number of different FAC-sorted samples analyzed. (b) In utero TCF/LEF luciferase assay showing that Chd8 is a positive regulator of canonical Wnt signaling (one-way ANOVA followed by Bonferroni’s multiple comparison test; DF = 3, F = 47.29, P < 0.0001; Control; n = 17, Chd8 sh2; n = 5, hCHD8; n = 5, Chd8 sh2 + hCHD8; n = 6; n, number of different brain samples analyzed). (c) TCF/LEF luciferase assay in N2a cell line using both Chd8 shRNAs and rescue with human CHD8. Both Chd8 shRNAs resulted in reduction of Wnt activity in N2a cell line, which could be rescued via overexpression of human CHD8 (one-way ANOVA followed by Bonferroni’s multiple comparison test; DF = 6, F = 53.25, P < 0.0001 for +Wnt3A; DF = 2, F = 46.64, P = 0.0001 for -Wnt3A; Control; n = 65, Chd8 sh1; n = 4, Chd8 sh2; n = 4, Control + Wnt; n = 65, Chd8 sh1 + Wnt; n = 26, Chd8 sh2 + Wnt; n = 30, hCHD8 + Wnt; n = 39, Chd8 sh1 + hCHD8 + Wnt; n = 14, Chd8 sh2 + hCHD8 + Wnt; n = 14; statistical analysis was run separately when comparing Control condition with Wnt3a- and Wnt3a+ conditions). n, number of different cultured cell samples analyzed. (d) TCF/LEF luciferase assay in HEK293T cell line shows increased Wnt signaling activity following CHD8 knockdown (one-way ANOVA followed by Bonferroni’s multiple comparison test; DF = 2, F = 96.27, P < 0.0001; Control; n = 14, Control + Wnt; n = 14, CHD8 shA + Wnt; n = 14). n, number of different cultured cell samples analyzed. (e) TCF/LEF luciferase assay using cultured embryonic mouse neural progenitors showing decreased activity following Chd8 knockdown (one-way ANOVA followed by Bonferroni’s multiple comparison test; DF = 3, F = 13.19, P < 0.0001; Control; n = 7, Control + Wnt; n = 7, Chd8 sh1 + Wnt; n = 4, Chd8 sh2 + Wnt; n = 7). n, number of different cultured cell samples analyzed. (f) TCF/LEF luciferase assay using cultured human neural progenitors showing decreased activity following Chd8 knockdown (one-way ANOVA followed by Bonferroni’s multiple comparison test; DF = 2, F = 17.32, P < 0.0001; n = 12 for all conditions). n, number of different cultured cell samples analyzed. *P < 0.05; **P < 0.01; ***P < 0.001; ns., nonsignificant. Results are presented as mean ± s.e.m. (a,b,e and f). Shown are median, 25th and 75th percentile, and min and max value (c,d).
control or Chd8 shRNA expression constructs and co-expressed either control empty vector or stabilized β-catenin constructs, respectively. Following FAC-sorting of GFP+ cells at E15, the samples were subjected to RNA-seq as described above. Consistent with the effects of stabilized β-catenin on neurogenesis and Wnt luciferase activity, the expression of many dysregulated genes was normalized upon β-catenin stabilization (Supplementary Fig. 10a and Supplementary Data Set 11). This is consistent with the findings that LEF1 consensus motif (CTTGTG) is the most significantly enriched transcription factor binding motif among genes downregulated in response to Chd8 sh2 knockdown (Supplementary Data Set 5). Additionally, expression of both Ezh2 and Su(z)2 was restored to control levels following the expression of stabilized β-catenin (Supplementary Fig. 10b and Supplementary Data Set 11).

Together, these data underscored the role of Chd8 in maintaining Wnt signaling in embryonic cortical progenitors and indicated that impaired Wnt signaling following Chd8 knockdown underlies abnormal cortical progenitor proliferation, neuronal differentiation defects and transcriptional dysregulation of genes important for proper cortical development.

**Reduced Chd8 expression in upper-layer cortical neurons in developing brain results in behavioral abnormalities in adult mice**

To evaluate the possible outcome of reduced Chd8 function during cortical development in adult animals, we performed bilateral in utero electroporation at E15, targeting neurons destined to populate cortical layers 2/3, with either control or Chd8 sh2 along with membrane-bound GFP or cytoplasmic GFP. Bilateral targeting of upper-layer cortical neurons was examined in these animals following behavioral testing (Supplementary Fig. 11a,b). We observed similar basic locomotor activity between the groups (Supplementary Fig. 11d–h). Similarly, contextual fear conditioning tests revealed no differences in learning behavior between the two groups (Supplementary Fig. 11i). We then examined the conflict between exploration and risk avoidance using a light–dark box test. The Chd8 shRNA group exhibited significant reductions in entries to and amount of time spent in the light chamber compared to controls (Fig. 6a,b), suggesting increased aversion following Chd8 loss-of-function. We further employed the elevated-plus maze.

The Chd8 shRNA mice entered and spent significantly less time in the open arms of the maze compared to controls (Fig. 6c,d), further supporting the notion that reduced Chd8 expression in late cortical progenitor cells resulted in altered exploratory drive in adult mice.

One of the most commonly observed behavioral deficits in people with ASD and in other psychiatric patients is abnormal social interaction. We used the three-chamber social arena assay to examine the sociability of control and Chd8-knockdown mice. In this assay mice are allowed to explore the empty arena during an initial habituation period, followed by a period during which a novel mouse (stranger) is held inside a wire cage in one of the lateral chambers and an identical cage with a novel inanimate object (empty) is placed in the opposing lateral chamber. During the habituation period, both control and Chd8 shRNA mice explored the two lateral chambers equally (Supplementary Fig. 12a). When the novel mouse was introduced, as expected the control mice displayed a preference for the lateral chamber with the novel mouse, whereas the Chd8 shRNA group did not show a preference for either chamber (Fig. 6e). Furthermore, whereas control mice spent significantly more time in close interaction with the cage containing the novel mouse (within 5 cm), Chd8-knockdown mice did not show any preference for either cage (Fig. 6f). We did not observe any deficits in exploratory behavior as both groups spent similar amount of total time interacting with both the wire cages, suggesting that social interaction deficits in the Chd8 shRNA group cannot be attributed to lack of exploratory behavior (Supplementary Fig. 12b). These data suggest that knockdown of Chd8 in neural progenitors destined for upper layer cortical neurons manifests in reduced exploratory drive and abnormal social interaction behavior in adult mice.

Given that β-catenin overexpression could rescue the defects in Wnt signaling activity and cortical progenitor proliferation downstream of Chd8 loss-of-function (Fig. 5), we asked whether increased Wnt–β-catenin signaling could also rescue the behavioral abnormalities associated with Chd8 knockdown. To test this hypothesis, we performed in utero electroporation at E15 and expressed stabilized β-catenin alongside Chd8 shRNA (Supplementary Figure 11c). Co-expression of stabilized β-catenin rescued the number of entries to light chamber (Fig. 6a), amount of time spent in light chamber (Fig. 6b), number of entries to open arms (Fig. 6c) and time spent in open arms (Fig. 6d). Furthermore, the rescue group spent significantly more time in the chamber with novel social mouse (Fig. 6e) and more
time in close interaction (Fig. 6f). These results suggest that impaired Wnt–β-catenin signaling could underlie the behavioral deficits associated with Chd8 loss-of-function.

To determine whether knockdown of Chd8 expression in the developing cortex affects long-term neuronal abnormalities in the adult brain, we examined the number, localization, dendritic complexity...
Chd8 Knockdown in upper cortical layer neurons results in behavioral deficits, which can be rescued via induction of Wnt signaling. (a) Chd8-knockdown mice show reduced number of entries to the light chamber in light–dark chamber assay (one-way ANOVA followed by Bonferroni's multiple comparison test; DF = 2, F = 18.70, P < 0.0001; Control; n = 17, Chd8 sh2; n = 8, Chd8 sh2 + S/A-β-cat; n = 10). n, number of animals used. (b) Chd8-knockdown mice spent less time in the light chamber (one-way ANOVA followed by Bonferroni’s multiple comparison test; DF = 2, F = 9.521, P = 0.0006; Control; n = 17, Chd8 sh2; n = 8, Chd8 sh2 + S/A-β-cat; n = 10). n, number of animals used. (c) Number of entries to the open arms in elevated plus maze assay was significantly reduced in Chd8-knockdown mice (one-way ANOVA followed by Bonferroni’s multiple comparison test; DF = 2, F = 23.27, P < 0.0001; Control; n = 16, Chd8 sh2; n = 12, Chd8 sh2 + S/A-β-cat; n = 10). n, number of animals used. (d) Chd8 knockdown mice spent significantly less time in the light chamber (one-way ANOVA followed by Bonferroni's multiple comparison test; DF = 2, F = 16.54, P < 0.0001; Control; n = 16, Chd8 sh2; n = 12, Chd8 sh2 + S/A-β-cat; n = 10). n, number of animals used. (e) In the 3-chamber social interaction assay, Chd8-knockdown animals did not spend significantly more time in the chamber with the novel social animal (Stranger) relative to the chamber with the empty wire cage (Empty) compared to control or to the Chd8 sh2 + S/A-β-cat group (One-way ANOVA followed by Bonferroni’s multiple comparison test; DF = 2, F = 43.09, P < 0.0001 for Control; DF = 2, F = 3.167, P = 0.0552 for Chd8 sh2; DF = 2, F = 19.81, P < 0.0001 for Chd8 sh2 + S/A-β-cat; Control; n = 13, Chd8 sh2; n = 12, Chd8 sh2 + S/A-β-cat; n = 10). n, number of animals used. (f) Social interaction in close proximity (within 5 cm of the wire cages). Chd8-knockdown animals did not show preference for either the Stranger or the Empty cage (Two-tailed unpaired student’s t-test; t = 6.942, DF = 10, P < 0.0001 for Control; t = 0.4274, DF = 14, P = 0.6756 n = 8 for Chd8 sh2; t = 9.930, DF = 18, P < 0.0001, n = 10 for Chd8 sh2 + S/A-β-cat). (g) Images of upper cortical neurons from 5-month-old mouse brains transfected at E15 with either control shRNA or Chd8 shRNA and membrane-bound GFP. Scale bars: 20 µm. (h) Chd8 knockdown reduced the complexity of dendritic arborization assessed via Sholl analysis (two-way ANOVA followed by Bonferroni’s multiple comparison test; DF = 2, sum-of-squares = 4,226, mean square = 2,113, F = 269.5, P < 0.0001 for genotype factor; Control; n = 24 cells from 5 animals, Chd8 sh2; n = 52 cells from 7 animals, Chd8 sh2 + S/A-β-cat; n = 17 cells from 5 animals). * P < 0.05; **P < 0.01; ***P < 0.001; n.s., nonsignificant. Results are presented as mean ± s.e.m.
and spine density of neurons. For these studies, in utero electroporation was performed using shRNA constructs coelectroporated with either membrane-bound GFP or cytoplasmic GFP. We observed a significant reduction in the number of GFP+ cells in Chd8-knockdown brains (P = 0.0055; Supplementary Fig. 13a,b). Furthermore, Chd8 knockdown also resulted in mislocalization of GFP+ neurons in the adult brain (Supplementary Fig. 13a,c). Sholl analysis of dendritic arborization of layer 2/3 neurons revealed markedly reduced dendritic branching in Chd8 knockdown samples compared to control neurons (Fig. 6g,h). Additionally, measurement of total spine density on the secondary branches of apical dendrites showed significant reduction following Chd8 knockdown (P < 0.001; Supplementary Fig. 13d,e). Finally, increased Wnt–β-catenin signaling was sufficient to restore the complexity of dendritic arborization (Fig. 6g,h). Reduced dendritic spine density in Chd8-knockdown neurons was substantially increased following induction of Wnt–β-catenin signaling; however, it did not reach control levels (Supplementary Fig. 13d,e). These results are consistent with previous findings showing that induced Wnt–β-catenin signaling increases dendritic complexity and spine density41,42. In sum, our observations suggest that Chd8 plays a crucial role in establishing proper cortical circuitry and that the disruption of neuronal morphology and neuronal connectivity due to Chd8 loss-of-function could contribute to the observed behavioral abnormalities in adult mice.

**DISCUSSION**

Chd8 is essential for cortical progenitor proliferation and differentiation

Our results indicate that the chromatin remodeler Chd8 plays a crucial role in mammalian cortical development by promoting the proliferation of neural progenitors in two distinct ways. On the one hand, Chd8 directly binds to the promoters of cell cycle genes and facilitates their expression, while on the other, Chd8 also regulates neurogenic divisions by targeting the PRC2 complex. Previous studies have shown that the absence of Ezh2 drives neural progenitors in the developing cortex toward neurogenic division28. Together, these activities ensure the generation of appropriate numbers of neurons during cortical development.

An overwhelming body of literature on the role of CHD8 and other CHD proteins supports our findings. Depletion of Chd8 resulted in reduced cell proliferation in mammalian cell lines11,15,43,44. Chd8 is closely related to Chd6, 7 and 9. Chd7, an interacting partner of Chd8, has been shown to be necessary for proliferation of inner ear neuroblasts45 and NSCs in the olfactory epithelium46 of mice, as well as cellular proliferation in multiple tissues of zebrafish47. Deletion of ChdC in Dictyostelium, an ortholog of the mammalian Class III CHD group that contains Chd5–9, impairs cell growth due to reduced expression of cell cycle genes48. Interestingly, knockdown of chd8 in zebrafish caused an increase in the number of newborn neurons and resulted in macrocephaly, although there was a reduction in the number of enteric neurons in the gastrointestinal tract19,16. The reason for these differences is presently unclear. A more detailed and direct analysis of neural progenitor proliferation in zebrafish should clarify this matter. Our results suggest that embryonic Chd8 knockdown would result in reduced number of neurons in the adult brain. However, because our experiments are limited to analysis of NPCs, we cannot elucidate the role of Chd8 in development of other cell types such as astrocytes. It is possible that dysregulation of Chd8 in other cells could alter the size or number of these cells, contributing to the macrocephaly phenotype observed among people with ASD carrying CHD8 mutations.

**Chd8 positively regulates Wnt signaling in neural progenitor cells**

Canonical Wnt signaling plays important roles in brain development through the regulation of cell cycle progression and dendritic morphogenesis23. Defects in Wnt signaling have been strongly implicated in the development of ASD. Mutations in several pathway components have been identified in people with ASD, and convergent pathway analyses indicate that many apparently unrelated ASD mutations also impinge on the Wnt signaling pathway49. Earlier work on the function of Chd8 demonstrated that it negatively regulates Wnt signaling. Unexpectedly, however, we found that multiple components of canonical Wnt signaling were downregulated upon loss of Chd8 in neural progenitors. Our efforts to resolve these differences have led to the discovery that Chd8 regulates Wnt signaling in a cell-type-specific manner: whereas Chd8 loss-of-function increases Wnt signaling in non-neuronal cells, its loss attenuates Wnt signaling in neural progenitors. Cell-type-specific regulation by Chd8 is not unprecedented. The Drosophila ortholog of Chd8, Kismet, regulates hedgehog (hh) expression in the wing imaginal disc when expression of the repressor, Ci-75, is low50. However, Kismet is not necessary for the regulation of hh expression when Ci-75 expression is high, suggesting a context-dependent function50. A similar context-dependent mechanism could account for differences in regulation of Wnt signaling by Chd8. Overall, our observations suggest that whereas Chd8 promotes cell proliferation in both neural and non-neural cells, it selectively promotes Wnt signaling in neural progenitors.

**Sustained expression of Chd8 in the developing cortex is necessary for normal adult behavior**

Abnormal social interaction, anxiety and intellectual disability are some of the behavioral deficits observed among people with ASD carrying CHD8 mutations5. Our results indicate that restricted Chd8 loss-of-function in developing mouse brain also produces behavioral deficits such as abnormal social interaction. We found that normal adult behavior and proper cortical development were restored upon overexpression of stabilized β-catenin, indicating that behavioral abnormalities likely stem from abnormal cortical development. We did not observe any deficits in learning and memory; however, Chd8 knockdown was confined to specific layers of the developing cortex. It is possible that Chd8 loss-of-function in other regions of the developing brain may affect additional behaviors. Future studies on the effects of whole brain Chd8 loss-of-function on adult behavior would be of importance. Thus, it remains to be determined whether the mechanisms described here apply to germline loss-of-function mutations observed in ASD subjects.

In sum, our observations suggest that in the developing mouse brain, Chd8 maintains neural progenitor proliferation through regulation of cell cycle genes. Furthermore, our results raise the possibility that some of the ASD associated behavioral abnormalities in subjects carrying CHD8 mutations may be caused by impaired development of cerebral cortical regions. Though Chd8 may be regulating neural progenitor proliferation through different ways, induction of Wnt–β-catenin signaling is sufficient to mitigate cortical development and adult behavioral deficits caused by Chd8 knockdown.

**METHODS**

Methods, including statements of data availability and any associated accession codes and references, are available in the online version of the paper.

**Accession Codes:** NCBI Gene Expression Omnibus (GEO): GSE72442.
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**ONLINE METHODS**

**DNA constructs.** Control nontargeting shRNA (Sigma, SHC002), sh1 and 2 targeting Chd8 were obtained from Sigma (sh1, Clone ID: NM_201637.1-14391s1c1 and sh2, Clone ID: NM_201637.3-33423c1c1). The sequences can be found in **Supplementary Table 1**. Full length hCHD8 was cloned into mammalian expression pCMV-TOPFLASH, a gift from Dr. R. Moon (University of Washington, WA) and a renilla-Luc-TK reporter (pRL-TK, Promega) were used for testing TCF/LEF transcriptional activity. pcAGIG-Venus was provided by Dr. Z. Xie (Boston University, MA). The membrane-bound GFP (pcAGIG-GAP 43-GFP) construct was gift from A. Gartner (University of Leuven, Belgium).

**PCAG-β-catenin (gift of Dr. Karun Singh, McMaster University, Canada) was constructed by cloning a β-catenin DNA sequence containing a stabilizing S37A mutation upstream of the IRES in pcAGIG.**

**Antibodies and reagents.** The following primary antibodies were used in this study: rabbit anti-CHD8 antibody (A301-224A, Bethyl Laboratories; ChIP), rabbit anti-Chd8 antibody (noncommercialized, Cell Signaling; 1:1,000 dilution; WB), chicken anti-gFP antibody (GFP-1020, Aves Labs; 1:1,000 dilution; IHC), mouse anti-BrdU antibody (M0744, Clone Bu20a, DakoCytomation; 1:500; IHC), rabbit anti-Ki67 antibody (Clone SP6, Lab Vision/Thermo Scientific; 1:500 dilution; HIC), rabbit anti-phospho-histone H3 (Ser10) antibody (06-570, Millipore; 1:500 dilution; IHC), mouse anti-Tuj1 antibody (MMS-435P, Covance; 1:500 dilution; IHC) and mouse anti-EZH2 antibody (AC22, Active Motif; 1:500 dilution, WB). Alexa-conjugated secondary antibodies (Jackson ImmunoResearch; 1:1,000 dilution) were used for IHC and ICC. Recombinant mouse Wnt1 (gift of Dr. Sh TAKEI, Japan) and mouse EGF (Santa Cruz) were used at 1:1,000 dilution. Secondary antibodies used for western blot were anti-rabbit (1:10,000, Santa Cruz) and anti-goat (1:5,000, Santa Cruz) antibodies. Primary antibodies used for western blot were anti-Smad3 (Cell Signaling) and anti-pSmad3 (Cell Signaling).

**Animals.** All animal work was approved by the Committee for Animal Care of the Division of Comparative Medicine at the Massachusetts Institute of Technology. Swiss Webster pregnant female mice were purchased from Taconic (Hudson, NY, USA) for *in utero* electroporation. Mice were housed in groups of 3–4 on standard 12-h light / 12-h dark cycle.

**In situ hybridization probe design.** RNA antisense probes were generated by PCR-amplifying mouse cDNA with primers to amplify exons 3–10 of the *Chd8* gene (Supplementary Table 1). The reverse primer contains a T7 RNA polymerase recognition sequence (TAATACGACTCACTATAGGG; *Supplementary Table 1*). The resulting PCR product was gel extracted and in vitro transcribed using a DIG-RNA labeling kit (Roche).

**In situ hybridization.** E12 embryos and E16 brains were fixed overnight in RNase-free 4% formaldehyde. Brains were embedded in 30% sucrose–PBS and frozen in O.C.T. (TissueTek). Cryosections (10 µm) were incubated with the DIG-labeled RNA antisense probe (1:1,000 in hybridization buffer) overnight at 65 °C, washed in 1× SSC/50% formamide/0.1% Tween-20 three times for 30 min each at 65 °C followed by 1× MABT for 30 min at room temperature (22–25 °C). Sections were blocked with 20% heat-inactivated sheep serum/2% blocking reagent (Roche)/1× MABT for 1 h and then incubated with anti-DIG antibody (Roche; 1:2,000 overnight). Sections were washed with 1× MABT four times for 20 min each, preblotted with 100 mM NaCl/50 mM MgCl2/100 mM Tris pH 9.5/0.1% Tween-20 twice for 10 min, followed by staining with NBT/BCIP (Roche; 4.5 µl/mL and 3.5 µl/mL, respectively, in prebluent buffer) for 2 h. Sections were washed with 1× PBS three times for 15 min, incubated in xylene three times for 5 min, and mounted with VectaMount (Vector Laboratories).

**In utero electroporation.** The Institutional Animal Care and Use Committee of Massachusetts Institute of Technology approved all experiments. In *in utero* electroporation was performed as described elsewhere51. Briefly, pregnant Swiss Webster mice were anesthetized by intraperitoneal injections of ketamine 1% / 10% (Sigma) and delivered 3–4 on standard 12-h light / 12-h dark cycle.

**Immunohistochemistry.** Brain sections. Following dissection, embryonic cortical brains were drop-fixed overnight in 4% paraformaldehyde (PFA) and then transferred to 30% sucrose/PBS solution at 4 °C. Brains were embedded in O.C.T. compound (Electron Microscopy Sciences) and sliced into 14-µm sections using cryostat. Cryosections were rehydrated with 1× PBS and blocked for 1 h with blocking solution (1× PBS + 10% donkey serum + 0.3% Triton-X). Following blocking, the cryosections were incubated with primary antibodies overnight at 4 °C. Incubation with secondary antibodies was performed for 1 h at room temperature. Finally, cryosections were mounted using ProLong Gold Antifade Reagent (Invitrogen).

**Fluorescent-activated cell sorting (FACS).** GFP-transfected cortices from E15 embryos electroporated at E13 were microdissected using a fluorescent dissection microscope to increase concentration of GFP-labeled cells. Cortices from 3–5 embryos were pooled and cells were enzymatically dissociated and resuspended in HBSS. GFP-positive cells were isolated using the FACs Aria 1 (BD Biosciences) system. Gating for GFP fluorescent cells was set using nontransfected cortical cells. Cells were directly sorted into RLT (QIAGEN RNeasy Plus Kit) buffer.

**Genome-wide RNA-sequencing and bioinformatic analyses.** Following total RNA isolation from FAC-sorted GFP-positive cells using RNeasy Plus Kit (Qiagen), RNA was quality controlled and quantified using an Agilent 2100 Bioanalyzer. Next, poly-A purified samples were converted to cDNA using the Clontech Low Input Library Prep Kit (Cat#: 634947) by MIT BioMicro Center. High-throughput sequencing was performed using the Illumina HiSeq 2000 platform at the MIT BioMicro Center. The raw FASTQ data files of 40-bp or 75-bp paired-end reads were collected for downstream analysis. Sequencing reads were aligned to the mouse genome assembly (mm9) using TopHat2 (version 2.0.12)52 with inner distance between mate pairs = 200, segment length = 200, no coverage search to speed up the process and default values for other parameters. Differential expression analysis was performed using the Cuffdiff module of Cufflinks (version 2.1.1)53 with default parameters, and FPKM values were normalized by the geometric method that Cuffdiff recommends. Significantly altered genes were the genes with both fold change greater than 1.2 and P-values less than 0.05 between two groups.
The numbers of genes defined as ‘NOTEST’ (not enough alignments for testing, threshold 10) were 10,500 for sh1 and 9377 for sh2. No genes were defined as ‘LOWDATA’ (too complex or shallowly sequenced; the default setting of Cuffdiff) for sh1 or for sh2. One gene defined as ‘HIDATA’ (too many fragments in locus, threshold 500,000) for sh1 and none for sh2. The numbers of genes defined as ‘FAIL’ (ill-conditioned covariance matrix or other numerical exception preventing testing; a default value) were 3 for sh1 and 1 for sh2. The numbers of filtered genes defined as ‘OK’ for the differential expression test were 12,731 for sh1 and 13,857 for sh2. The number of tested genes with at least a 1.2-fold change between WT and KD groups was 5,615 for sh1 and 5,245 for sh2. The number of differentially expressed genes with at least 1.2 fold change and P < 0.05 was 3,762 for sh1 and 5,245 for sh2.

Comparisons of each biological replicate showed Pearson coefficients > 0.65 (Supplementary Fig. 4b). To compare to human data sets, mouse gene names were converted to human homologs using MGI annotation database (http://www.informatics.jax.org/homology.shtml). Gene ontology was assessed using TopGene (https://toppgene.cchmc.org/) and DAVID (https://david.ncifcrf.gov/) web servers. The raw data are available online (NCBI GEO GSE72442).

Human brain data link to Chd8 KD RNA-seq data. Human developmental transcriptome data set (Gencode v10, summarized to genes) was acquired from BRAINSPAN database (http://www.brainspan.org/static/download.html). CHD8 expression patterns from early fetal stage to childhood were extracted for comparison. The SFARI ASD-risk gene list was acquired from the SFARI gene database (http://gene.sfari.org). The statistical significance of overlap between human ASD risk genes and human homologs of mouse Chd8 knockdown RNA-seq genes was calculated using a hypergeometric probability test.

Mouse E12 forebrain chromatin state analysis. Mouse E12 forebrain ChIP-seq raw data for seven histone marks (H3K4me1, H3K4me2, H3K4me3, H3K27ac, H3K27me3, H3K9ac and H3K36me3) were downloaded from ENCODE (https://www.encodeproject.org/), contributed by the Bing Ren group at UCSD and mapped to mouse genome assembly (mm9) using the BWA aligner (version 5, samse option)34. Duplicate reads were removed using Samtools35. Mouse genome segmentation based on combinatorial histone marks was trained using ChromHMM33 with the default parameters. In brief, sequencing reads were counted in non-overlapping 200-bp bins across the whole genome; each bin was binarized with either 1 for enriched or 0 for nonenriched based on a Poisson P-value (threshold 1 × 10−4) that compares ChIP-seq signals against whole-cell extract signals. Models with different numbers of states (from 10 to 14 were tested), and a model with 13 states was chosen. Annotation of those chromatin states was based on known classification of histone marks and relative enrichment of genomic and nonneural regions of reference genes. Mouse Chd8, Ezh2 and Suz12 ChIP-seq peak files were directly downloaded or called using raw data files downloaded from GEO database (GSE57369 (ref. 12), GSE31655 (ref. 34), GSE27148 (ref. 35), mm9 assembly) for chromatin state enrichment analysis. Human CHD8 ChIP-seq peak files (GSE57369 (ref. 12) and GSE61487 (overlap of three antibodies)34) were converted to mm9 assembly using the liftover tool for enrichment analysis.

Overlap of ChIP-seq data with Chd8 KD RNA-seq data. ChIP-seq peaks were further associated to the nearest annotated genes based on the distance to the transcription start sites using HOMER36 for overlap analysis with RNA-seq data. Histone ChIP-seq signal intensity files (bedgraph format, normalized against input data) were generated using MACS2 program37. A custom Perl script embedded into NeuEpi-Analyzer (http://bioinfopil46.mit.edu/neuepi/) was used to calculate ChIP-seq signal intensity around transcription start sites (TSS) for all annotated genes. Intensity signals in each 100-bp bin were averaged for a detection window ranging from −5 kb to 5 kb around TSS. To compare ChIP-seq signals for different gene groups, aggregated average bin signals across the genes in a particular group were computed and plotted using a custom R script. Unless specified, overlapped genes were counted using NeuVenn-Analyzer (http://bioinfopil46.mit.edu/neueven/).

Chromatin immunoprecipitation (ChIP). Chromatin immunoprecipitation was performed using SimpleChIP Plus Enzymatic Chromatin IP Kit (Magnetic Beads, 9005, Cell Signaling Technology) as described by the manufacturer. Briefly, tissue from 6 mouse cortices (embryonic day 12) were pooled for each experiment. Cortical cell samples were finely minced and cross-linked with formaldehyde (1.5% final concentration) for 20 min at room temperature, chilled on ice with 10x glycine and washed once with ice cold PBS. Samples were passed through a 30G needle to homogenize and create single cell suspension. Cells were resuspended in 1x Buffer A containing 0.1 mM DTT and incubated on ice for 10 min, followed by incubation in 1x Buffer B containing 0.1 mM DTT, Micrococal Nuclease was added to mix and incubated for 20 min at 37 °C, vortexing every 3 min. The reaction was stopped with 50 mM EDTA (final concentration). The centrifuged pellet was resuspended in 1x ChIP buffer containing protease inhibitor cocktail. Resuspended nuclei were sonicated using a bioruptor (medium power, 30 s on/30 s off for 10 cycles). Sheared chromatin was immunoprecipitated with 5 µg antibodies against Chd8 (A301-224A, Bethyl Laboratories) and IgG overnight at 4 °C. Next day, ChIP-grade protein-G magnetic beads were added and incubated for 2 h at 4 °C. Cross-linked protein–DNA complexes were eluted from the beads using 1x ChIP elution buffer and incubated 65 °C for 30 min, followed by reversion of crosslinking with NaCl and Proteinase K (65 °C for 2 h). Finally, DNA was purified and samples were subjected to qRT-PCR using the indicated primer (Supplementary Table 1).

Western blot analysis. Transfected cells were lysed and run on 6% sodium dodecyl sulfate-polyacrylamide gels at 60–120 constant voltage to separate them, and transferred onto Immobilon-P PVDF membranes (Millipore) at a constant voltage (80 V for 6 h). Membranes were blocked using 3% bovine serum albumin prepared in TBS-T (50 mm Tris-HCl (pH 7.4), 150 mm NaCl, 0.1% Tween-20). Membranes were incubated with the primary antibodies overnight at 4 °C. Next, they were washed three times with TBS-T, followed by incubation with horseradish peroxidase-conjugated secondary antibodies (GE Healthcare, Pittsburgh, PA, USA) for 1 h at room temperature. Following washing with TBS-T, immunoreactivity signals were detected by enhanced chemiluminescence (Perkin-Elmer, Waltham, MA, USA).

Luciferase assay. Luciferase assays were performed as described elsewhere38.

In vitro. N2a and HEK293T cells at 1 × 105, mNPC and hNPC at 3 × 105 cells per well density were plated into 24-well plates without antibiotics. Cells were transfected with 0.8 µg of shRNA plasmid along with 50 µg of Super8KTOPFLASH and 10 ng of pRL-TK. The media were replaced with one containing antibiotics 2 h after transfection. 36 h after transfection, cells were stimulated with recombinant mouse Wnt3a for 12 h, in Wnt-stimulated condition. For the rescue experiments with hCHD8, 0.2 µg of hCHD8 was cotransfected with 0.6 µg of Chd8 shRNA. For the rescue experiments with S/A β-catenin, 0.1 µg of S/A β-catenin was cotransfected with 0.7 µg of Chd8 shRNA.

In vivo. The following ratio was used; shRNA or overexpression constructs (15) to pCAGG-Venus (3) to 8XSuperTOPFLASH (3) to pRL-TK (1). In vitro electroporation was done at E13 and the GFP-positive cortical region was dissected using fluorescent dissection microscopy to enrich for luciferase activity. Tissue was lysed using 1× Passive Lysis Buffer (E1941, Promega). TCF/LEF reporter activity was measured using the Dual-Luciferase Reporter System (Promega). Firefly luciferase activity was normalized to Renilla luciferase activity in all conditions.

Behavioral experiments. For behavioral analysis of Chd8 knockdown and Control mice, 10-week-old animals were used. Detailed information on the ages of animals can be found on Supplementary Table 2. All animals were handled for 3 d before each experiment. All behavioral experiments were performed during the light cycle. The investigator was blinded to the group allocation both during the experiment and when assessing outcomes. Ear-tag identification numbers were used until outcome analyses were finalized. Mice that were tested on multiple behavioral paradigms were given a minimum 1-week resting period between experiments. Three Control animals and 4 shRNA animals were excluded from the study due to unsuccessful bilateral targeting of cortex (i.e., GFP+ cells were found only in one hemisphere or no transfection occurred), and randomization of experimental groups was not required.

Open field test. Mice were placed in an open field arena (40 cm × 40 cm × 30 cm) and activity was measured over a 10-min period with VersaMx software.

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Total time moving, total distance moved and time spent in center and margin were measured in 5-min bins using a grid of infrared light beams and analyzed using the software.

**Light–dark exploration test.** An apparatus (40 cm × 40 cm) consisting of two chambers, a black box and a light chamber each covering half of the arena (20 cm × 20 cm each), was used for the light/dark exploration test. A mouse was placed into the dark side and allowed to explore the arena for 10 min. The number of visits to light chamber and the time spent in the light and dark chambers were measured.

**Elevated plus maze.** Mice were allowed to explore the elevated plus maze apparatus, consisting of four arms (two open and two closed arms; each arm 45 cm × 9.5 cm; the height of closed arms 29.5 cm). The activity of each mouse was recorded for 10 min. Time spent and number of entries to each arm was hand-scored following the experiment. An entry was defined as a mouse having front paws and half of the body on the arms.

**Three-chamber social interaction.** The three-chamber social interaction apparatus consisted of a black acrylic box with three chambers (20 cm × 35 cm). Side chambers were connected to the middle chamber through 5-cm openings that can be closed or open. Wire cages placed in the side chambers were cylindrical with bottom diameters of 10 cm and bars spaced 1 cm apart. For testing, test animals were placed into the middle chamber and allowed to habituate/explore the empty arena (with empty wire cages in place) for 10 min. Following the first 10 min, an age-matched novel mouse (Stranger) was placed into one of the wire cages, and an inanimate object in the other wire cage. The test animal was allowed to explore for an additional 10 min. The activity of each test mouse was video recorded. Time spent in each chamber and time spent in close interaction with wire cages (within 5 cm and facing the cages) were hand-scored using the recorded videos.

**Confocal imaging.** Images were taken with a Zeiss LSM 510 confocal microscope. Brightness and contrast were adjusted using ImageJ where needed.

**Statistics.** All plots were generated and statistical analyses were performed using Graphpad Prism 5.0 software. Results are presented as mean ± s.e.m. Sample size was not predetermined but numbers of samples are consistent with previous publications. Two-tailed t-tests were used for comparison of two data sets. One-way ANOVA followed by either Dunnett’s (when comparing to control conditions) or Bonferroni multiple comparison tests were used for experiments with three or more data sets. Equal variances between groups and normal distributions of data were presumed but not formally tested. Molecular and biochemical analyses were performed using a minimum of three biological replicates per condition. Behavioral experiments require larger data sets due to increased variability. A minimum of 6 animals per group was used for behavioral testing. Significance threshold was set to $P = 0.025$ to correct for two parameters used for each behavioral test.

A Supplementary Methods Checklist is available.

**Data availability.** Additional data that support the findings of this study are available upon reasonable request.

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