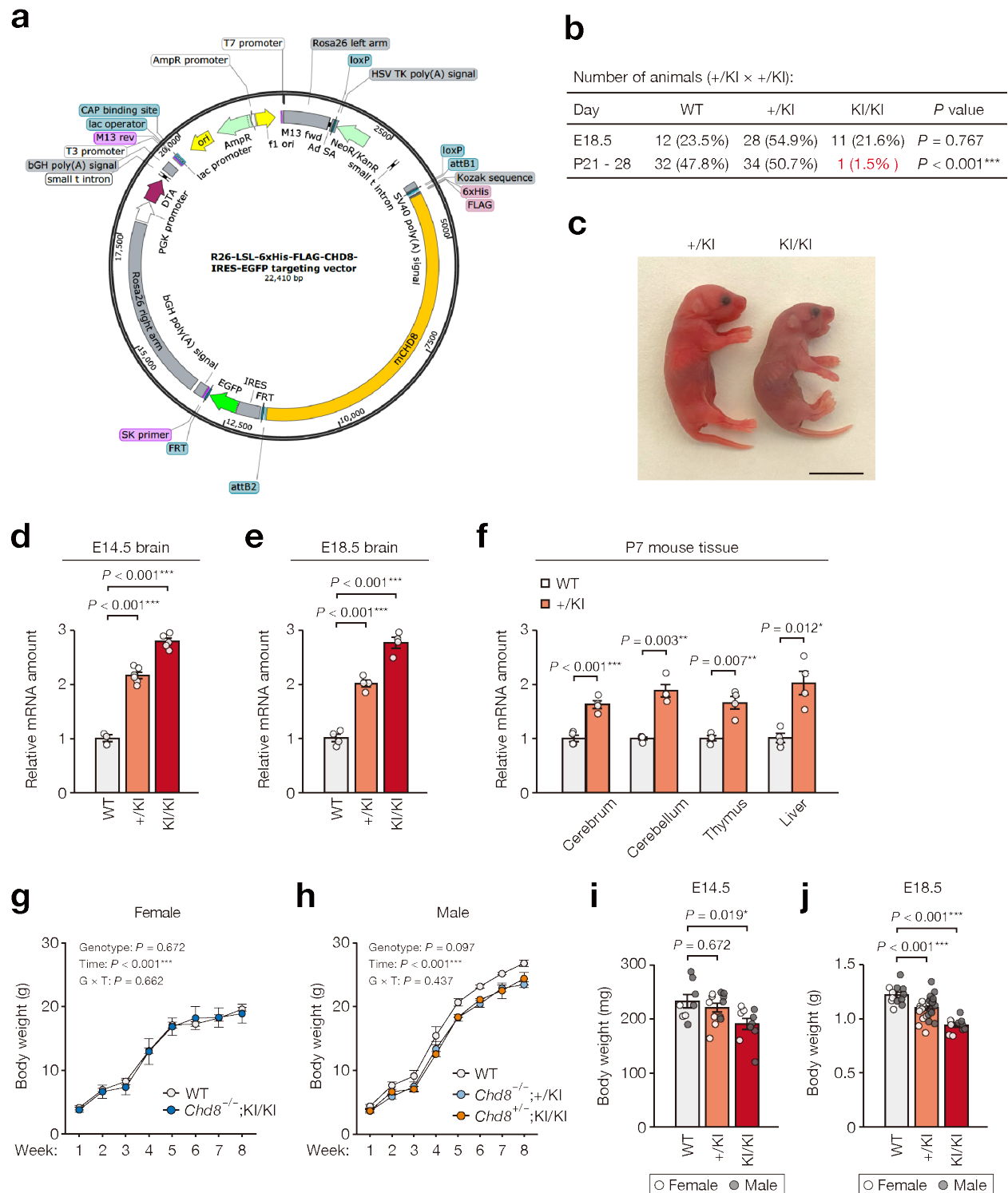


Supplementary Information

Duplication of the autism-related gene *Chd8* leads to behavioral hyperactivity and neurodevelopmental defects in mice

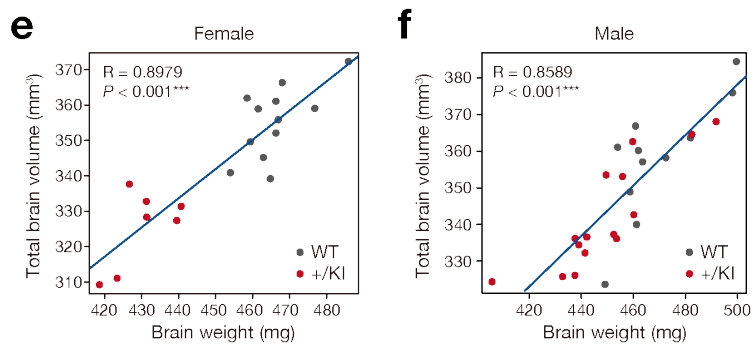
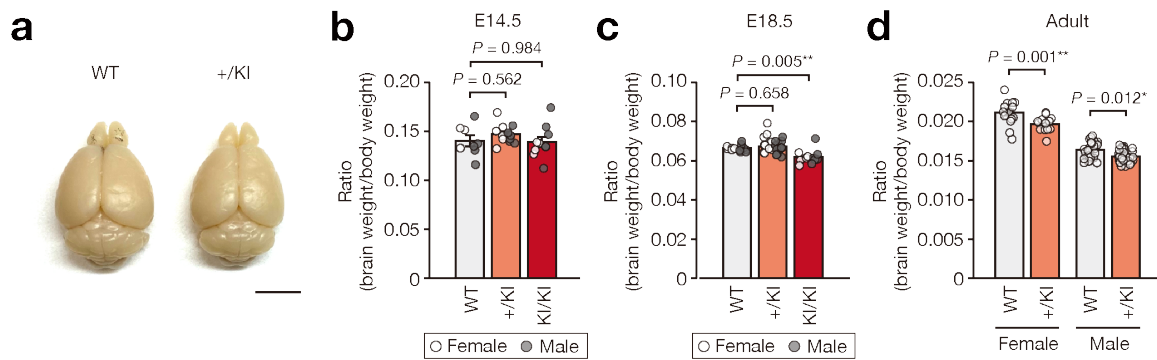
Atsuki Kawamura, Kazuki Fujii, Kota Tamada, Yoshifumi Abe, Kenta Nitahara, Tomoya Iwasaki, Sho Yagishita, Kenji F. Tanaka, Toru Takumi, Keizo Takao & Masaaki Nishiyama

Supplementary Figures 1–11

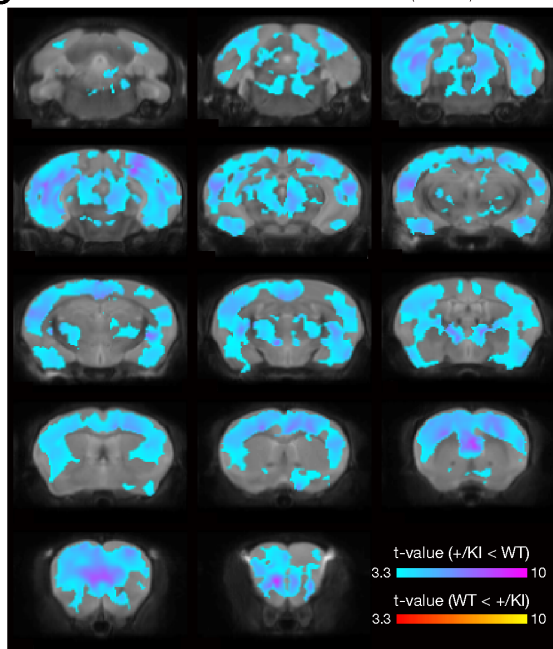


Supplementary Fig. 1 *Chd8* KI/KI mice die after birth and manifest growth retardation. **a**, Map for the targeting vector that includes a splice acceptor site (SA), a neomycin resistance gene

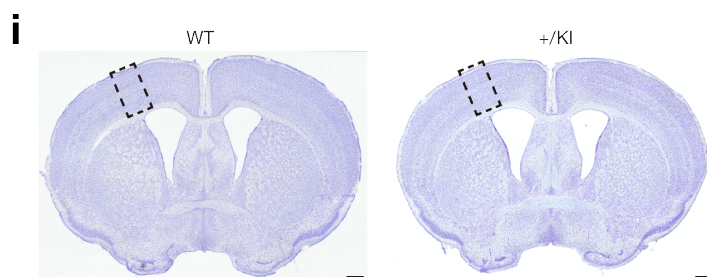
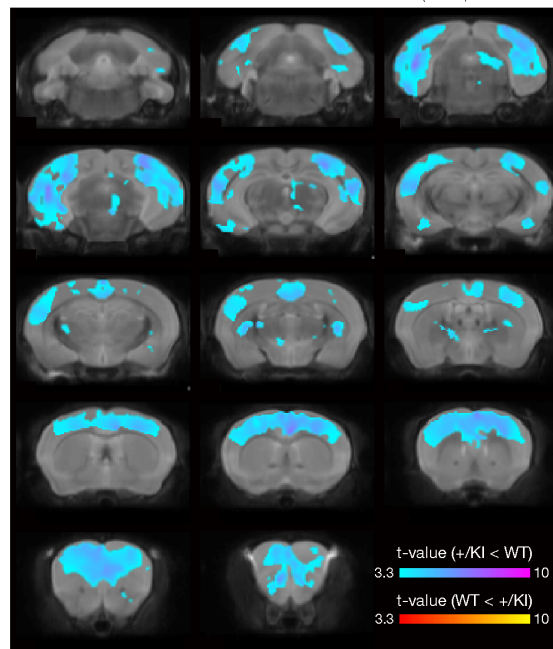
(neo) with a stop cassette flanked by loxP sequences, cDNA encoding hexahistidine- and FLAG-tagged mouse CHD8, a frt-flanked IRES-EGFP cassette, and a bovine polyadenylation sequence (pA). Plasmid map was generated using SnapGene software (from Dotmatics; available at snapgene.com). **b**, Genotype frequencies for progeny of *Chd8* KI mouse intercrosses at E18.5 and postnatal day (P) 21 to 28. Most KI/KI mice die immediately after birth, although rare animals survive to adulthood. **c**, Gross appearance of *Chd8* KI and KI/KI littermates at P0.5. Scale bar, 1 cm. **d,e**, RT and real-time PCR analysis of *Chd8* mRNA abundance in the brain of WT ($n = 3$), *Chd8* KI ($n = 6$), and KI/KI ($n = 5$) mice at E14.5 (**d**) and of WT ($n = 4$), *Chd8* KI ($n = 4$), and KI/KI ($n = 4$) mice at E18.5 (**e**). **f**, RT and real-time PCR analysis of *Chd8* mRNA abundance in the cerebrum, cerebellum, thymus, and liver of WT and *Chd8* KI mice at P7 ($n = 4$ mice per genotype). **g,h**, Body weight for WT ($n = 4$) and *Chd8*^{-/-};KI/KI ($n = 2$) female mice (two-way repeated-measures ANOVA with Tukey's post hoc test: genotype effect, $P = 0.672$; time effect, $P < 0.001$; $G \times T$, $P = 0.662$) (**g**) and for WT ($n = 3$), *Chd8*^{-/-};+/KI ($n = 4$), and *Chd8*^{+/-};KI/KI ($n = 2$) male mice (two-way repeated-measures ANOVA with Tukey's post hoc test: genotype effect, $P = 0.097$; time effect, $P < 0.001$; $G \times T$, $P = 0.437$) (**h**) from 1 to 8 weeks of age. **i,j**, Body weight for WT ($n = 3$ female, 5 male), *Chd8* KI ($n = 6$ female, 6 male), and KI/KI ($n = 4$ female, 6 male) mice at E14.5 (two-way ANOVA with Tukey's post hoc test: genotype effect, $P = 0.018$; sex effect, $P = 0.466$; $G \times S$, $P = 0.197$) (**i**) and for WT ($n = 5$ female, 7 male), *Chd8* KI ($n = 11$ female, 17 male), and KI/KI ($n = 5$ female, 6 male) mice at E18.5 (two-way ANOVA with Tukey's post hoc test: genotype effect, $P < 0.001$; sex effect, $P = 0.023$; $G \times S$, $P = 0.818$) (**j**). All quantitative data with the exception of those in **b** are means $\pm$ s.e.m. P values were determined by the chi-square test in **b**, by one-way ANOVA with Tukey's post hoc test in **d** and **e**, and by the unpaired Student's t test in **f**. See Supplementary Data 3 for more detailed statistics. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.



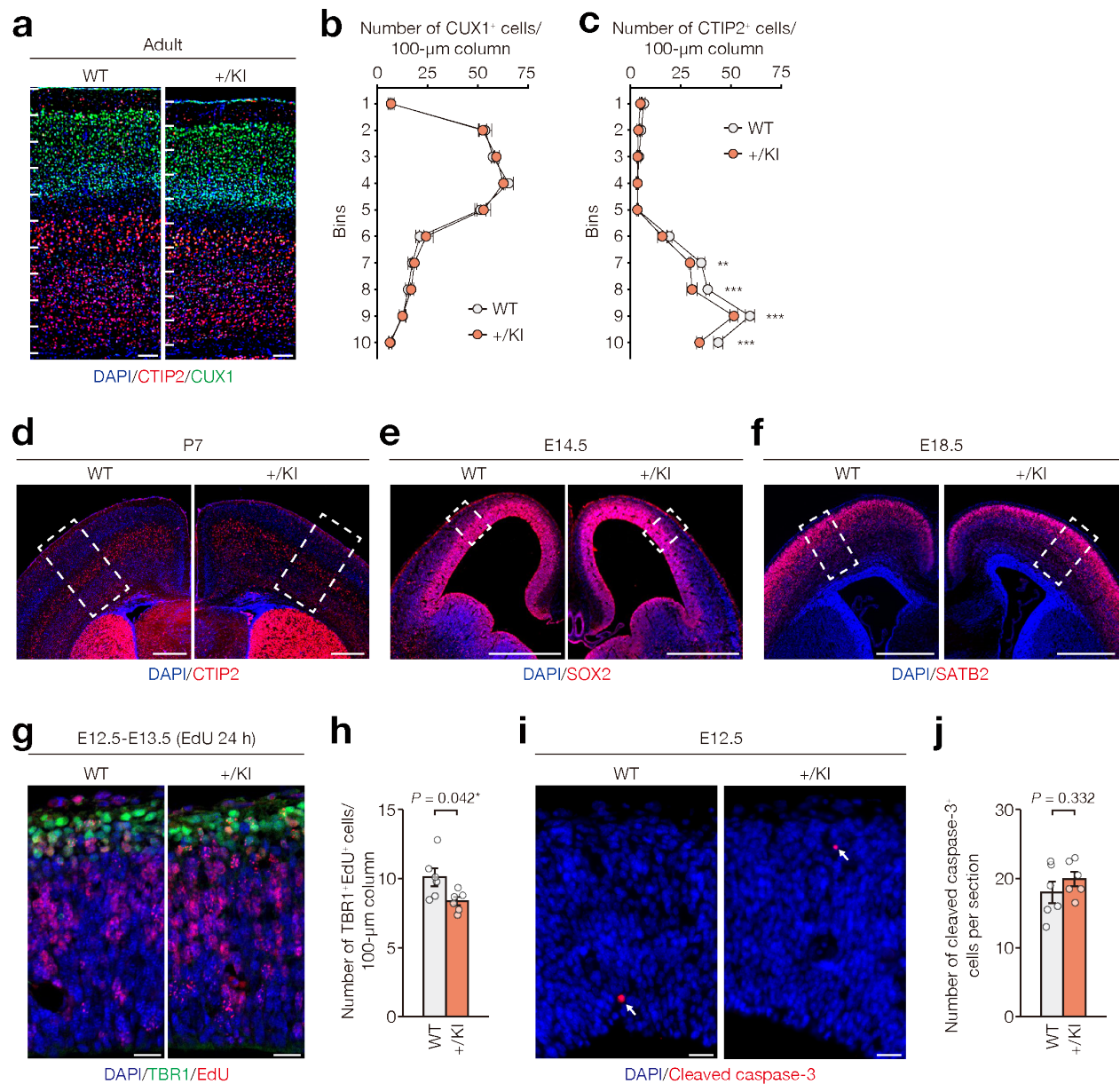
g Brain volume differences: +/KI vs. WT (female)



h Brain volume differences: +/KI vs. WT (male)

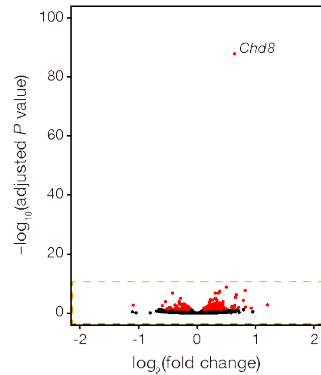
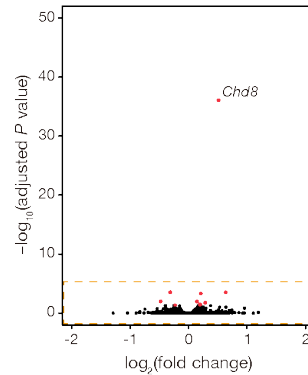
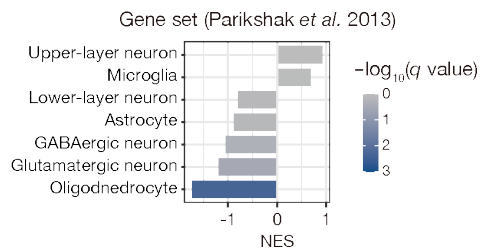


Supplementary Fig. 2 *Chd8* KI mice manifest microcephaly. **a**, Gross appearance of the brain for WT and *Chd8* KI mice at 10 weeks of age. Scale bar, 5 mm. **b–d**, Brain to body weight ratio for WT ($n = 3$ female, 5 male), *Chd8* KI ($n = 6$ female, 6 male), and KI/KI ($n = 4$ female, 6 male) mice at E14.5 (two-way ANOVA with Tukey's post hoc test: genotype effect, $P = 0.390$; sex effect, $P = 0.863$; $G \times S$, $P = 0.399$) (**b**), for WT ($n = 5$ female, 7 male), *Chd8* KI ($n = 11$ female, 17 male), and KI/KI ($n = 5$ female, 6 male) mice at E18.5 (two-way ANOVA with Tukey's post hoc test: genotype effect, $P < 0.001$; sex effect, $P = 0.215$; $G \times S$, $P = 0.156$) (**c**), and for WT ($n = 16$) and *Chd8* KI ($n = 15$) female mice and WT ($n = 30$) and *Chd8* KI ($n = 30$) male mice at 13 to 15 weeks of age (two-way ANOVA with Tukey's post hoc test: genotype effect, $P < 0.001$; sex effect, $P < 0.001$; $G \times S$, $P = 0.199$) (**d**). **e,f**, Correlation between brain weight and total brain volume determined by MRI analysis for *Chd8* KI and WT mice at 8 weeks of age. Pearson correlation coefficient: $R = 0.897$, $P < 0.001$ for female (**e**); $R = 0.858$, $P < 0.001$ for male (**f**). **g,h**, Whole-brain voxel-wise comparisons of gray matter volume for WT ($n = 12$) and *Chd8* KI ($n = 7$) female mice (**g**) and for WT ($n = 13$) and *Chd8* KI ($n = 13$) male mice (**h**) at 8 weeks of age. The significance threshold for the voxel-wise whole-brain analysis was set at a P value corrected for cluster-level family-wise error (FWE) of <0.05 , with an individual voxel threshold of an uncorrected P value of 0.001. **i**, Nissl staining of coronal sections for the brain of WT and *Chd8* KI mice at 12 weeks of age. Scale bars, 500 μm . The region enclosed by the black dashed lines indicates the area where cortical thickness was measured (as in Fig. 1n,o). Quantitative data in **b** to **d** are means $\pm$ s.e.m. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

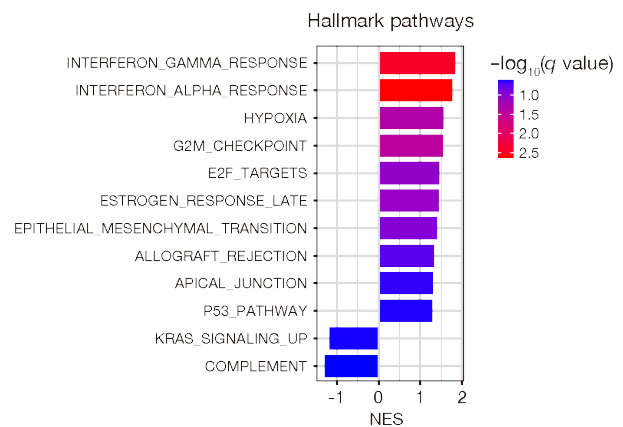
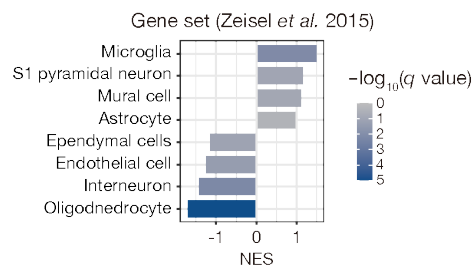
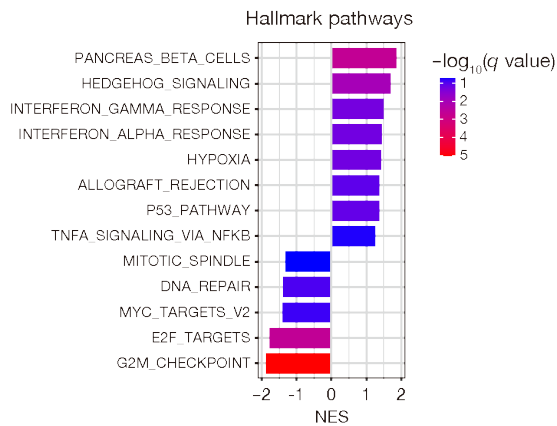


Supplementary Fig. 3 *Chd8* overexpression impairs generation of cortical neurons. a–c, Immunofluorescence staining of CTIP2 (a marker for deep-layer neurons) and CUX1 (a marker for upper-layer neurons) (a) and distribution of the number of CUX1⁺ cells (b) and CTIP2⁺ cells (c) across cortical bins per 100-μm column of coronal sections for the somatosensory cortex of WT and *Chd8* KI male mice at 11 to 15 weeks of age ($n = 5$ per genotype). Two-way repeated-measures ANOVA with Tukey's post hoc test: genotype effect, $P = 0.597$; bin effect, $P < 0.001$;

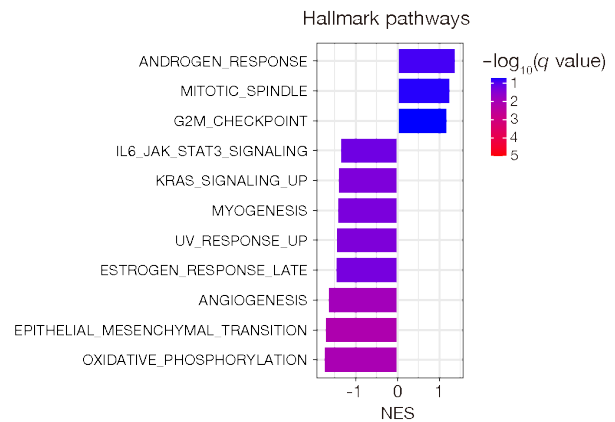
$G \times B$, $P = 0.956$ (**b**); genotype effect, $P < 0.001$; bin effect, $P < 0.001$; $G \times B$, $P = 0.001$ (**c**). Nuclei were stained with DAPI. Scale bars, 100 μm . **d–f**, Immunofluorescence staining of CTIP2, SOX2, and SATB2 in coronal sections for the brain at P7 (**d**), E14.5 (**e**), and E18.5 (**f**), respectively. The region enclosed by the white dashed lines indicates the area as shown in Figure 2c, e, and k, respectively. Nuclei were stained with DAPI. Scale bars, 500 μm . **g,h**, Immunofluorescence staining of TBR1 and chemical staining of EdU (**g**) as well as quantification of cells positive for TBR1 and EdU per 100- μm column (**h**) in coronal sections of the lateral cortex of WT ($n = 1$ female, 5 male) and *Chd8* KI ($n = 2$ female, 4 male) mice at 24 h after pulse-labeling with EdU at E12.5. The P value was determined with the unpaired Student's t test. Nuclei were stained with DAPI. Scale bars, 20 μm . **i,j**, Immunofluorescence staining of cleaved caspase-3 (a marker for apoptotic cells) (**i**) and quantification of cells positive for cleaved caspase-3 (**j**) in the brain of WT ($n = 2$ female, 4 male) and *Chd8* KI ($n = 3$ female, 3 male) mice at E12.5. The P value was determined with the unpaired Student's t test. Arrows indicate cells positive for cleaved caspase-3. Nuclei were stained with DAPI. Scale bars, 20 μm . All quantitative data are means $\pm$ s.e.m. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

a +/KI vs. WT (E14.5 forebrain)**b** +/KI vs. WT (E18.5 forebrain)**c** +/KI vs. WT (adult cortex)**e**

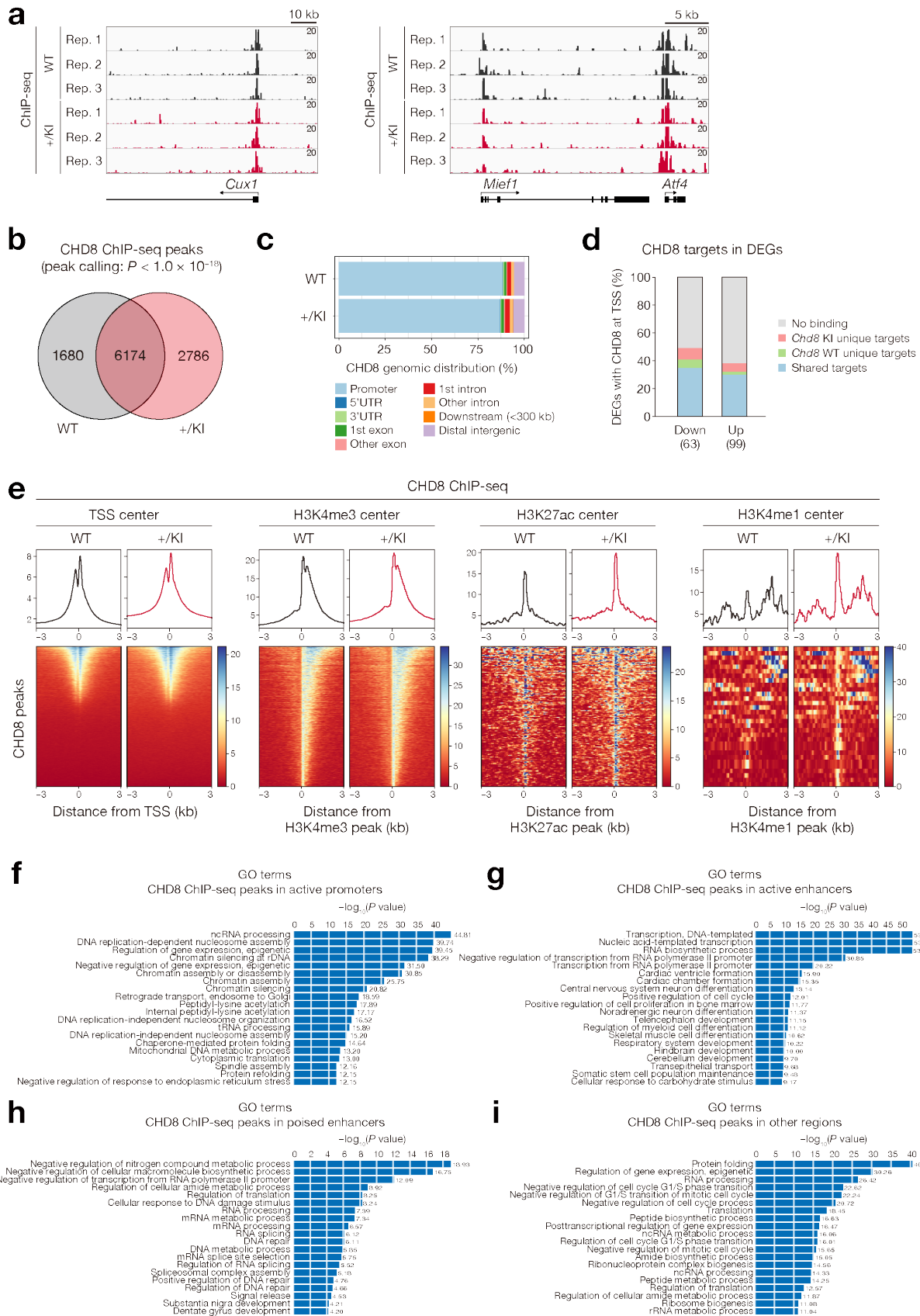
+/KI vs. WT (E14.5 forebrain)

**d** +/KI vs. WT (adult cortex)**f** +/KI vs. WT (E18.5 forebrain)**g**

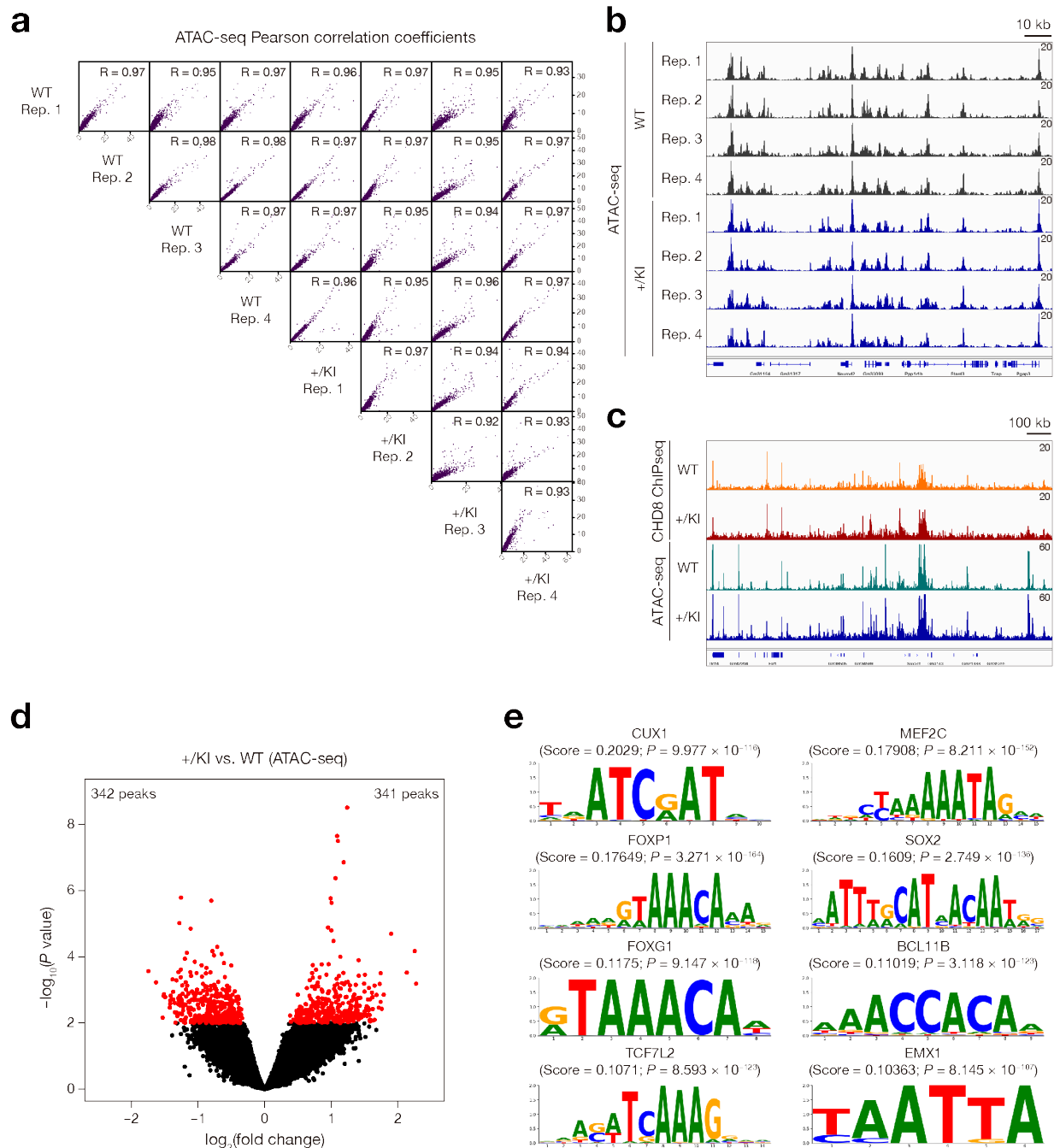
+/KI vs. WT (adult cortex)



Supplementary Fig. 4 *Chd8* duplication alters the expression of interferon response– and cell cycle–related genes. **a,b**, Volcano plots for DEGs in the forebrain of *Chd8* KI mice compared with that of WT mice ($n = 5$ mice per genotype) at E14.5 (**a**) or E18.5 (**b**). The area below the orange dashed line in each panel is shown enlarged in Figure 3a and b, respectively. DEGs (FDR-adjusted P value of <0.05) are shown in red. Source data are provided in Supplementary Data 1. **c,d**, GSEA for cell type–specific gene sets from Parikshak *et al.*⁶² (**c**) or Zeisel *et al.*⁶³ (**d**) in the cortex of *Chd8* KI mice compared with that of WT mice at 8 weeks of age. **e–g**, GSEA for hallmark pathways in the forebrain of *Chd8* KI mice compared with that of WT mice at E14.5 (**e**) or E18.5 (**f**) as well as in the cortex of *Chd8* KI mice compared with that of WT mice at 8 weeks of age (**g**).

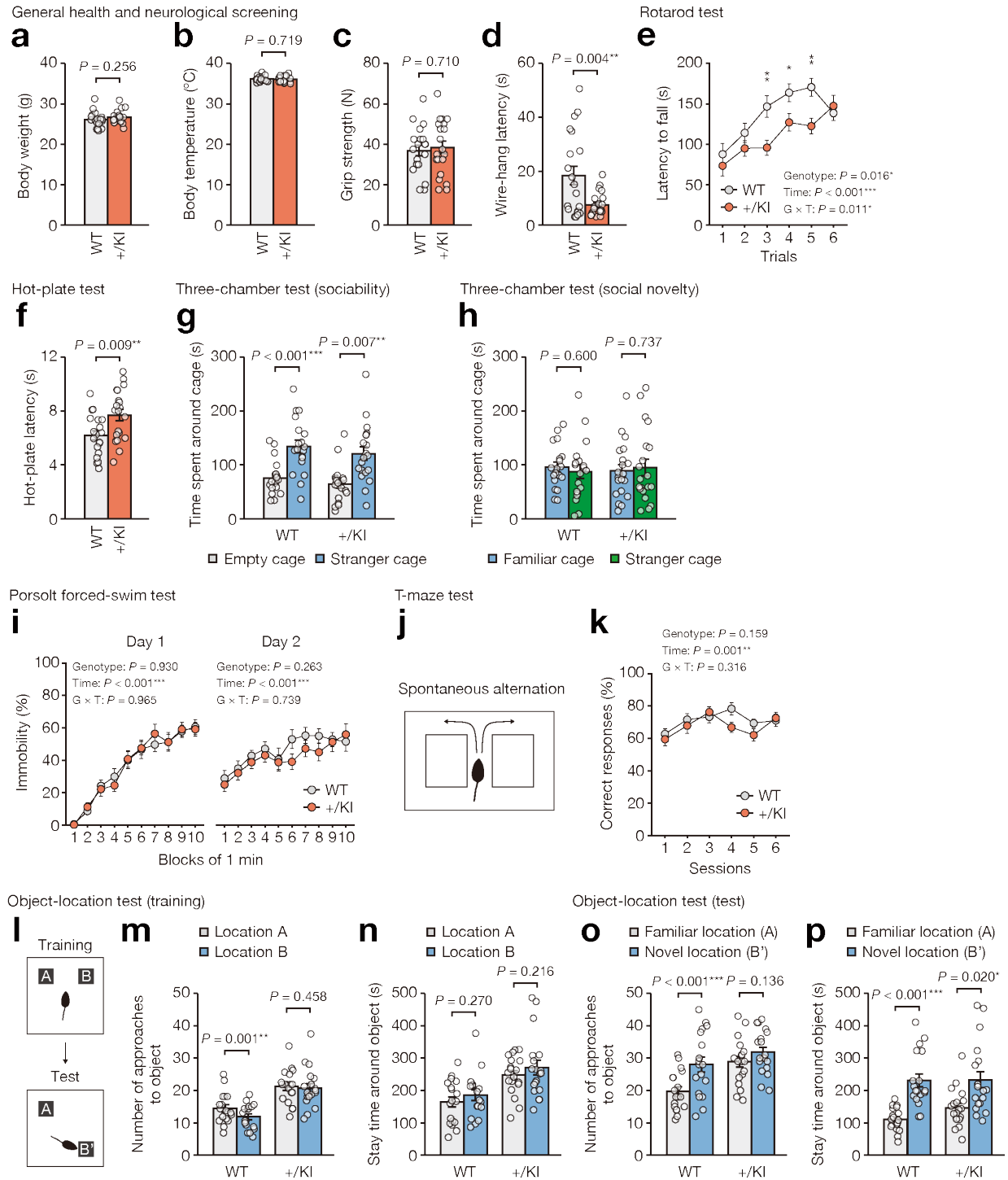


Supplementary Fig. 5 Aberrant CHD8 binding to enhancer regions related to neuron development in *Chd8* KI mice. **a**, CHD8 ChIP-seq data for representative genes in each replicate as viewed in the Integrative Genomics Viewer browser. **b–d**, Venn diagram showing overlap of CHD8 ChIP-seq peaks between the WT and *Chd8* KI mouse forebrain at E14.5 (**b**), composition of CHD8 binding peaks (**c**), and binding of CHD8 to the TSS of DEGs identified for the forebrain of *Chd8* KI versus WT mice at E14.5 (**d**). These analyses were performed for CHD8 peaks identified with the use of a more stringent peak-calling threshold ($P < 1 \times 10^{-18}$) than that adopted in Figure 4a–c. **e**, Heat map clustering and signal density for CHD8 ChIP-seq data derived from the forebrain of WT and *Chd8* KI mice at E14.5. The region spanning 3 kb upstream to 3 kb downstream of the center of the TSS or of H3K4me3, H3K27ac, and H3K4me1 peaks was compared. **f–i**, GREAT analysis characterizing the biological terms most significantly associated with genes proximal to CHD8 binding peaks in active promoter (**f**), active enhancer (**g**), poised enhancer (**h**), and other (**i**) regions.



Supplementary Fig. 6 Motif enrichment analysis of ATAC-seq footprints identifies changes in transcription factor binding in the embryonic forebrain of *Chd8* KI mice. **a**, Scatter plot analysis comparing four independent biological replicates (Rep. 1–4) for ATAC-seq profiles in the forebrain of WT and *Chd8* KI mice at E14.5. The Pearson correlation coefficient (R) is

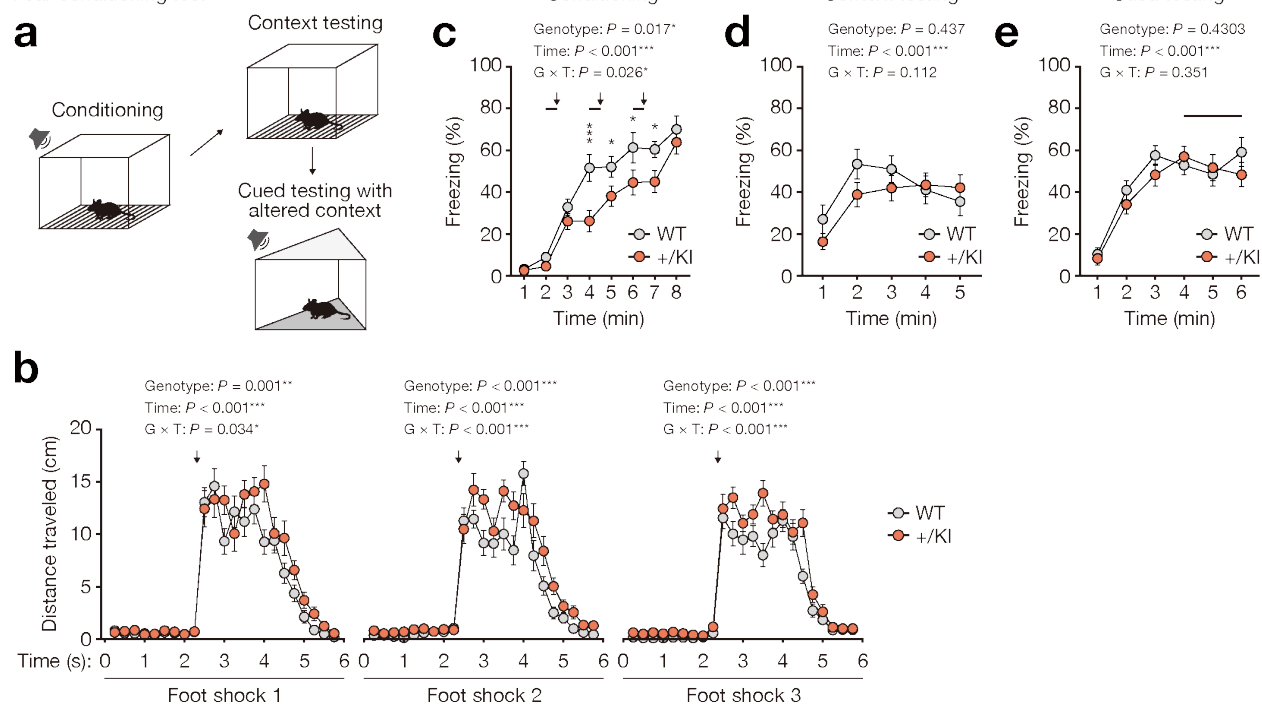
shown for each plot. **b**, ATAC-seq data for representative genes in each replicate as viewed in the Integrative Genomics Viewer browser. **c**, CHD8 ChIP-seq and ATAC-seq data for representative genes viewed in the Integrative Genomics Viewer browser. **d**, Volcano plot for accessible regions identified by ATAC-seq analysis in the forebrain of *Chd8* KI mice compared with that of WT mice at E14.5 ($n = 4$ mice per genotype). DARs (P value of <0.01) are highlighted in red. **e**, Motifs of representative ASD-related transcription factors identified from differential footprinting analysis of ATAC-seq signals in the forebrain of *Chd8* KI versus WT mice at E14.5.



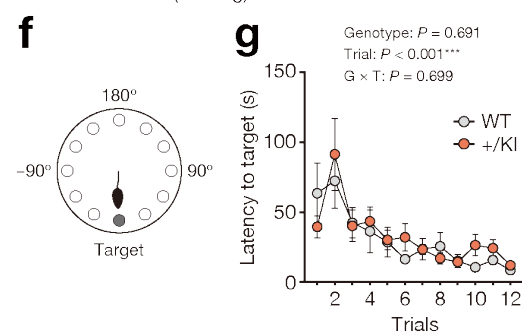
Supplementary Fig. 7 *Chd8* KI mice manifest defects in nociception and motor function. **a–d**, Body weight (**a**), body temperature (**b**), grip strength (**c**), as well as latency to falling in the

wire-hang test (**d**) for male mice of the indicated genotypes at 11 weeks of age ($n = 20$ per genotype). **e**, Latency to falling for 12- to 13-week-old *Chd8* KI ($n = 20$) and WT ($n = 19$) male mice in the rotarod test. **f**, Latency to the first hind-paw response for 12-week-old *Chd8* KI ($n = 20$) and WT ($n = 19$) male mice in the hot-plate test. **g,h**, Time spent around each cage for the three-chamber test of sociability (**g**) or social novelty (**h**) as performed with *Chd8* KI ($n = 20$) and WT ($n = 19$) male mice at 13 weeks of age. **i**, Percentage of immobility time in the Porsolt forced-swim test performed with *Chd8* KI ($n = 19$) and WT ($n = 20$) male mice at 13 to 14 weeks of age. **j,k**, Schematic representation of the experimental procedure for (**j**) and percentage correct responses during the first six training sessions in (**k**) the T-maze spontaneous-alternation task performed with *Chd8* KI ($n = 18$) and WT ($n = 19$) male mice at 27 to 28 weeks of age. **l–n**, Schematic representation of the experimental procedure for (**l**) as well as the number of encounters with each object (**m**) and time spent around each object (**n**) during the training sessions in the novel object–location test performed with *Chd8* KI ($n = 18$) and WT ($n = 19$) male mice at 31 to 32 weeks of age. **o,p**, The number of encounters with each object (**o**) and time spent around each object (**p**) during the test session in the novel object–location test performed with *Chd8* KI ($n = 18$) and WT ($n = 19$) male mice at 31 to 32 weeks of age. All data are means $\pm$ s.e.m. *P* values were determined by the unpaired Student's *t* test (**a–d,f**), two-way repeated-measures ANOVA with Tukey's post hoc test (**e,i,k**), or the paired Student's *t* test (**g,h,m–p**). **P* < 0.05, ***P* < 0.01, ****P* < 0.001. See Supplementary Data 3 for more detailed statistics.

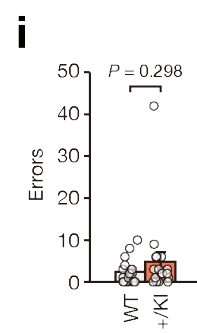
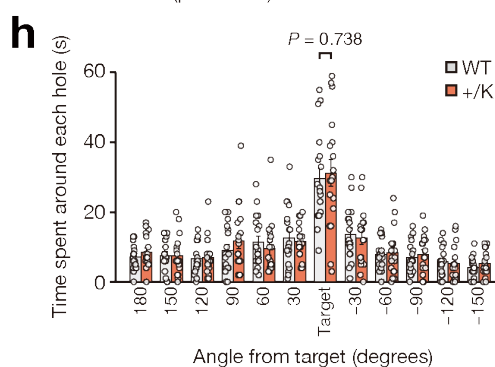
Fear-conditioning test



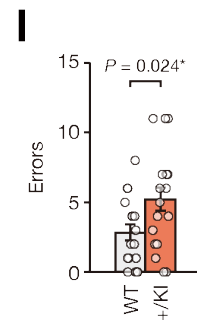
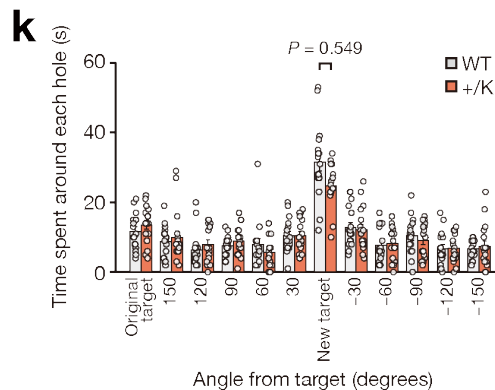
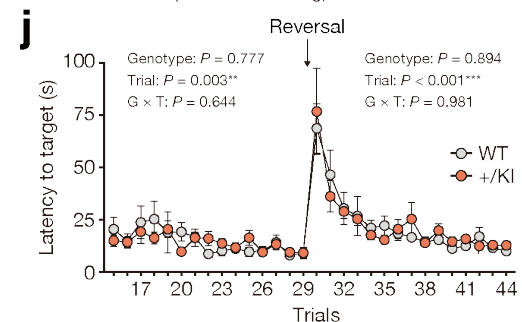
Barnes maze test (training)



Barnes maze test (probe trial)

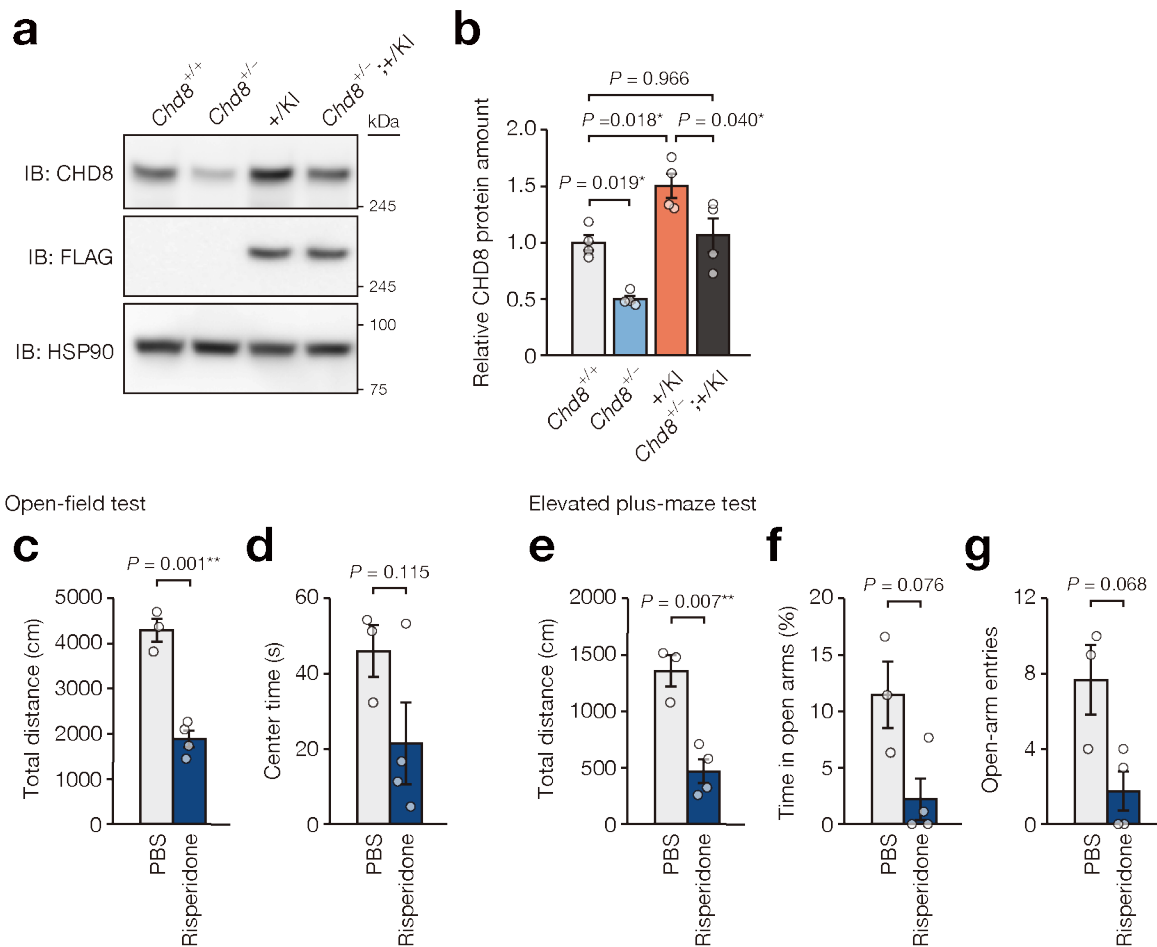


Barnes maze test (reversal learning)



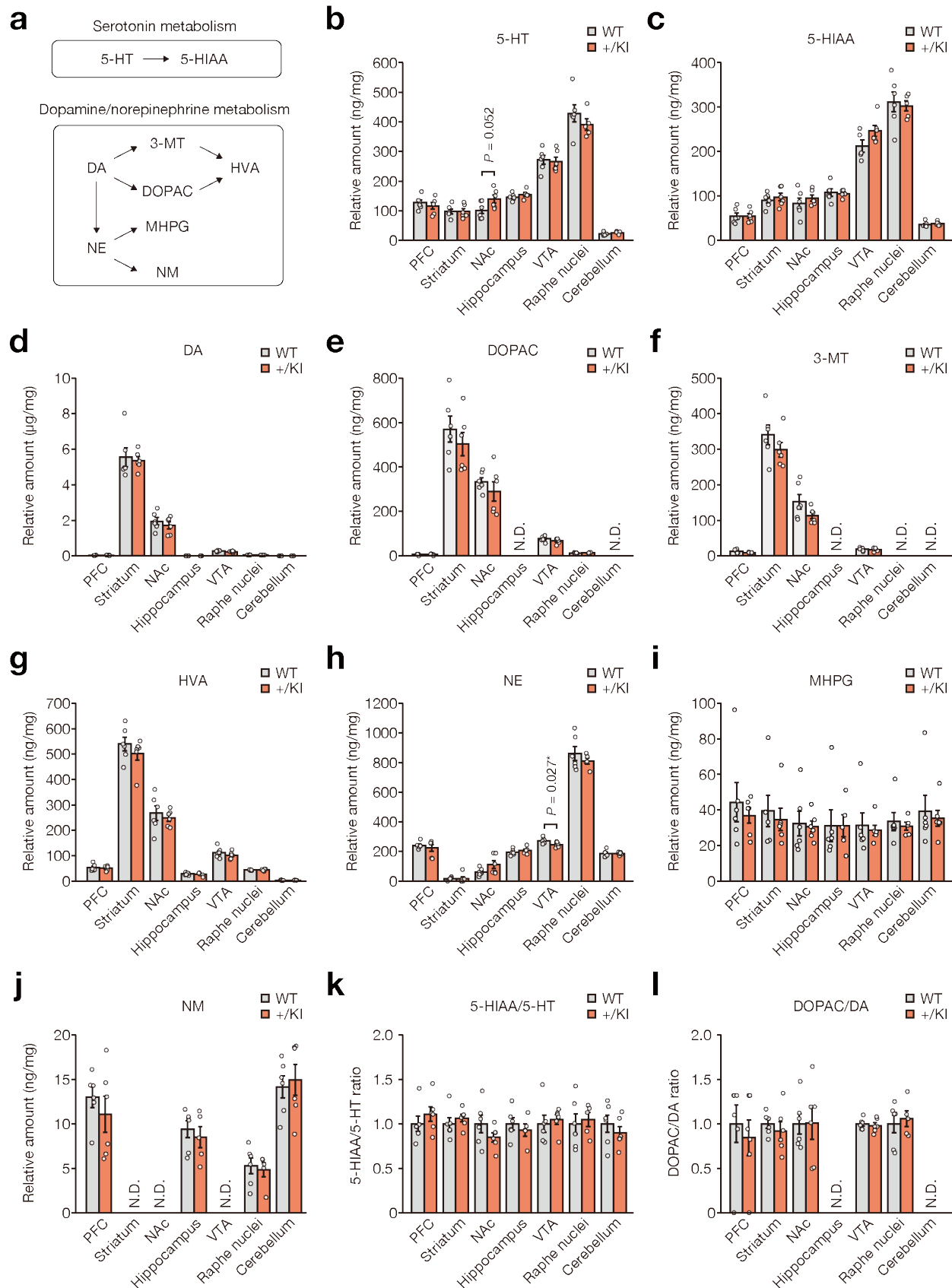
Supplementary Fig. 8 *Chd8* KI mice manifest mild memory deficits and behavioral

inflexibility. a–e, Schematic representation of the experimental procedure for (a) as well as the distance traveled during foot shock (b) and percentage freezing time for each minute of conditioning (c), context testing (d), and cued testing with an altered context (e) in the contextual and cued fear-conditioning test performed with *Chd8* KI ($n = 17$) and WT ($n = 17$) male mice at 66 to 67 weeks of age. Black bars and arrows represent tone and foot shock, respectively. **f,g**, Schematic representation of the experimental procedure for (f) and latency to reach the target hole during the 12 training trials in (g) the Barnes maze test performed with *Chd8* KI ($n = 18$) and WT ($n = 18$) male mice at 53 to 54 weeks of age. **h,i**, Time spent around each hole (h) as well as the number of errors before reaching the target hole (i) after removal of the escape box for the probe trial of the Barnes maze test performed the day after completion of training trials with the mice in g. **j–l**, Latency to reach the target in reversal training trials (j) as well as the time spent around each hole (k) and number of errors before reaching the new target hole (l) in the probe trial for the reversal phase of the Barnes maze test performed with *Chd8* KI ($n = 17$) and WT ($n = 17$) male mice at 57 to 60 weeks of age. All data are means $\pm$ s.e.m. *P* values were determined by two-way repeated-measures ANOVA with the Tukey post hoc test (b–e,g,j) and the unpaired Student's *t* test (h,i,k,l). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. See Supplementary Data 3 for more detailed statistics.

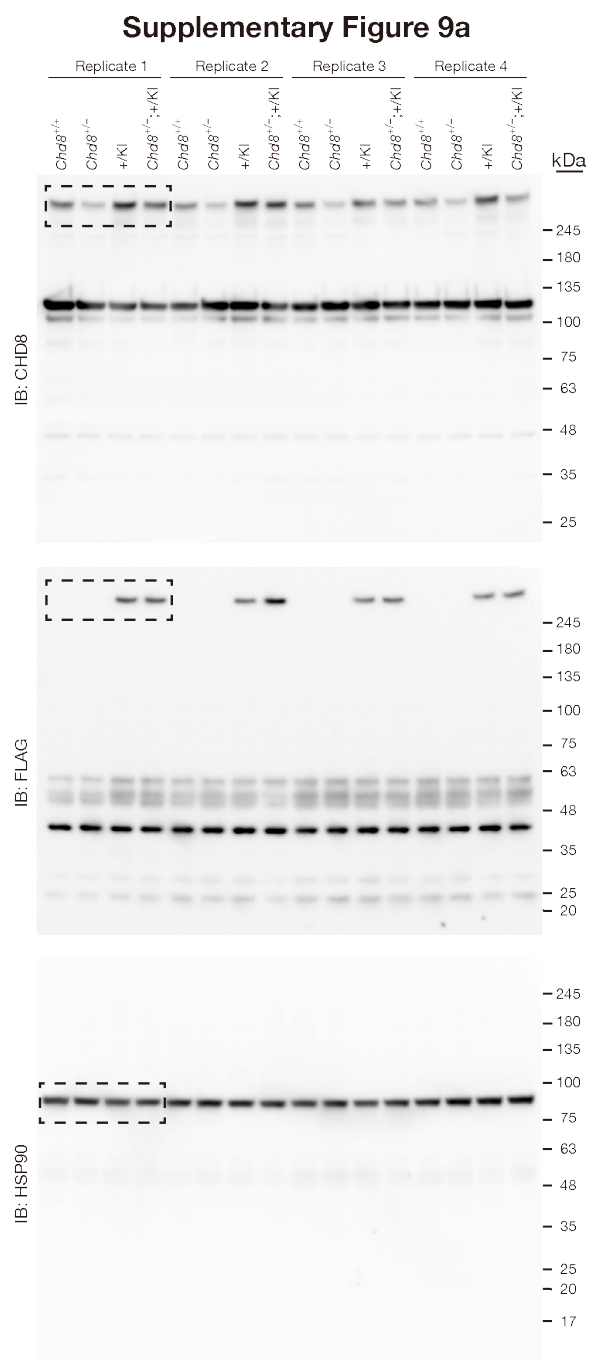
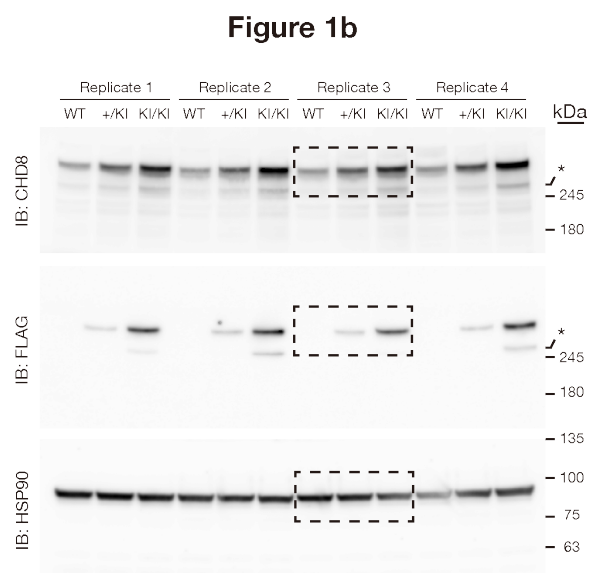


Supplementary Fig. 9 Expression level of CHD8 in *Chd8*^{+/-}; *Chd8* KI mice is similar to that in WT mice. **a**, Representative immunoblot (IB) analysis of CHD8, FLAG, and HSP90 (loading control) for the whole brain of WT, *Chd8*^{+/-}, *Chd8* KI, and *Chd8*^{+/-}; *Chd8* KI mice at E14.5. **b**, Quantification of the abundance of CHD8 (CHD8/HSP90 band intensity ratio) for $n = 4$ mice per genotype. The P values were determined by one-way ANOVA with Tukey's post hoc test. Source data are provided in Supplementary Figure 11. **c,d**, Total distance traveled (**c**) and time spent in the central area (**d**) for the open-field test performed with WT mice treated with PBS vehicle or risperidone. **e–g**, Total distance traveled (**e**), time spent in the open arms (**f**), and number of entries into the open arms (**g**) for the elevated plus-maze test performed with WT mice treated with vehicle or risperidone. Risperidone (0.2 mg/kg) was administered by daily intraperitoneal

injection for 7 consecutive days, with the final injection being administered 1 h before behavioral analysis. All data are means $\pm$ s.e.m. P values were determined with the unpaired Student's t test. $*P < 0.05$, $**P < 0.01$.



Supplementary Fig. 10 Measurement of monoamine content in brain regions of *Chd8* KI and WT mice. **a**, Serotonin and dopamine/norepinephrine metabolites analyzed. 5-HT, 5-hydroxytryptamine (serotonin); 5-HIAA, 5-hydroxyindoleacetic acid; DA, dopamine; DOPAC, 3,4-dihydroxyphenylacetic acid; 3-MT, 3-methoxytyramine; HVA, homovanillic acid; NE, norepinephrine; MHPG, 3-methoxy-4-hydroxyphenylglycol; NM, normetanephrine. **b–l**, Concentrations of 5-HT (**b**), 5-HIAA (**c**), DA (**d**), DOPAC (**e**), 3-MT (**f**), HVA (**g**), NE (**h**), MHPG (**i**), and NM (**j**) as well as turnover of 5-HT (5-HIAA/5-HT ratio) (**k**) and dopamine (DOPAC/DA ratio) (**l**) in the prefrontal cortex (PFC), striatum, nucleus accumbens (NAc), hippocampus, ventral tegmental area (VTA), raphe nuclei, and cerebellum of WT and *Chd8* KI mice at 9 to 12 weeks of age. All data are expressed as nanograms or micrograms of metabolite per milligram of protein and are means $\pm$ s.e.m. *P* values were determined with the unpaired Student's *t* test. **P* < 0.05. N.D., not detected.



Supplementary Fig. 11 Uncropped immunoblots for the data shown in Figure 1b and Supplementary Figure 9a. Boxes indicate regions shown in Figure 1b and Supplementary Figure 9a. Asterisks indicate degradation products of CHD8.