



Published in final edited form as:

*Nat Genet.* 2017 August ; 49(8): 1239–1250. doi:10.1038/ng.3906.

## Kmt1e regulates a large neuron-specific topological chromatin domain

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### Abstract

We report locus-specific disintegration of megabase-scale chromosomal conformations in brain after neuronal ablation of *Kmt1e/Setdb1* histone H3-lysine 9 methyltransferase, including a large

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**Competing Financial Interest:** The Authors declare no conflicts of interest.

**Author Contributions:** Performed experiments: Y.J., P.Raj., T.H., B.K., B.H., S-M.H., B.J., L.K., R.P., S.C., C.D., C.P., J.TCW, B.S. Conceived and designed experiments: Y.J., S.A. Performed statistical analyses: Y.J.. Bioinformatics and genomic analyses: E.L., P.Raj., W.L., P.Rou., L.S.. Contributed materials: A.S., B.R. (G9a/Glp transcriptome data); B.L., E.N. (mouse stress model/transcriptome data). Supervised the DNA methylation analysis: B.T. Supervised research: H.M., K.B., T.Y., L.S., S.A.. Wrote the paper: Y.J., Y-H. E.L., B.T., L.S., S.A. with contributions from co-authors.

topologically associated 1.2Mb domain conserved in human and mouse and encompassing >70 genes at the *clustered Protocadherin (cPcdh)* locus. TAD<sup>*cPcdh*</sup> in mutant neurons showed abnormal accumulations of CTCF transcriptional regulator and 3D genome organizer at cryptic binding sites, converted into permissive state with DNA cytosine hypomethylation and histone hyperacetylation. Broadly upregulated expression across *cPcdh* included defective S-type *Protocadherin* single-cell stochastic constraint. Setdb1-dependent loop formations, bypassing 0.2–1Mb of linear genome, radiated from TAD<sup>*Pcdh*</sup> fringes towards *cPcdh* cis-regulatory sequences, counterbalanced shorter-range facilitative promoter-enhancer contacts and carried loop-bound polymorphisms associated with genetic risk for schizophrenia. We show that KRAB-zinc finger Setdb1 repressor complex, shielding neuronal 3D genomes from excess CTCF binding, is critically required for structural maintenance of TAD<sup>*cPcdh*</sup>.

## Introduction

A significant portion of the chromosomal material is compartmentalized into ‘topologically associated domains’ (TADs), typically encompassing several hundred kilobases of linear genome folded upon itself with regulatory proteins including cohesion and the multifunctional CCCTC-binding factor CTCF constraining expression of TAD-associated genes<sup>1–4</sup>. TAD-like structures in brain<sup>5, 6</sup> were implicated in the genetic risk architecture of psychiatric disease<sup>7</sup> but regulatory mechanisms remain unexplored. Here, we report that neuronal maintenance of a subset of very large ‘superTADs’ critically requires *Set-domain-bifurcated 1 (Setdb1/Eset/Kmt1e)* histone H3-lysine 9 methyltransferase<sup>8</sup>. *Kmt1e/Setdb1* is important for prenatal development and pup survival<sup>9</sup> and broadly regulates retroelement suppression and transcriptional silencing in stem cells<sup>10–12</sup>. Little is known about its essential functions in differentiated cells including neurons. Cell type-specific 3D genome, CTCF, DNA methylation and histone modification profilings, in conjunction with targeted epigenomic editing and conditional mutagenesis uncovered a Setdb1-dependent ‘shield’ protecting genomes from excess CTCF binding and unique locus-specific epigenomic vulnerabilities triggering higher order chromatin collapse on a megabase-scale.

## Results

### Locus-specific TAD disintegration in Setdb1 mutant neurons

To explore higher order chromatin in *Setdb1*-deficient brain, we generated a mouse line for CK-Cre driven recombination in postnatal forebrain neurons with exon 3 deletion, frameshift and premature stop upstream of the critical Tudor, methyl-CpG binding (MBD) and catalytic SET domains. *CK-Cre<sup>+</sup> Setdb1<sup>2lox/2lox</sup>* mutant, in comparison to *CK-Cre<sup>-</sup> Setdb1<sup>2lox/2lox</sup>* control mice showed grossly normal brain cytoarchitecture. However, adult mutants consistently showed a reduction in brain weight, without premature death or neuronal loss as assessed by flow cytometry-based nuclei counts and COMET nuclear DNA damage assays (Figure 1a, Supplementary Figure 1). We conducted in situ Hi-C<sup>1</sup>, or genome-scale DNA-DNA proximity mappings in formalin-fixed, restriction-digested, religated NeuN<sup>+</sup> neuronal nuclei collected by fluorescence-activated sorting (to avoid signal contribution from non-neuronal nuclei) in adult *CK-Cre<sup>+</sup> Setdb1<sup>2lox/2lox</sup>* and *CK-Cre<sup>-</sup> Setdb1<sup>2lox/2lox</sup>* cortex (Figure 1B). However, chromosomal contact mappings at 40kb

resolution (N=2 in situ Hi-C libraries/genotype, with 250–300M aligned reads/library, Figure 1b and Supplementary Figure 2a) showed that mutant neurons were not affected by a generalized disorganization of the 3D genome. For example, length distributions and numbers of autosomal TADs, assessed by TADtree<sup>1</sup>, were indistinguishable and minimally different between genotypes, with ~200kb median length as expected for mammalian genomes<sup>1</sup> (Figure 1c, Supplementary Figure 2b). We then assessed longer-range chromosomal contacts, spanning >200kb of linear genome. We identified genome-wide 110 long-range loop contacts affected in mutant neurons (DESeq2  $P < 0.05$ ). Unexpectedly, the large majority, 84/110 or 76%, represented clustered, locus-specific ‘loop aggregates’ showing massive weakening, or complete loss, after neuronal *Setdb1* ablation (Figure 1d, Supplementary Figure 2c). These included a singular hotspot on chromosome 18 with remarkable chromosome-wide enrichment (1Mb sliding window (1Mb<sup>sw</sup>) Poisson test  $P = 1.2 \times 10^{-24}$ , Figure 1d,e), fully engulfing the *clustered Protocadherin* (*cPcdh*) locus harboring 77 genes including 58 cell adhesion molecules linearly arranged as three gene clusters (*Pcdh*  $\alpha$ ,  $\beta$ ,  $\gamma$ ), regulating neuronal connectivity<sup>13, 14</sup>. Closer inspection of the wildtype *cPcdh* domain revealed multiple small (~100kb length) cluster-specific subTADs nested into a massive superTAD encompassing at least 1.2Mb of linear genome. TADtree analyses confirmed that this superTAD completely disintegrated after *Setdb1* ablation, leaving behind only subTAD remnants in mutant neurons (Figure 1e, Supplementary Figure 2d). Additional loci, including distal portions of chromosomes 5 and 7, showed partial loss of large-sized TADs (Figure 1d, Supplementary Figure 2e). Insulation, measuring the strength of physical segregation of neighboring chromosomal sequences, informs about functional compartmentalization of chromatin<sup>15</sup>. We quantified contact insulation across multiple DNA-DNA contact scales or ‘bands’ (Figure 1f) defined by increasing genomic distance<sup>16</sup>. Indeed, wildtype neurons showed very strong insulation scores at the fringes of the *cPcdh* locus. However, insulation of corresponding sequences in mutant neurons was dramatically weakened across multiple bands (Figure 1f). Therefore, multiple computational approaches, including (i) TADtree, (ii) long-range contact mapping and (iii) insulation analysis reveal structural disintegration of the superTAD<sup>*cPcdh*</sup> after *Setdb1* ablation.

### Setdb1 shields neuronal genomes from excess CTCF occupancy

How could neuronal *Setdb1* ablation trigger such highly localized alterations in chromosomal conformations? To explore the role of *Setdb1*-regulated repressive histone methylation, we charted the *Setdb1* product, trimethyl-histone H3-lysine 9 (H3K9me3), in NeuN<sup>+</sup> and, for comparison, NeuN<sup>-</sup> nuclei sorted from adult *Cre<sup>+</sup>Setdb1<sup>2lox/2lox</sup>* and *CK-Cre<sup>-</sup>Setdb1<sup>2lox/2lox</sup>* cortex. DiffRep-based analysis with 1kb<sup>sw</sup> revealed that 75% of 2,021 differentially H3K9me3-tagged sequences were hypomethylated in mutant neurons. These deficits were specific, because ChiP-seq profiling for open chromatin-associated acetyl-H3-lysine 27 (H3K27ac) showed that 96.4% of 1,112 differentially tagged sequences were hyperacetylated in *Setdb1*-deficient neurons (Figure 2a, Supplementary Tables 1,2). Furthermore, *cPcdh* emerged genome-wide as top scoring locus for H3K9me3 hypomethylation (1Mb<sup>sw</sup>: H3K9me3, 35-fold enrichment, observed/expected 21/0.61, Poisson test  $P = 3.19 \times 10^{-25}$ ) (Figure 2b), with the densely concentrated H3K9me3 deficit readily visible in whole chromosome 18 browser views (Figure 2c). In contrast, NeuN<sup>-</sup>

nuclei sorted from the same cortical specimens were only minimally affected (Figure 2a, Supplementary Figure 3, Supplementary Tables 3,4).

We then analyzed motifs in H3K9me3 hypomethylated sequences in *Setdb1*-deficient neurons. Strikingly, 3/5 top scoring motifs matched to the transcriptional regulator and key 3D genome organizer CTCF, including the CTCFL/BORIS paralog (HOMER enrichment<sup>17</sup>,  $P < 10^{-60}$ ) (Figure 2d, Supplementary Table 5). Furthermore, we uncovered in published *Setdb1* ChIP-seq data from stem cells and CD19<sup>+</sup> B lymphocytes significant CTCF motif enrichment (Supplementary Figure 4a, Supplementary Tables 6,7). Of note, other types of H3K9 methyltransferase, including G9a/Glp, completely lacked CTCF motif enrichment (Supplementary Figure 4b). We therefore predicted altered CTCF occupancy in the *Setdb1*-deficient neuronal genome. Strikingly, ChIP-seq on NeuN<sup>+</sup> from adult *CK-Cre<sup>+</sup>Setdb1<sup>2lox/2lox</sup>* and *CK-Cre<sup>-</sup>Setdb1<sup>2lox/2lox</sup>* cortex showed that 99.4% (3059/3078) of sequences with altered CTCF binding represented up-regulated and *de novo* peaks (Figure 2e, Supplementary Table 8), including many promoters and enhancers (Supplementary Figure 5). There was extreme over-representation for CTCF motifs (HOMER enrichment  $P < 10^{-1000}$ ) (Figure 2f, Supplementary Table 9) independent of filter conditions (Supplementary Figure 6, Supplementary Table 10), affecting cis-regulatory elements and H3K9me3 hypomethylated sequences (Supplementary Table 11). Therefore, *Setdb1* shields mature neuronal genomes from excess CTCF occupancy at cryptic binding sites. Of note, *cPcdh* again emerged as top scoring locus genome-wide (1Mb<sup>sw</sup>, CTCF NeuN<sup>+</sup> up-peaks: 18.7-fold enrichment, Poisson test  $P = 1.32e^{-21}$ , Figure 2g,h). Additional localized enrichments of excess CTCF occupancies matched to loci on chromosomes 5 and 7 affected by loss of long-range chromosomal contacts and H3K9 hypomethylation (Figure 2g).

Given that CTCF—a key regulator of higher order chromatin including domain insulation<sup>18</sup>—is upregulated at thousands of positions in the *Setdb1*-deficient neuronal genome, we assessed genome-wide domain insulation in the in situ Hi-C datasets from our mutant and wildtype cortical neurons. We first focused on CTCF *de novo* peaks, filtered for (i) proximity to TAD boundary (20% of total TAD length) and (ii) vicinity ( $\pm 100$ kb) of sequences with altered H3K9me3 after *Setdb1* ablation. Of these, 52–57% of *de novo* CTCF showing stronger insulation scores in mutants across 8/9 insulation bands, covering 80–1040kb contact distance (Supplementary Figure 7). At sites with conserved CTCF peaks, insulation scores showed very minimal differences between mutant and control neurons (Supplementary Figure 8). Therefore, excess of CTCF on a genome-wide scale conveys a subtle shift towards increased insulation strength in mutant neurons, with the notable exception of *Setdb1*-sensitive superTADs affected by structural collapse and loss of insulation. Our findings, in conjunction with recent genome-scale studies reporting loss of domain insulation in glioma cells due to decreased CTCF binding<sup>19</sup>, suggest that spatial architectures of chromosomes are highly sensitive to bidirectional changes in CTCF occupancies. Next, we explored alterations in A/B compartments, defined as multi-Mb chromosomal segments representing ‘A’ open/‘B’ condensed chromatin tending to interact with other loci sharing similar levels of chromatin accessibility<sup>2</sup>. Because A/B compartments are defined on a continuum<sup>2</sup> (as opposed to a biphasic signal), we quantified ‘compartment-ness’ from the intrachromosomal contact matrices generated by HiC-Pro at 100kb bin resolution (see Online Methods). Of note, the total number of A/B-specific

compartment bins was only minimally different between genotypes. Strikingly, however, 6032/11048 or 54% of ‘A’ and 8468/12977 or 65% of ‘B’ bins had higher ‘compartment-ness’ scores in mutant compared to wildtype neurons (Fisher’s exact  $P < 10^{-210}$ ) (Supplementary Figure 9a). However, *Setdb1*-sensitive superTADs did not follow this genome-wide trend, as exemplified by the weakened ‘B’ signal at *cPcdh* in mutant neurons (Supplementary Figure 9b).

Which molecular mechanisms contribute to the CTCF excess at the H3K9me3 hypomethylated sites? Of note, the majority of *cPcdh* sequences affected show coordinate increases in CTCF binding and histone hyperacetylation (Figure 2h), suggesting a shift towards open/permissive chromatin states. To this end, alterations in DNA cytosine methylation—reducing CTCF’s DNA affinity<sup>20, 21</sup> via interaction with the seventh of CTCF’s 11 zinc fingers<sup>22</sup>—could play a key role, because *Setdb1* functions as upstream regulator for DNA methylation<sup>23</sup>. To explore, we quantified by bisulfite sequencing (bis-seq)<sup>24</sup> levels of cytosine <sup>m</sup>C5 methylation with 43 PCR amplicons targeting 13 *cPcdh* sites including cortical and striatal NeuN<sup>+</sup> and NeuN<sup>-</sup> nuclei from adult *CK-Cre<sup>+</sup>Setdb1<sup>2lox/2lox</sup>* and *CK-Cre<sup>-</sup>Setdb1<sup>2lox/2lox</sup>* brain, plus cerebellar tissue as additional control, comprising 46 individual samples altogether (Supplementary Table 12). As expected, <sup>m</sup>C5 in non-neuronal nuclei and cerebellum remained unaltered. However, in *Setdb1*-deficient neurons, intergenic and promoter sequences affected by excess/de novo CTCF showed significant <sup>m</sup>C5 deficits. In contrast, <sup>m</sup>C5 levels were extremely low while CTCF peaks were very robust, independent of genotype, at sites harboring strong *cPcdh* enhancers elements including HS5-1 and HS16<sup>25–29</sup> (Figure 3, Supplementary Tables 12,13). Therefore, excess CTCF in *Setdb1*-deficient neuronal genomes is associated with ‘open’ chromatin state conversion at cryptic CTCF binding sites. This includes reduced DNA methylation levels, and weakening of regulatory mechanism designed to prevent excess CTCF binding.

### TAD-specific regulation of gene expression

To explore whether disintegration of superTAD<sup>*cPcdh*</sup> affects gene expression, we mapped transcriptomes and neuronal H3K27ac in *Cre<sup>+</sup>Setdb1<sup>2lox/2lox</sup>* and *CK-Cre<sup>-</sup>Setdb1<sup>2lox/2lox</sup>* cortex. Consistent with *Setdb1*’s repressor function<sup>8</sup>, the majority of transcripts altered in mutant were up-regulated (208/321) (Supplementary Table 14). Importantly, 20% of the entire pool of *Setdb1*-sensitive genes located to *cPcdh*, affected Protocadherins and non-Protocadherins, resulting in a unique, 543-fold enrichment on a genome-wide scale (Poisson test  $P=2.32e^{-89}$ ; 1Mb<sup>sw</sup> sliding windows applied to  $N=208$  transcripts) (Figure 4a,b). Similarly, among the 1070 sequences with histone hyperacetylation in mutant neurons (DiffRep 1kb<sup>sw</sup>, adj.  $P < 0.05$ , Supplementary Table 2), the *cPcdh* locus was uniquely affected with 96-fold enrichment on a genome-wide scale (1MB<sup>sw</sup> observed/expected 38/0.42, Poisson test  $P=6.06e^{-60}$ ) (Figure 4a).

To confirm that such extremely locus-specific accumulation of up-regulated transcripts and H3K27ac hyperacetylated sequences is indeed driven by neuronal ablation of *Setdb1*, we reintroduced full length *Setdb1*, via a *CK-Setdb1* transgene<sup>30</sup>, into the conditional mutant line (Supplementary Figure 10). Parallel testing of four genotypes, or *CK-Cre<sup>+</sup>Setdb1<sup>2lox/2lox</sup>* and *CK-Cre<sup>-</sup>Setdb1<sup>2lox/2lox(2lox/wt)</sup>* each with and without *CK-Setdb1* ( $N=6$

mice/genotype) confirmed complete rescue with return to baseline expression for 33/43, or 76% of  $\alpha$ ,  $\beta$  and  $\gamma$  *Protocadherins* and of additional (non-Protocadherin) genes in *cPcdh* (Figure 4c, Supplementary Table 15). To further test whether *cPcdh*'s unique vulnerability is specific for *Setdb1* deletions in postnatal neurons, we profiled adult cortical and striatal transcriptomes after *CK-Cre* ablation of neuronal *G9a/Glp*, encoding a H3K9 methyltransferase complex essential for normal brain function<sup>31, 32</sup>. In addition, we profiled transcriptomes from embryonic day E15.5 Nestin-Cre<sup>+</sup>, *Setdb1*<sup>2lox/2lox</sup> cortex. None of these various transcriptome sets showed local enrichment at *cPcdh* (Supplementary Figure 11). Therefore, *Setdb1* exerts unique transcriptional control across the *cPcdh* domain specifically in mature neurons. However, this regulatory layer is not representative for other types of H3K9 methyltransferase, or for the prenatal *Setdb1*-deficient brain.

Of note, 31/53 S-type (single-neuron stochastically expressed) *Protocadherin*  $\alpha$ ,  $\beta$  and  $\gamma$  genes, critically important for neuronal diversity and connectivity<sup>33, 34</sup>, showed increased expression after neuronal *Setdb1* ablation (Figure 4b,c). We studied S-type expression patterns by in situ hybridization providing single cell resolution, using probes specific for individual S-type *Pcdha1*, *Pcdha8*, *Pcdhb22* and *Pcdhga7*. Strikingly, brain sections from *Setdb1* conditional mutants but none of the three control genotypes including transgenic rescue showed massively increased numbers of robustly stained neurons diffusely distributed across cortical layers II-VI and hippocampus (Figure 4C). Cerebellar cortex, which in contrast to forebrain is lacking CK-Cre expression, remained unaffected in conditional mutant brain (Figure 4c, Supplementary Figures 12–15). Therefore, S-type single neuron stochastic constraint is severely compromised in *Setdb1*-deficient neurons, contributing to up-regulated expression at the *cPcdh* locus. Given the critical importance of orderly *cPcdh* expression—including single-cell stochastic constraint of S-type *Pcdh* genes—for neuronal morphology and connectivity<sup>13, 33, 34, 49</sup>, we quantified spine densities and diameters from layer III apical dendrites from *Setdb1*<sup>wt/wt</sup> and *Setdb1*<sup>2lox/2lox</sup> mice, crossed into a conditional line expressing membrane-bound GFP (GFP-F)<sup>50</sup> for Golgi-like labeling after low-titer AAV8<sup>hSYN1-CreGFP</sup> delivered to adult PFC. Indeed, spines from *Setdb1*-deficient neurons showed 40–50% increased density, and overall decreased size (Supplementary Figure 1g), providing a morphological correlate for dysregulated *cPcdh* expression.

### Balanced facilitative and repressive conformations at cPcdh

Next, we wanted to gain deeper mechanistic insight into the molecular mechanisms mediating the unique position of the *cPcdh* locus within the *Setdb1*-sensitive transcriptome and epigenome space. Because CTCF associates with RNA polymerase subunits<sup>35</sup> and transcriptional activators<sup>36, 37</sup>, CTCF-upregulation at H3K27ac-hyperacetylated S-type *Pcdh*  $\alpha/\beta/\gamma$  promoters in *Setdb1*-deficient neurons could facilitate expression, including loss of single cell-stochastic constraint. However, promoter-bound CTCF alone is not sufficient to up-regulate transcription because from genome-wide 63 genes with excess CTCF around the transcription start site, only transcripts within the *cPcdh* locus were increased. Of note, promoter-enhancer loopings furnished by the CTCF-cohesin scaffolding complex contribute to transcriptional regulation of *cPcdh* genes<sup>20</sup> and therefore, excess CTCF occupancy in *cPcdh* sequences from *Setdb1*-deficient neurons could trigger alterations in higher order chromatin. Indeed, excessive CTCF binding at the *cPcdh* locus of *Setdb1* mutant neurons

was not limited to promoters, because multiple CTCF peaks emerged *de novo* in intergenic DNA upstream from  $\alpha$ , and within the  $\gamma$  cluster (peaks A-C in Figure 2h). Importantly, these *de novo* peaks were surrounded by broad >100–200kb stretches of H3K9me3-tagged chromatin that underwent significant ‘shrinkage’ after neuronal *Setdb1* ablation (labeled ‘R1’ and ‘R2’ in Figure 5a). Importantly, ‘R1’ and ‘R2’ marked the anchor regions of massive bundles of long-range chromosomal conformations in wildtype neurons. Thus, densely spaced H3K9me3-tagged ‘R1’ loopings, emanating from 100–200kb wide blocks of repressive chromatin upstream of *Pcdh $\alpha$*  genes, radiated towards many sites within *cPcdh*, even reaching the distal-most *Pcdh $\gamma$*  sequences. However, these long-range loopings became completely dissolved upon structural disintegration of the superTAD<sup>*cPcdh*</sup> (Figure 1e). Among H3K9me3-tagged conformations lost after neuronal *Setdb1* deletion were multiple loopings interconnecting R1 and R2 with two DNase I hypersensitive enhancer elements, HS16- and HS5-1, previously shown to broadly facilitate *cPcdh* expression<sup>25–29</sup>. These defects in HS16/HS5-1 bound long-range contacts were highly specific, because mutant neurons fully maintained shorter-range loopings from *Protocadherin* gene promoters to HS16/HS5-1 enhancers within the subTADs (Figure 1e). We confirmed these Hi-C findings, including specific weakening of long-range R1—HS16, R1—R2 and R2—HS5-1 and preservation of shorter-range contacts, in neuron-specific chromosome conformation capture (3C) PCR assays from adult mutant and control cortex (Figure 5b, Supplementary Figure 16a). These studies, taken together, would suggest that in wildtype, HS16 and HS5-1 enhancer sequences are ‘locked’ into H3K9me3-tagged repressive chromatin. Upon *Setdb1* deletion, loss of R1/R2 repressive loop formations could release the ‘epigenomic brake’, thereby shifting the balance from repressive towards facilitative contacts furnished by HS16 and HS5-1-bound promoter-enhancer loopings, thereby triggering increased expression across the *cPcdh* locus (Figure 5c). To test this hypothesis, we transfected NG108 neuroblastoma cells with small RNA guided (sgRNA) Cas9-SunTag protein scaffolds<sup>38</sup> designed to load ten copies of the potent transcriptional activator, VP64, onto single HS16 sites (Figure 5d). Therefore, such type of HS16<sup>Cas9-SunTag(10xVP64)</sup> ‘epigenomic superactivation’ could, like the loss of R1—HS16 and R2—HS5-1 repressive loopings after *Setdb1* ablation, increase transcription at multiple positions across the entire 1Mb *cPcdh* locus via promoter-enhancer contacts and other mechanisms. Dual labeled cells (*sgRNA-dCas9-10xGCN4-SunTag*-blue fluorescent protein and *scFV(recognizing GCN4 epitope)*-superfold GFP-VP64<sup>β8</sup>) were compared to controls expressing exactly the same types of vectors but without the sgRNA. Indeed, HS16 epigenomic superactivation was associated with increased expression of 3/6 *cPcdh* transcripts (pre-selected for consistent baseline expression in neuroblastoma cells), closely mimicking the transcriptional phenotype in *Setdb1* mutant cortex (Figure 5e).

### Conserved regulation of human and mouse superTAD<sup>CPCDH</sup>

The linear arrangement of  $\alpha$ ,  $\beta$  and  $\gamma$  clusters with S- and C-type *Protocadherin* genes is highly conserved across vertebrate genomes<sup>13</sup>. We showed that higher order chromatin, including broad >100–200kb stretches of intergenic *Setdb1*-regulated H3K9me3-tagged sequence associated with repressive loop bundles, critically regulates transcription across the *cPcdh* locus. We asked whether such types of 3D genome conformations, just like the linear genome, could be conserved across mammalian lineages. To examine, we generated *in situ*

Hi-C interaction matrices in human glutamatergic neurons differentiated from induced pluripotent stem cell-derived neural precursors by controlled expression of Neurogenin 2, and compared the 3D genome map to wildtype mouse cortex NeuN<sup>+</sup> nuclei (of which >80% are from excitatory neurons). Indeed, TAD landscapes surrounding the *cPcdh/PCDH* locus (*Mm10* chr. 18; *Hg19* chr. 5) showed startling similarities between mouse cortex NeuN<sup>+</sup> nuclei and human neurons, including complete preservation of cluster-specific subTADs nested into a large Mb-scale superTAD<sup>*cPCDH*</sup>. In addition, human and mouse neuronal chromatin exhibited highly similar-shaped H3K9me3 landscapes, including the broadly stretched aforementioned Setdb1-sensitive ‘R1’ at the superTAD’s 5’ end and ‘R2’ around the 5’ end of the  $\gamma$  cluster (Figure 6a). We were surprised to discover that ‘R1’ near-perfectly matched a risk haplotype (*chr5:140,023,664-140,222,664*) of the Psychiatric Genomics Consortium<sup>39</sup>. This haplotype (no. 108 in reference<sup>39</sup>, referred to as ‘PGC’ hereafter) significantly contributes, independently from another 107 loci genome-wide, to schizophrenia heritability<sup>39</sup>, with a small INDEL as the lead polymorphism (rs111896713 *chr5:140,143,664*<sup>39</sup>). This risk polymorphism matched to robust Setdb1 peaks conserved in human and mouse cells including brain, but ‘replaced’ by *de novo* CTCF peaks upon *Setdb1* ablation (Figure 6a, Supplementary Figure 16b,d). Therefore, we predicted that higher order chromatin organization at these positions will be highly conserved in human brain cells, and specifically in neurons, with long-range loopings radiating from ~200kb R1 towards *cPCDH* promoter and enhancers primarily anchored in chromatin at and around the Setdb1 peak. To explore, we surveyed with 40kb resolution the *cPCDH*-bound chromosomal contacts in our in situ Hi-C datasets generated from human neurons and their isogenic neural precursors cells (NPC) and NPC-differentiated astrocytes. Strikingly, 40kb bins within PGC showed a step-wise progression in contact intensities with *cPCDH* sequences, culminating in massively increased contact frequencies at the bin harboring a robust Setdb1 peak (‘PGC-3’ in Figure 6b). This effect was pronounced in neurons and NPC, while corresponding loopings were much weaker or missing altogether in our contact maps from astrocytes, indicating strong cell type-specific regulation of local 3D genome architectures (Figure 6b). Next, we asked whether these within-PGC haplotype differences in *cPCDH* interaction frequencies translate into differential repressive potential. To this end, we introduced small guide RNAs (sgRNA) into two stable NPC lines, expressing 1. dCas9-KRAB fusion protein tethering KAP1 (KRAB-associated protein 1)-Setdb1 repressor complex<sup>8, 40</sup>, or 2. dCas9-VP64 to dock the VP64 activator at different positions within the schizophrenia risk haplotype. We then measured expression levels for S-type  $\gamma$ Pcdh genes expressed in NPC at comparatively high levels at baseline (data not shown). Interestingly, KRAB recruited to sequences close to the Setdb1 peak at the risk haplotype’s lead polymorphism (‘PGC-3’ in Figure 6c), was consistently associated in 3/3 experiments with a robust multifold decrease in expression of *PCDHGB6* (but not *PCDHGA3*). In contrast, dCas9-KRAB docked to a non-Setdb1 binding site (‘PGC-2’ in Figure 6c) or to a scrambled control sequence (‘Scr’ in Figure 6c) remained ineffective and did not suppress *PCDHGB6* and *PCDHGA3* expression (Figure 6c). Of note, VP64 epigenomic editing at ‘PGC-3’ and neighboring ‘PGC-2’ was associated with increased expression of a subset of *cPCDH* genes (Figure 6c). These findings, taken together, suggest that repressive effects on Protocadherin gene expression are specific for loop-bound KRAB positioned at intergenic ‘PGC-3’ sequences upstream of the *cPCDH* gene clusters.



It is remarkable that KRAB—a critical module in KRAB-zinc finger proteins (KRAB-ZNF/Zfp) important for sequence-specific docking of the KAP1-Setdb1 repressor complex<sup>8, 40</sup>—inhibits *cPCDH* expression via long-range loopings, bypassing 644kb linear genome in case of *PCDHGB6* (Figure 6c). Therefore, intergenic R1/risk haplotype-bound Setdb1 is likely to function as key transcriptional regulator at the *cPCDH* locus. These intergenic sequences harbor in the ENCODE database matching peaks for Setdb1, KAP1 and multiple KRAB-ZNF proteins including ZNF 274<sup>41</sup> and ZFP143 (Figure 6a). Importantly, ZFP143 recognition sequences emerged as top scoring zinc finger motifs enriched at sites with excessive CTCF binding in *Setdb1*-deficient mouse neurons (Figure 6d, Supplementary Tables 9,10). ZNF143, like CTCF and cohesion considered a key organizer for the 3D genome<sup>42, 43</sup>, co-assembles with positive and negative regulators of transcription depending on local chromatin context<sup>44</sup>. Unsurprisingly then, ZNF143 at the *cPCDH* locus occupies in addition to R1 repressive chromatin also promoters and HS16/HS5-1 enhancers (Figure 6a). Therefore, alterations in ZNF143 supply, affecting facilitative and repressive chromatin, could destabilize *cPcdh* expression. Indeed, small RNA-mediated *Zfp143* knock-down in mouse neuroblastoma cells was associated with decreased expression of multiple *cPcdh* genes (Figure 6d). Our studies, taken together, suggest that 1. regulatory 3D genome architectures at the *cPCDH* locus are highly conserved between mouse and human, 2. include SETDB1-KRAB-ZNF143 and CTCF as key organizers of local repressive and facilitative chromosomal conformations, 3. ‘bundles’ or ‘aggregates’ of Setdb1-dependent long-range repressive loopings radiating from intergenic DNA (‘R1’, ‘R2’) function as ‘epigenomic brakes’ for transcriptional control, counterbalancing facilitative shorter range promoter-enhancer contacts (Figure 6e).

## Discussion

Neuronal *Setdb1* ablation triggers structural disintegration of megabase-scale TADs, including the *Protocadherin*  $\alpha/\beta/\gamma$  locus as the only Setdb1-sensitive TAD harboring a gene cluster. TADs affected in mutant neurons showed shrinkage of broadly stretched H3K9me3-tagged chromatin, in conjunction with localized hotspots of excess and *de novo* CTCF binding. Setdb1-regulated long-range repressive *cPcdh* loopings were highly enriched in neurons as compared to their isogenic precursors, and carried DNA polymorphisms conferring liability for schizophrenia. 3D genome conformations at *cPCDH* could have even broader relevance for neuropsychiatric disease, given that *SETDB1* microdeletions and structural variants are associated with neurodevelopmental delay<sup>51, 52</sup>, with CpG hypermethylation reported for orthologous CTCF binding sites within the *PCDH* gene cluster in Down syndrome (trisomy 21) including the mouse model<sup>53</sup>, and *cPcdh* DNA promoter methylation linked to depression and anxiety<sup>54–56</sup>. Furthermore, mice exposed to chronic variable stress, a preclinical paradigm frequently implied in psychiatric disease<sup>57</sup>, show hyperexpression of  $\beta$  *Protocadherin* genes (Supplementary Figure 17).

We show that Setdb1, maintaining high levels of DNA methylation and low levels of histone acetylation at sequences in close vicinity or partial overlap with potential CTCF binding sites, critically shields neuronal genomes from uncontrolled CTCF docking at thousands of cryptic binding sites genome-wide. However, upon neuronal *Setdb1* ablation, the shield becomes defunct, triggering collapse of vulnerable TADs. Remarkably, our findings on

excessive *cPcdh* CTCF occupancies and increased *cPcdh* expression and the resulting increase in spine densities in *Setdb1*-deficient neurons are perfect opposites to the previously reported *decreases* in *cPcdh* expression and spine densities after neuronal *Ctcf* ablation<sup>58</sup>. Likewise, switching ‘reverse-forward’ strand orientations in CTCF binding sequences<sup>25</sup> could disrupt promoter-enhancer loopings and broadly dampen transcription across  $\alpha/\beta/\gamma$  clusters (figure 2d in reference<sup>25</sup>). These findings strongly point to delicate regulatory mechanisms governing chromosomal conformations, with genomes excessively populated by CTCF, including the *Setdb1*-deficient neurons of the present study, showing disintegration of higher order chromatin in locus-specific manner. The structural collapse of the *Setdb1*-sensitive superTADs was highly specific, given that the genome-wide excess of CTCF binding in mutant neurons triggered a genome-wide *increase* in insulation strength and ‘compartment-ness’.

Future work will clarify whether neuronal *Setdb1* overexpression<sup>30, 59</sup>, or loss of other proteins assigned with regulation of *cPcdh* expression, including DNMT3b cytosine methyltransferase<sup>34</sup> and SMCHD1<sup>60</sup> and WIZ<sup>61</sup> repressors, could trigger TAD-specific 3D genome changes in neurons. We note that *Setdb1* is primarily located towards the 5’ and 3’ ends of superTAD<sup>*cPcdh*</sup> (Figure 6a,e, Supplementary Figure 18). In any case, the TAD-specific phenotypes in *Setdb1* mutant neurons point towards unexplored modular complexities in the regulatory mechanisms governing the 3D genome. Thus, rewiring or disintegration of specific TAD units may not be exclusive to chromosomal microdeletion and –duplication events<sup>62, 63</sup>, because as shown here, loss of *Setdb1* function triggers the disintegration of highly select subset of neuronal TADs. With each chromosome furnishing hundreds of TAD-like structures, it will be an exciting and challenging task to dissect ‘TAD-by-TAD’ and in cell-type specific manner, the multilayered mechanisms governing locus-specific higher order chromatin in highly differentiated brain cells.

## ONLINE METHODS

### Human Stem Cell Lines

All work with human induced pluripotent stem cell lines has been approved by the Institutional Review Board of the Mount Sinai School of Medicine, in accordance with Mount Sinai’s Federal Wide Assurances (FWA#00005656, FWA#00005651) to the Department of Health and Human Services. No new stem cell lines had been generated for the work presented here. Informed consent had been obtained from all participating subjects. See Supplementary Methods for differentiation into neural progenitors, glutamatergic neurons and astrocytes.

### Animal studies

All animal work was approved by the Institutional Animal Care and Use Committee of the Icahn School of Medicine at Mount Sinai. Mice were held under specific pathogen-free conditions with food and water being supplied ad libitum in an animal facility with a reversed 12 h light/dark cycle (light off at 7:00 am) under constant conditions (21 ± 1°C; 60% humidity). All animals were group housed (2–5/cage).

## Generation of *Setdb1* conditional mutant and rescue mice

*Setdb1*<sup>2lox/2lox</sup> mice were generated by Ozgene, Australia. In brief, two loxP sites were inserted to endogenous *Setdb1* locus (*Setdb1* ENSMUSG00000015697; SET domain, bifurcated 1, *MGI:1934229*) flanking exon 3. To generate conditional *Setdb1* knockout mice, *Setdb1*<sup>2lox/2lox</sup> mice were first crossed with *CK-Cre*<sup>+/-</sup> transgenic mice to generate *Setdb1*<sup>2lox/+</sup>, *CK-Cre*<sup>+/0</sup> heterozygous, which were further crossed with *Setdb1*<sup>2lox/2lox</sup> mice to generate *Setdb1*<sup>2lox/2lox</sup>, *CK-Cre*<sup>+/0</sup> homozygous. Cre recombinase mediated excision of *Setdb1* exon 3 causes frame shift and generates a stop codon at the new junction of exon 2 and exon 4, and results in early termination of *Setdb1* translation. Gender and age matched littermates with genotype *Setdb1*<sup>2lox/2lox</sup>, *CK-Cre*<sup>0/0</sup> were used as controls with wildtype SETDB1 levels. *CK-Setdb1* transgenic mice, described previously<sup>64</sup>, express full-length mouse *Setdb1* cDNA driven by CK promoter in postnatal and adult mouse forebrain. To generate *Setdb1* rescue mice, the *CK-Setdb1* transgene was introduced into *Setdb1*<sup>2lox/2lox</sup> conditional knockout background and *CK-Setdb1*<sup>+/0</sup>, *Setdb1*<sup>2lox/2lox</sup> mice were crossed with *Setdb1*<sup>2lox/+</sup>, *CK-Cre*<sup>+/0</sup> to generate *Setdb1*<sup>2lox/2lox</sup>, *CK-Cre*<sup>+/0</sup>, *CK-Setdb1*<sup>+/0</sup> rescue mice. Gender and age matched littermates with genotype *Setdb1*<sup>2lox/2lox</sup>, *CK-Cre*<sup>0/0</sup>, *CK-Setdb1*<sup>0/0</sup> were used as wildtype controls. All genetically engineered lines were backcrossed to the C57BL6/J line for at least 10 generations.

See Supplementary Methods for information on Nestin-Cre conditional mutagenesis, Mendelian survival ratios, Histology, Comet-assay and RNA quantifications including RNA-seq.

## Chromatin assays

Chromatin assays (ChIP-seq, in situ Hi-C, 3C-PCR) and RNA-seq were conducted in young adult mice, at 3 months of age ( $\pm$  2 weeks). Supplemental Tables 1–4, 8 and 12 provide additional information for each chromatin assay, including number of animals and sex ratios.

**Nuclei preparation, immunotagging and fluorescence-activated sorting**—For fluorescence-activated nuclei sorting, nuclei were extracted from mouse cerebral cortex or human prefrontal cerebral cortex (control, PFC, male, PMI 17) and anterior cingulate cortex (control, ACC, female, PMI 27) as described before<sup>65</sup>. In brief, brain tissue was homogenized in hypotonic lysis solution, purified by ultra-centrifugation, and then re-suspended in 1 ml DPBS containing 0.1% BSA, 1:1000 Anti-NeuN antibody, clone A60, Alexa Fluor®488 conjugated (EMD MILLIPORE CORP MAB377X). Samples were incubated for at least 45 min by rotating in the dark at 4°C. DAPI was added before FACS to label all the nuclei. Sorting was done on Flow Cytometry Center at Mount Sinai. Nuclei were separated into NeuN+ and NeuN- population and then pelleted for following applications. For XChIP, 3C and *in situ* HiC experiments, 10 minutes of 1% formaline fixation at room temperature was incorporated right after brain homogenization. Cross-linking was quenched by incubating with 125 mM glycine. Nuclei were then purified, stained and sorted as described above.

**ChIP-seq**—Native immunoprecipitation (NChIP) was performed as described<sup>65</sup>. In brief, NeuN<sup>+</sup> (neuronal) and NeuN<sup>-</sup> (non-neuronal) nuclei were pelleted post-FACS and then

resuspended in 300  $\mu$ l of MNase digestion buffer (10 mM Tris, pH 7.5; 4 mM MgCl<sub>2</sub>; and 1 mM Ca<sup>2+</sup>), digested with 3  $\mu$ l of MNase (0.2 U/ $\mu$ l) for 5 min at 28°C to obtain mono-nucleosomes. Reaction was stopped with 50mM EDTA, pH 8. Nuclei were swollen to release chromatin after addition of hypotonization buffer (0.2 mM EDTA, pH 8, containing PMSF, DTT, and benzamidine). Chromatin was incubated with anti-H3K9me3 (Abcam AB8898) and anti-H3K27ac (Active Motif, #39133) antibodies overnight at 4°C. The DNA-protein-antibody complexes were captured by Protein AG Magnetic Beads (Thermo Scientific™ 88803) by incubating at 4°C for 2 hours. Magnetic beads were then washed with low-salt buffer, high-salt buffer, and TE buffer. DNA was eluted from the beads, and treated with RNase A followed by proteinase K digestion. DNA was purified by phenol-chloroform extraction and ethanol precipitation.

For XChIP on crosslinked preparations, formaldehyde-fixed NeuN<sup>+</sup> nuclei after FACS were resuspended in lysis buffer containing 0.1% SDS, sonicated (Bioruptor® Plus sonication device, Diagenode) at the ‘high’ setting for 30 minutes on ice. The size of DNA fragmentation was between 100 bp to 500 bp with an average size of 300bp. Chromatin was then incubated with anti-CTCF (EMD Millipore, # 07729) or anti-SETDB1 (Santa Cruz H-300X #sc-66884 X) or anti-SETDB1 (Thermofisher 5H6A12 #MA5-15722) and captured with Protein AG Magnetic Beads. After washing and elution, DNA was incubated at 65°C overnight for reverse cross-linking, followed by RNase A, proteinase K treatment and DNA precipitation. Setdb1 occupancies were measured by conventional ChIP-PCR. For CTCF ChIP-seq library preparation, ChIP DNA was end repaired (End-it DNA Repair kit; Epicentre) and A tailed (Klenow Exo-minus; Epicentre). Adaptors (Illumina) were ligated to the ChIP-DNA (Fast-Link kit; Epicentre) and then PCR amplified using Illumina TruSeq ChIP Library Prep Kit. Library DNA with expected size (NChIP, ~275bp; XChIP, 350bp to 500bp) was selected by Pippin and submitted to New York Genomic Center and sequenced with Illumina HiSeq 2000, 75bp, paired end. The ChIP-seq data was first checked for quality using the various metrics generated by FastQC (v0.11.2). Raw sequencing reads were then aligned to the mouse mm10 genome (or Hg19 for human) using default settings of Bowtie (v2.2.0). Uniquely mapped reads were retained and the alignments were subsequently filtered using the SAMtools package (v0.1.19) to remove duplicate reads. Differential analysis between mutant and control samples was performed using diffReps with window size 1000 bp and moving step size 500 bp, and FDR<5% as significance cutoff<sup>66</sup> and data visualized on the genome using the Integrative Genomics Viewer (IGV) program<sup>67</sup>. For H3K27ac and CTCF ChIPseq, peak-calling was performed using MACS (v2.1.1) with a FDR cutoff of 0.05. Gene Ontology enrichment of annotated genes, with significant hits from diffReps within gene bodies or within 3Kb around transcriptional starting sites, was further analyzed using DAVID Functional Annotation Bioinformatics tools (Resources 6.7, National Institute of Allergy and Infectious Diseases, NIH). Significant hits from diffReps for decrease in H3K9me3 and increase in CTCF ChIPseq were subjected to motif analysis, using the Homer package (v4.8.3) at default settings<sup>68</sup>. Manhattan plots for genome-wide differential epigenetic profiling of conditional mutants and controls were constructed after the genome was divided into non-overlapping 1Mb bins, including the 1Mb bin spanning the clustered *Pcdh* genes chr18:36,870,001-37,870,000, mm10. The number of occurrences of each signal was tabulated within each bin. The probability of the number of occurrence of

each signal per 1Mb bin was then modeled using a Poisson distribution with the maximum likelihood estimator for the lambda parameter given by the calculated mean number of occurrences. The Poisson models for each signal were used to calculate the probability of occurrence of the signal observed in every 1Mb bin (including the *Pcdh* bin).

**Chromosome Conformation Capture (3C)**—3C was performed using standard protocols with minor modifications<sup>69</sup>. In brief, nuclei were fixed and extracted from mouse cerebral cortex and FACS sorted as described above. NeuN positive (neuronal) nuclei were then pelleted and digested with Hind III restriction enzyme (NEB) at 37°C overnight, washed, and treated with T4 DNA ligase at room temperature for 4 hr. 3C DNA was then incubated at 65°C overnight for reverse crosslinking followed by DNA purification and precipitation. 3C primers were listed in Supplementary Table 16. Sequence-verified PCR products were measured semi-quantitatively with UVP Bioimaging system/Labworks 4.5 software. Neighboring primers at *B2m* gene locus was used for normalization.

**in situ Hi-C including bioinformatical analyses**—Nuclei were fixed and extracted from mouse cerebral cortex and human postmortem anterior cingulate cortex and sorted into NeuN<sup>+</sup> (neuronal) and NeuN<sup>-</sup> (non-neuronal) populations, which were then processed using an *in situ* Hi-C protocol<sup>70</sup>, with minor modifications. Briefly, the protocol involves a restriction digest of the cross-linked chromatin within intact nuclei, followed by biotinylation of the strand ends, re-ligation, sonication and size selection for 300–500bp fragments, followed by standard library preparation for Illumina sequencing. The resulting data were mapped, filtered, and normalized using HiC-Pro<sup>71</sup> (v2.7.8) and visualized on the Washington University Epigenome Browser. To explore localized enrichments in the *in situ* Hi-C datasets, we tabulated for each 40kb bin along chromosome 18 the number of long-range interactions greater than 200kb that were disrupted (ie. significantly decreased in conditional CK-Cre mutants versus control, as detected using DESeq2 at P<0.05). The probability of observing the number of disrupted interactions at each bin was then modeled using a Poisson distribution with maximum likelihood of mean (0.165) calculated from the data. Topological associating domains (TAD) were predicted using TADtree<sup>70</sup> using the 20kb Hi-Cpro data as input with the following parameter settings: maximum size of TAD in bins (S) = 60; maximum number of TADs in each tad-tree (M) = 10; boundary index parameter (p) = 6; boundary index parameter (q) = 24; balance between boundary index and squared error in score function (gamma) = 500; number of TADs to use (N) = 400 (chr18) or 700 (chr5).

In addition, initial processing of the raw 2×125bp read pair FASTQ files was performed using the HiC-Pro analysis pipeline. In brief, HiC-Pro performs four major tasks: aligning short reads, filtering for valid pairs, binning, and normalizing contact matrices. HiC-Pro implements the truncation-based alignment strategy using Bowtie v2.2.3<sup>72</sup>, mapping full reads end-to-end or the 5' portion of reads preceding a GATCGATC ligation site that results from restriction enzyme digestion with MboI followed by end ligation. Invalid interactions such as same-strand, dangling-end, self-cycle, and single-end pairs are not retained. Binning was performed in 40kb and 100kb non-overlapping, adjacent windows across the genome

and resulting contact matrices were normalized using iterative correction and eigenvector decomposition (ICE) as previously described<sup>73</sup>.

Starting with the 20 kb resolution intra-chromosomal contact matrices generated by HiC-Pro, we first generated 100 kb resolution contact matrices by summing the interaction frequencies of the 20 kb bins within each 100 kb bin. We next generated the corresponding  $\log_2(\text{observed}/\text{expected})$  matrices, where the observed/expected values are the ratio of the contact values of each interaction bin to the average contact values of all interaction bins the same distance apart. The Pearson's correlation matrices were then calculated from the  $\log_2(\text{observed}/\text{expected})$  matrices, and PCA was performed on them. The first principal component (PC1) was then used to differentiate the compartments. When the first principal component value was positively correlated to gene density and gene expression (we found that the first principal component always correlates with both gene density and gene expression in the same direction), bins with positive PC1 values are assigned as compartment A while bins with negative PC1 values are assigned as compartment B. Conversely, when PC1 is negatively correlated to gene density and gene expression, bins with negative PC1 values are assigned as compartment A while bins with positive PC1 values are assigned as compartment B.

Higher-resolution topologically associated domain (TAD) calls were made following the procedure described by Dixon, et al.<sup>74</sup> using the directionality index (DI) metric. DI was calculated using raw interaction counts between 40kb or 100kb bins and respective window sizes of 2Mb or 5Mb to capture observed upstream or downstream interaction bias of genomic regions. A Hidden Markov model (HMM) was then trained to infer true bias states. TADs were defined by pairing adjacent regions of inferred downstream or upstream bias states.

To identify significantly enriched interactions involving a bin of interest, the expected interaction counts for each interaction distance were estimated by calculating the mean of all intrachromosomal bin-bin interactions of the same separation distance throughout the entire ICE-normalized contact matrix. We estimated the probability of observing an interaction between a bin-of-interest and some other bin by calculating the expected interaction between those two bins divided by the sum of all expected interactions between the bin-of-interest and all other intrachromosomal bins. We then calculated the p-value of observing the observed number of interaction counts or more between the bin-of-interest and some other bin using a binomial test where the number of successes was defined as the observed interaction count, the number of tries as the total number of observed interactions between the bin-of-interest and all other intrachromosomal bins, and the success probability as the probability of observing the bin-bin interaction estimated from the expected mean interaction counts. To control false discovery rate, the R package, *qvalue*, was used to estimate q-values from the calculated binomial p-values.

The Insulation Analysis was performed with reference to Crane et al<sup>75</sup> and Vietri Rudan et al<sup>76</sup>. Briefly, using the 20kb resolution HiC matrix, we calculated (at each 20kb bin) the average interaction frequency of the chromosomal bins within a certain distance band. The normalized "insulation" score along the chromosome for each band was then calculated as

the log<sub>2</sub> ratio of average interaction frequency at each 20kb bin to the average of all 20kb bins in that band. Regions along the chromosome that display a dip/valley/minima of normalized insulation values represent regions of reduced interactions, and can be interpreted as TAD boundaries or regions of high local insulation.

**DNA methylation**—Targeted bis-seq was utilized for fine-mapping of methylation patterns in *cPcdh* candidate sequences. Genomic DNA (1 µg) was bisulfite-converted using the EpiTect Bisulfite Kit (Qiagen). Primers were designed in MethPrimer<sup>77</sup> and bisulfite-converted DNA was amplified and multiplexed by high throughput PCR using a Fluidigm AccessArray instrument. PCRs were performed in duplicate and the duplicates pooled. Primers are listed in Supplementary Table 16. The library was diluted to a final concentration of 10pM with 35% of PhiX library. Paired-end reads (250 bp) were generated with an Illumina MiSeq sequencer. Fastq files were generated by the MiSeq sequencer. After trimming for low-quality bases (Phred score<30), Illumina and Fluidigm adaptors and reads with a length <40 bp with TrimGalore, the reads were aligned to the mouse genome (mm10) using Bismark<sup>78</sup> using the following settings: -D 50 -R 10 --score\_min L,0,-0.6. Since the sequences are PCR-based, reads were not deduplicated. Methylation calling was performed using Bismark extractor<sup>78</sup>. Net methylation was assessed when the coverage was at least 100X and reported by CpG and averaged across amplicon. Graphical representations of random samples of 50 sequenced DNA fragments were generated using R. Briefly, using CpG context output files generated by Bismark methylation extractor<sup>78</sup> which reports CpG methylation status for each individual sequenced DNA fragments (taking into account paired-end reads), methylation patterns for each DNA fragment were reconstructed based on the coordinates of the covered CpGs and their methylation status. Off-target reads mapping outside the amplicon coordinates were discarded. After random sampling of 50 sequences using the R sample function, their methylation patterns were plotted using the R plot function. Only CpGs present in the reference genome were represented and sequences were represented on positive strand. Circles represent consecutive CpGs, with each line being a unique DNA fragment. White circles are unmethylated CpGs and black circles are methylated CpGs.

To ensure sufficient library complexity, we pooled two PCRs for each amplicon. The median coverage per library ranged from 1000X to 2600X (Supplementary Figure 19A). Since we used a PCR based targeted bis-seq approach, the sequence start points are constrained for each amplicon. Therefore the constrained start sites generated duplication levels which should not be treated as technical duplicates nor removed by bioinformatics deduplication. These duplication levels will be reflected in the distribution of specific sequences that are identified using start sites and therefore not informative to assess the library complexity. Therefore, since some level of randomness of DNA methylation patterns in a cell population is present, to estimate the library complexity, we assessed the number of distinct methylation patterns (i.e. specific sequences based on C>T conversion) observed for each amplicon in a given library. Because of biological duplicates (genuinely distinct DNA molecules with the same methylation pattern), this metric provides highly stringent coverage information. In addition, to take into account sequencing errors, only methylation patterns representing more than 1% of the total reads covering a given amplicon were counted. Overall, the median of

methylation patterns per amplicon was 5 (range:1–12.5), with 81.2% of the amplicons with less than 5 methylation patterns covering fully methylated (>80% of methylation) or fully unmethylated (<20%) regions, which are expected to show lower randomness level (Supplementary Figure 19B).

### Lenti-shRNA knockdown of Zfp143

Mouse shRNA lentiviral particles targeting Zfp143 (4 unique 29mer target-specific shRNA, 1 scramble control) were purchased from Origene (TL502149V), with sequences in the shRNA expression cassettes are verified by the manufacturer to correspond to the target gene Zfp143 (Gene ID 20841) with 100% identity, to produce 70% or more gene expression knock-down provided a minimum transfection efficiency of 80%. NG108 cells (NG108-15 #108CC15, Vendor: ATCC, Organism: *Mus musculus* (neuroblastoma); *Rattus norvegicus* (glioma)) were seeded in 12-well plates 12 hours before viral transduction. 72 hours after transduction, cells were lysed with 500ul of Trizol, total RNA was extracted, reverse transcribed and *Pcdh* gene expression quantified by real-time PCR. Primers are listed in Supplementary Table 16.

### dCas9-SunTag(10xVP64) epigenomic editing

The CRISPR/dCas9\_SunTag\_VP64 two-plasmid system<sup>79</sup> was used for genomic editing on *Pcdh* locus in the NG108 neuronal cell line. For pLV-U6-sgRNA-CK-dCas9-10xGCN4-BFP construct, lentiviral backbone and dCas9 cassette were cloned from plasmid Lenti-dCAS-VP64\_Blast (Addgene, #61425), CamK-II promoter (1293 bp) was cloned from plasmid pAAV-CamKII-hChR2(T159C)-p2A-ETFP-WPR (a gift from Javier Maeso), and 10XGCN4-P2A-BFP cassette was cloned from pHRdSV40-dCas9-10xGCN4\_V4-P2A-BFP(Addgene, #60903). The U6-sgRNA cassette was inserted upstream of CamK-II promoter. For the pLV-CK-scFv-GCN4-sfGFP-VP64-GB1-NLS construct, the lentiviral backbone and CK promoter cloned as described above. The scFv-GCN4-sfGFP-RsrII-GB1-NLS (1875 bp) sequence was cloned from plasmid pHR-scFv-GCN4-sfGFP-GB1-NLS-dWPRE (Addgene, #60906), and the VP64 cassette was cloned from plasmid pHRdSV40-scFv-GCN4-sfGFP-VP64-GB1-NLS (Addgene, #60904). All plasmid construction was done by the VectorBuilder team from Cyagen Biosciences. Three sgRNAs (Supplementary Table 16) were designed to target mouse HS16 site at the *Pcdh* locus. For transfection, control cells received a two-vector system pLV-CK-dCas9-10xGCN4-BFP lacking the sgRNA cassettes and pLV-CK-scFv-GCN4-sfGFP-VP64-GB1-NLS; Cell cultures for HS16 superactivation were transfected with pLV-U6-sgRNA-CK-dCas9-10xGCN4-BFP plasmid that included the sgRNA cassettes targeting the HS16 site, and pLV-CK-scFv-GCN4-sfGFP-VP64-GB1-NLS. 72 hours after transfection, cells were harvested and sorted by FACS to collect BFP and GFP double positive cells. Total RNA was then extracted, reverse transcribed and *Pcdh* gene expression quantified by real-time PCR. Primers are listed. Primers are listed in Supplementary Table 16.

### dCas9-KRAB epigenomic editing

Human neural precursor cells (NPCs) were maintained at high density, grown on growth factor reduced Matrigel (BD Biosciences) coated plates in NPC media (Dulbecco's Modified Eagle Medium/Ham's F12 Nutrient Mixture (ThermoFisher Scientific), 1x N2, 1x



B27-RA (ThermoFisher Scientific) and 20 ng ml<sup>-1</sup> FGF2 (R & D Systems, 233-FB-10) and split 1:3 every week with Accutase (Millipore, Billerica, MA, USA). The hiPSC-NPC line 553-S1-1, as previously described and validated<sup>80,81</sup> was used in all NPC editing experiments. *Generation of stable dCas9-KRAB NPCs*: 3.5×10<sup>6</sup> NPCs per well were seeded onto growth factor reduced Matrigel coated 6-well plates in NPC media. The following day lentiviruses generated as above using either the lentiviral vectors dCas9:VP64-T2A-puro and dCas9:KRAB-T2A-puro were added and cultures were spinfected (1 hour, 1000xg, 25°C). Following spinfection, plates were transferred to a cell culture incubator for 3 hours. Media was then removed and replaced with fresh NPC media. The following day, fresh NPC medium containing 1 µg/ml puromycin (Sigma, #P7255) was added and cells were maintained in NPC medium containing 1 µg/ml puromycin for the remainder of the experiment. Stable NPC lines were validated via FACS using Cas9-AF488 antibody (Cell Signaling Technologies, 5µL/1×10<sup>6</sup> cells #34963S). *sgRNA design and cloning*: The sgRNAs were designed using the Optimized CRISPR Design tool (See URLs) at the genomic regions of interest. Guide RNAs were selected based on their specific locations at decreasing distances from region of interest as well as strand specificity and lack of predicted off targets. Synthetic oligonucleotides (Supplementary Table 16) were annealed (95°C for 5 min, ramp down to 25°C at 5°C per minute), diluted 1:100 and then ligated into BsmB1 digested lentiGuide-dTomato. *NPC lentiviral transduction and FACS*: 100,000 dCas9-KRAB NPCs per well were seeded onto growth factor reduced Matrigel coated 24-well plates in NPC medium containing 1 µg/ml puromycin. The following day lentiviruses with scrambleD sgRNAs and pooled sgRNAs targeting PGC-1, PGC-2 and PGC-3 region were added to cultures in the presence of polybrene. 48 hours after transduction, NPC cells were FACS sorted and live cell population with dTomato signal were collected directly into Trizol LS (Thermo Fisher, 10296028). Total RNA was extracted for RT-PCR.

### Data Availability

All next generation sequencing data for genome-scale analysis in this publication have been deposited in NCBI's Gene Expression Omnibus<sup>82</sup> and are accessible through GEO Series accession number GSE99363 (See URLs). All other data discussed are included in the publication and available from the Authors upon request.

### URLs

Optimized CRISPR Design tool, <http://crispr.mit.edu/>

GSE99363, <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE99363>

### Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

### Acknowledgments

Supported by National Institutes of Health, U.S.A. (NIH) grants R01MH106056 (S.A.), P50MH096890 (E.N.), R01MH101454, NIA U01P50AG005138-30-1 and U01AG046170 (K.B.), R01AG050986, R01MH109677, (P.Rou.), R01NS091574 (A.S.), and NIH training and fellowship awards 1F30MH113330 (P.Ra.) and T32-AG049688 (S.C.). Additional support was provided by a Grant-in-Aid for AMED-CREST, AMED, Japan and the

Japan-U.S. Brain Research Cooperation Program to T.Y., the Veterans Affairs Merit grant BX002395 (P.Rou.), the Brain & Behavior Research Foundation (Y.J., P.Rou.), the Alzheimer's Association (P.Rou.), the New York Stem Cell Foundation (K.B.) and the Brain Research Foundation (S.A.).

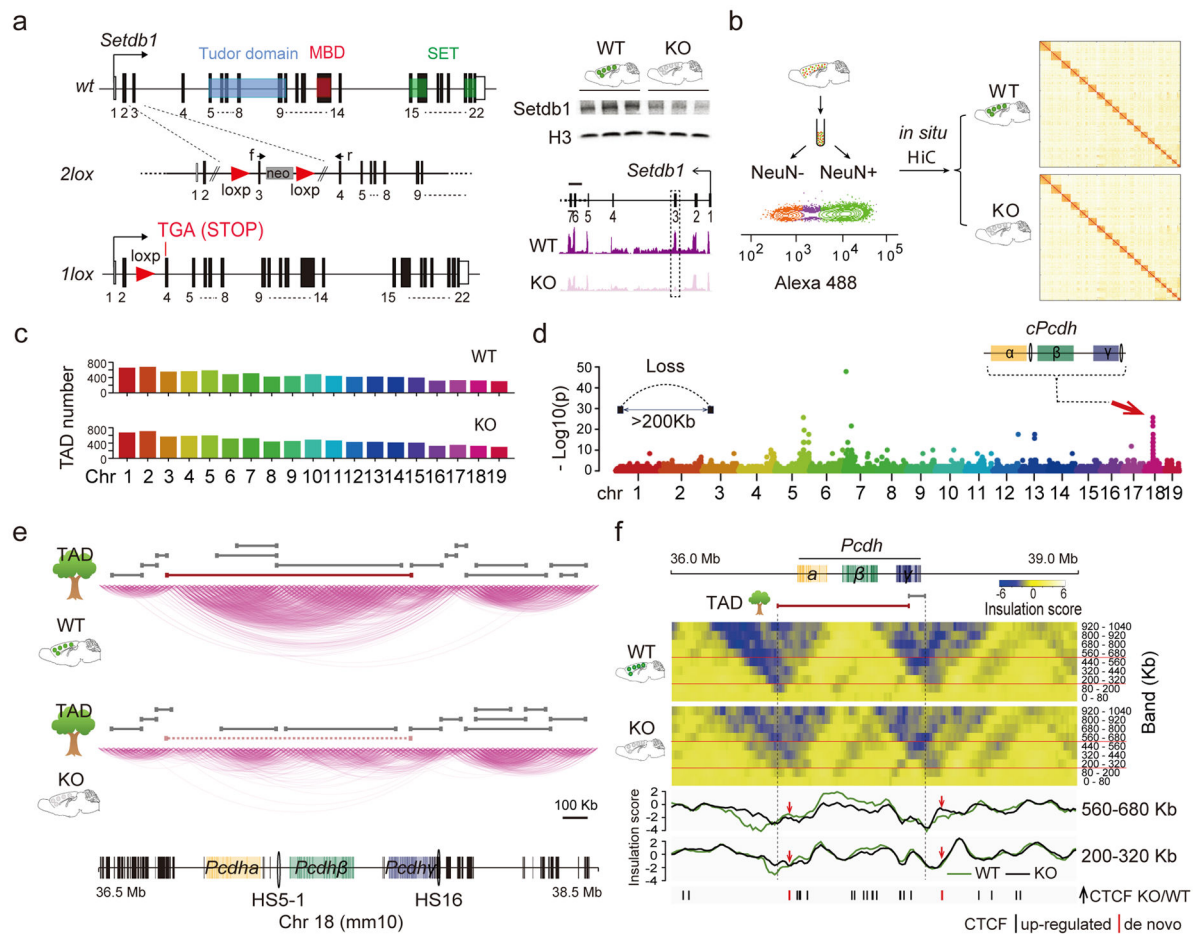
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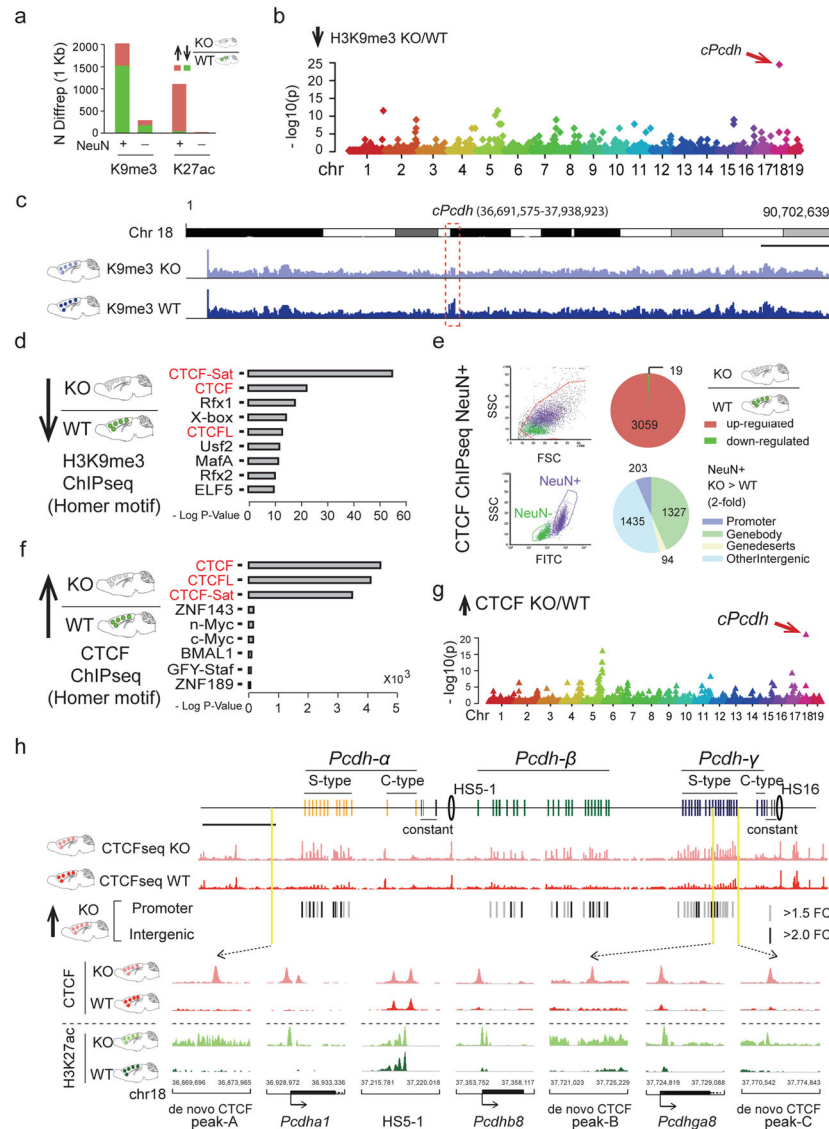
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**Figure 1. 3D genomes in *Setdb1*-deficient cortical neurons**

(a) (Left) Conditional *Setdb1* ablation with loxP sites surrounding exon 3. Recombination results in frame shift and premature stop (TGA) upstream of Tudor, methyl-CpG-binding (MBD) and catalytic SET domains. (Right) *Setdb1* immunoblot (histone H3 loading control) (complete blot shown in Supplementary Figure 1c) and RNA-seq from adult *CK-Cre<sup>+</sup> Setdb1<sup>2lox/2lox</sup>* mutant (K), in comparison to *CK-Cre<sup>-</sup> Setdb1<sup>2lox/2lox</sup>* (WT) cortex. (b) (Left) Flow cytometry-based sorting of adult cortex NeuN immunotagged nuclei. (Right) genome-scale *in situ* Hi-C contact matrix from WT and KO NeuN<sup>+</sup> nuclei. (c) TAD numbers per autosome for mutant (KO) and wildtype (WT) NeuN<sup>+</sup>, (N=2/genotype). (d) Manhattan plot summarizing loss of long-range DNA loop contacts bypassing >200kb linear genome in KO compared to WT NeuN<sup>+</sup>. Notice localized aggregates of densely spaced loop losses on chromosomes 5, 7 and 18. (e) *in situ* Hi-C 2Mb window showing chromosome 18 conformations in KO and WT at position marked by red arrow in Manhattan blot in panel D, with TADs called (TADtree) in both genotypes marked gray. Large 'superTAD' called in WT but lost in KO shown as red line. (f) Contact insulation map for 3Mb window centered on *cPcdh* locus. (Top) Heatmaps from WT and KO cortical neurons for 9 bands, from 0–80kb to 920–1040kb distance. KO shows loss of superTAD<sup>*cPcdh*</sup> insulation. (Bottom) Two representative insulation bands reveal *Setdb1*-sensitive insulation zones in KO neurons aligned with excess CTCF peaks, as indicated.



**Figure 2. Histone modification and CTCF landscapes in *Setdb1*-deficient neuronal nuclei**  
**(a)** DiffRep counts (1kb<sup>sw</sup>) for H3K9me3 methylation and H3K27ac acetylation (ChIP-seq) from KO compared to WT adult cortex, for NeuN<sup>+</sup> and NeuN<sup>-</sup>. H3K9me3 >1.5-fold; H3K27ac >2-fold; FDR P<0.05. **(b)** Manhattan plot with linear representation of autosomes, showing localized enrichments (1MB<sup>sw</sup>) for H3K9me3 hypomethylation in KO. Top-scoring chromosome 18 *cPcdh* locus corresponds to site affected by loss of long-range loop bundles (Figure 1d). **(c)** Mouse total chromosome 18 (mm10; merged fastQ N=3 animals) H3K9me3 landscape for NeuN<sup>+</sup> KO and WT, with ~1.2 Mb (chr18:36,691,575-37,938,923) *cPcdh* locus flagged. Scale bar, 10 Mb. **(d)** CTCF motif (red) enrichment in sequences H3K9me3 hypomethylated in KO. **(e)** (Left) FACS plots showing separation of crosslinked NeuN<sup>+</sup> from NeuN<sup>-</sup> nuclei (adult cortex) for cell-type specific CTCF ChIP-seq. (Right) Mutant NeuN<sup>+</sup> showed 3059 CTCF up- and only 19 CTCF down-regulated sequences, affecting primarily inter- and intragenic DNA (>2-fold KO/WT NeuN<sup>+</sup> nuclei, N=4 animals/group, FDR P <0.05). **(f)** Dramatic CTCF motif (red) enrichment among the 3059 CTCF-up

sequences. **(g)** Autosomal genome Manhattan plot showing localized clustering of CTCF up sequences in KO neurons, with *cPcdh* as top ranking locus (1Mb<sup>sw</sup>). **(H)** (Top) *cPcdh* CTCF landscapes in KO and WT NeuN<sup>+</sup> as indicated. Significantly up-regulated (KO>WT) promoter-bound and intergenic CTCF sequences marked separately. Scale bar, 100 kb. (Bottom) Coordinate increase of (red) CTCF and (green) H3K27ac at selected *cPcdh* promoters and intergenic DNA in KO neurons. In contrast, robust peaks at baseline, independent of genotype, at DNaseI hypersensitive HS5-1(HS5-1a+HS5-1b).

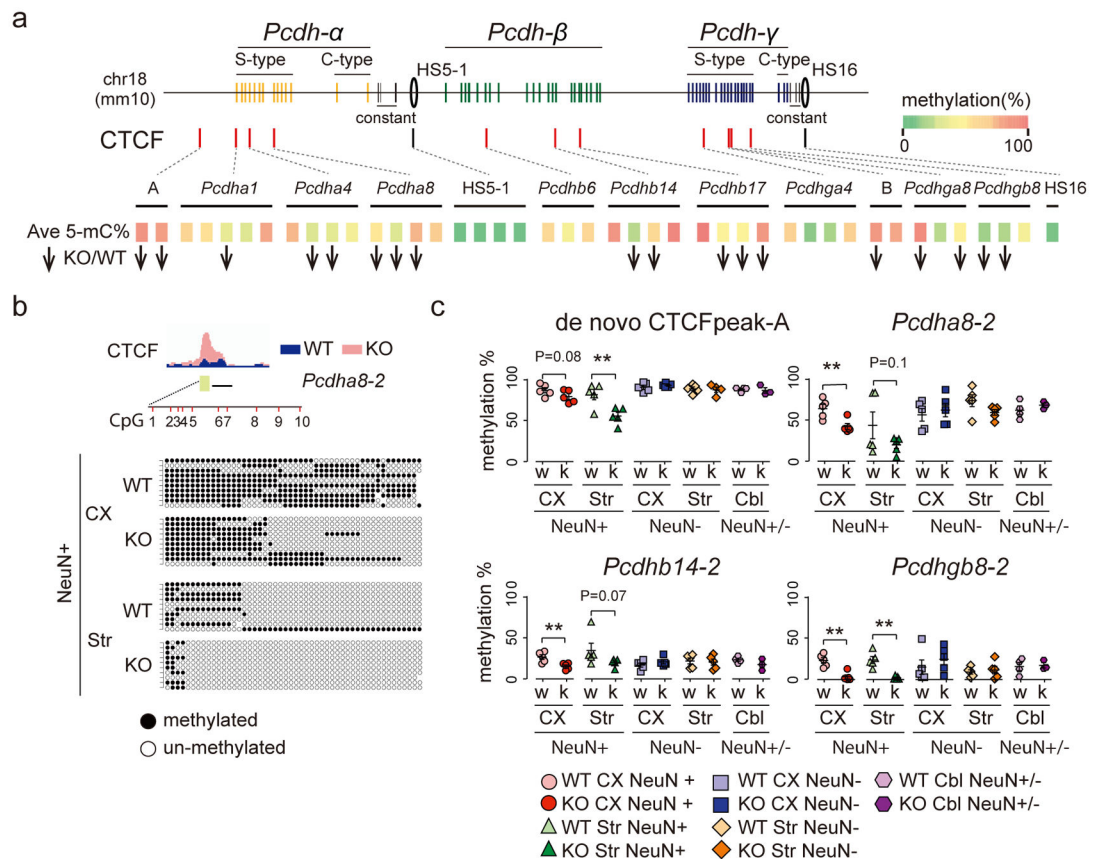
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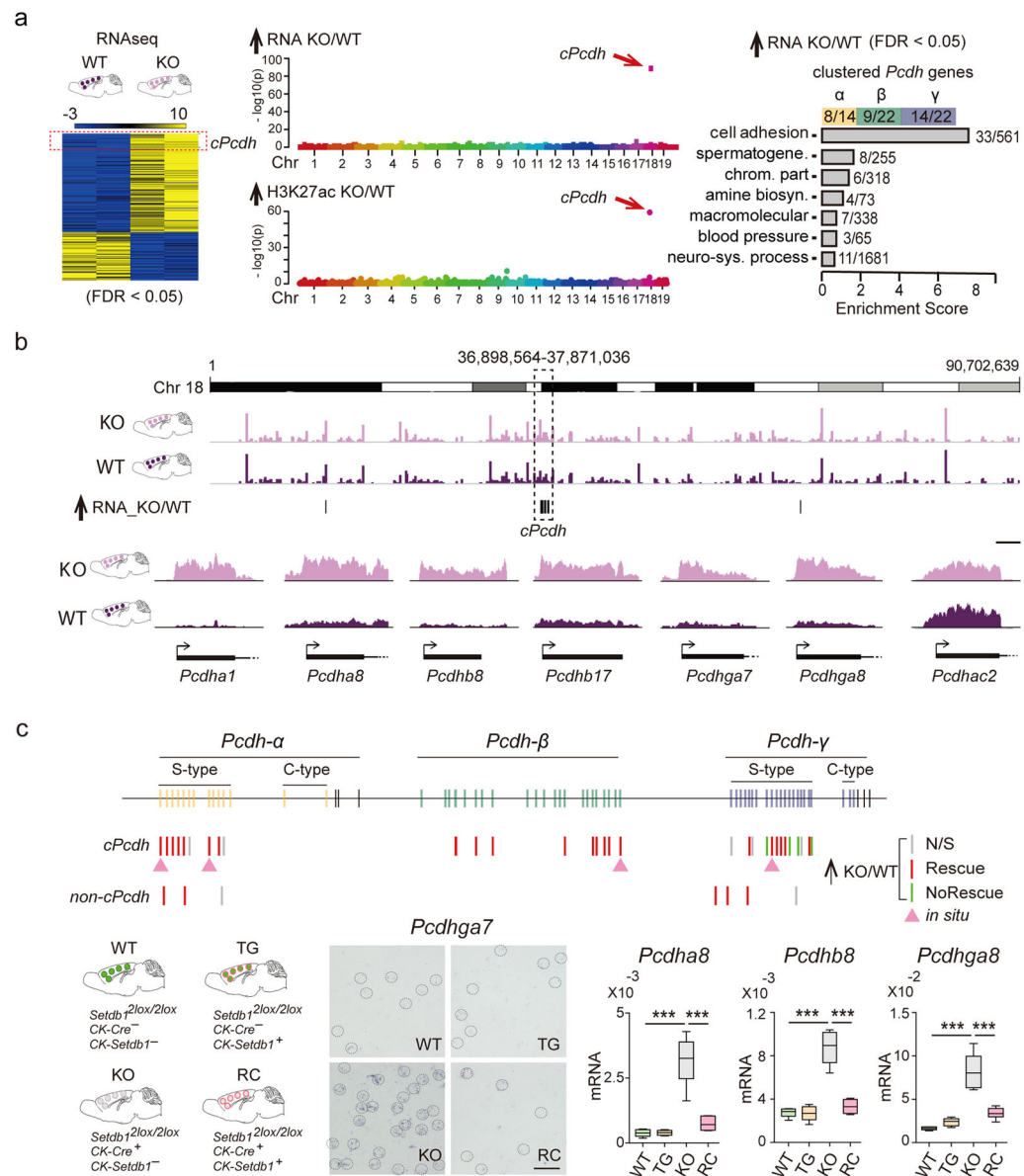
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**Figure 3. DNA methylation profiling at the *cPcdh* locus**

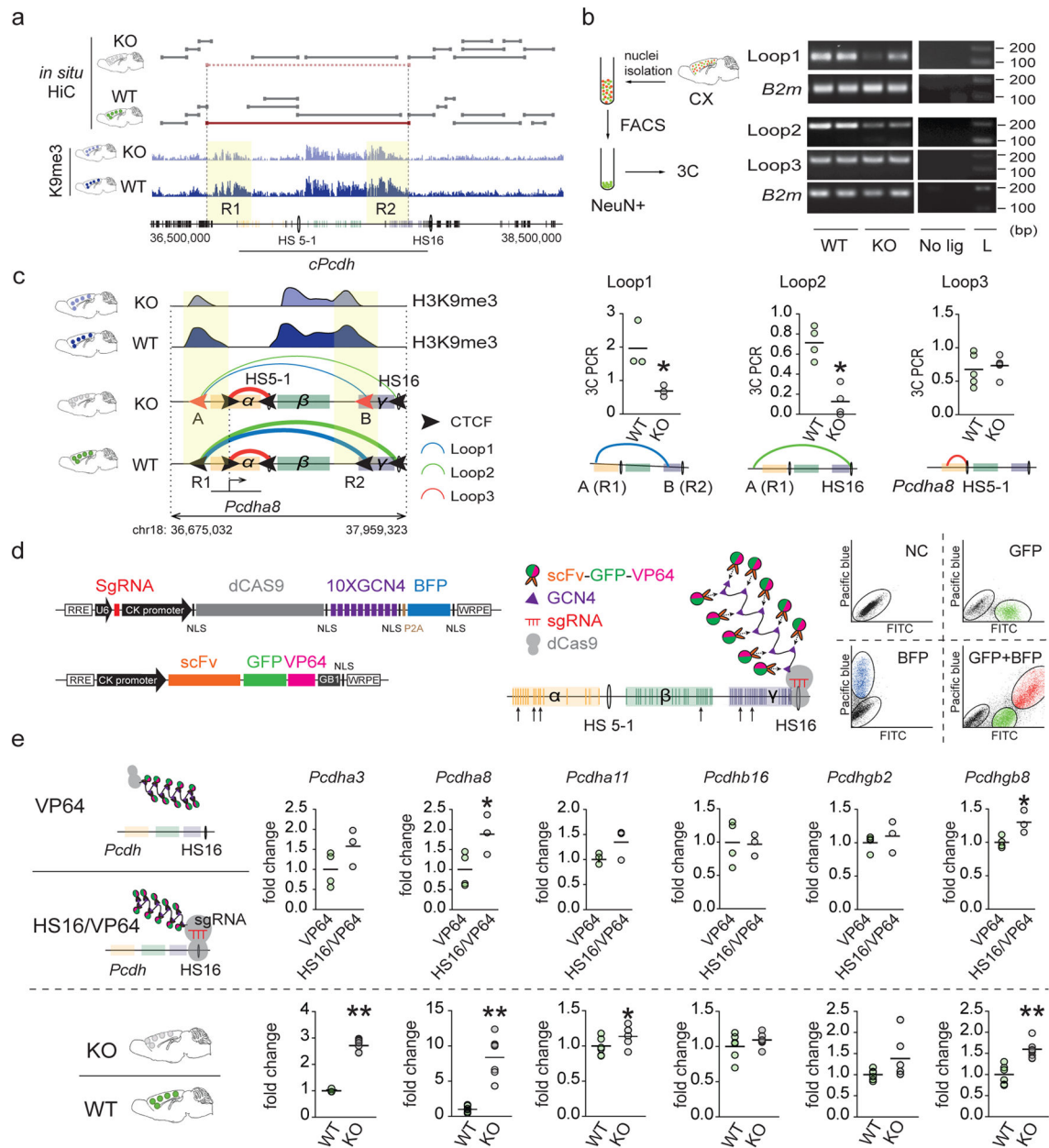
(a) (Top) red tick marks for 11 *cPcdh* promoters and 2 intergenic sequences (A,B) with excess/*de novo* CTCF occupancy in KO neurons; black tick marks for HS5-1 and HS16 with robust CTCF peaks in both KO and WT (see also Figure 2h). (Bottom) Averaged 5mC DNA methylation levels (green-red=0–100%) of 43 amplicons representing the set of 13 regulatory sequences show in Top panel. Bis-seq data were averaged across 47 DNA samples from cortical and striatal NeuN<sup>+</sup> and NeuN<sup>-</sup>, and cerebellar homogenate. Downward arrows: 18/43 bis-seq amplicons show <sup>3</sup>mC5 deficit in *Setb1*-deficient neurons. 0/43 show increase ( $P < 0.5 - 0.1$ /amplicon) (see Supplementary Table 13 for details on quantification). (b) Representative bis-seq example from *Pcdha8* amplicon no.2 capturing 10 CpG sites. Score cards from 50 randomly selected DNA molecules: circles black/white methylated/not methylated. (c) Quantification of bis-seq amplicons expressed as %methylated. \*,\*\* $P < 0.05(0.01)$  unpaired one-tailed t-test. Each symbol represents 1 sample from 1 animal. Note methylation deficits specifically for cortical (CX) and striatal (Str) NeuN<sup>+</sup> from *Setdb1*-deficient (k) neurons, compared to wildtype (w).



**Figure 4. Transcriptional dysregulation at the *cPcdh* locus**

(a) (Left) genome-wide RNAseq heatmap, blue-yellow range show average levels of expression (log 2), for transcripts with significant (FDR  $P < 0.05$ ) difference in expression of KO compared to WT PFC. (Right) Gene Ontology of differentially expressed genes (FDR  $P < 0.05$ ) highlight Setdb1-dependent regulation of *cPcdh* cell adhesion genes. (Middle) Manhattan plots for autosomal genome (mouse chromosomes 1–19), showing singular enrichment (1MB<sup>SW</sup>) for (Top) upregulated transcripts and (Bottom) histone hyperacetylated chromatin at chromosome 18 *cPcdh* locus, as indicated. (b) (Top) Whole chromosome 18 view on cortex RNA-seq, merged FastQ N=2KO (light purple) and N=2WT (dark purple). (Bottom) representative RNAseq tracks for first exon of specific *Pcdha*, *Pcdhb*, and *Pcdhy* genes. Scale bar, 500bp. Notice increased expression primarily from non-C type *Pcdh* genes with stochastic expression pattern (S-type), while C-type *Pcdh* genes remain unaffected. (c)

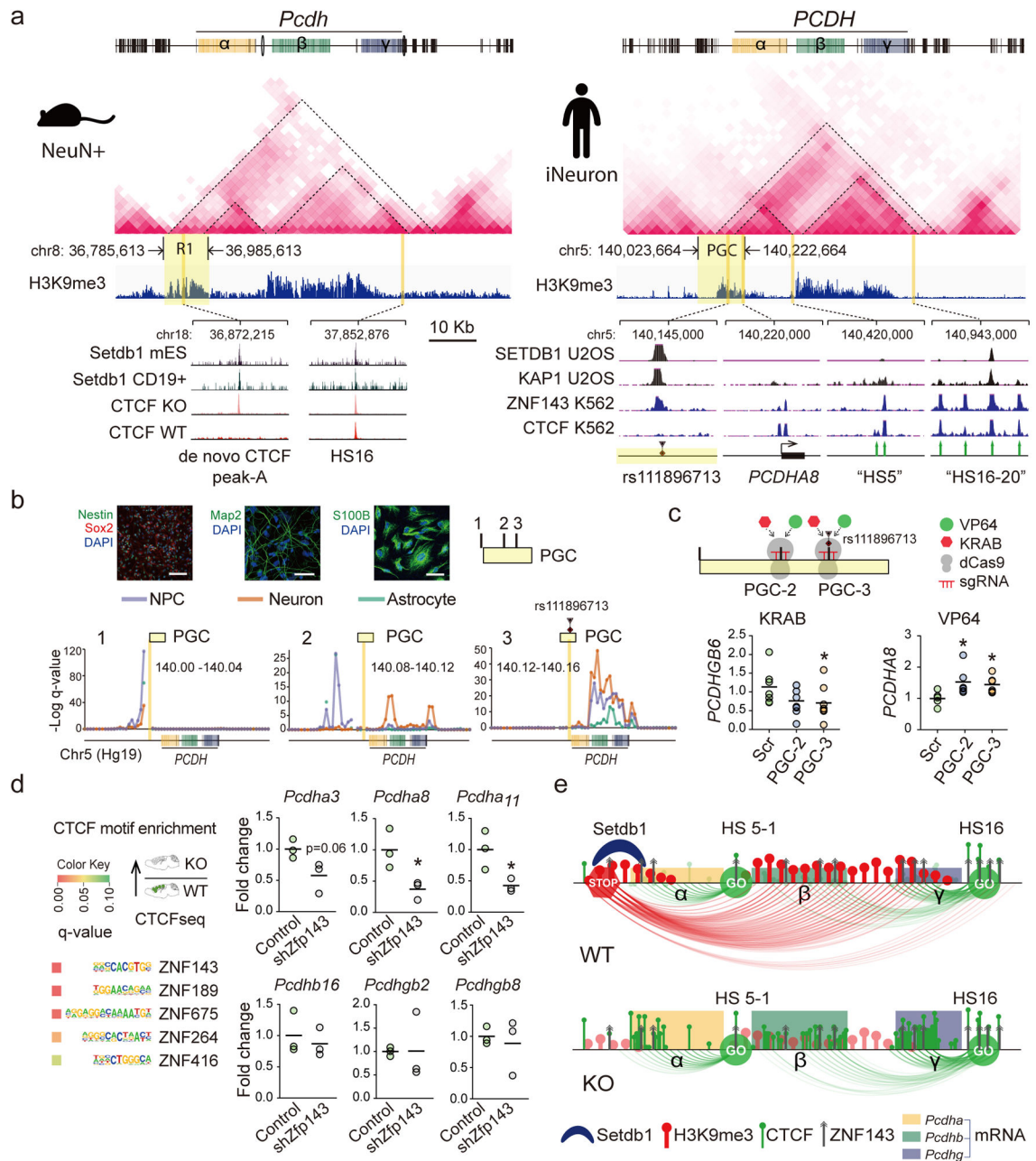
Top: *Pcdh* and non-*Pcdh* transcripts tested in adult cortex from mutant and transgenic rescue mice and their respective controls by qRT-PCR and *in situ* hybridization as indicated. Bottom: Transgenic rescue for representative S-type *Pcdh*, shown by ISH from middle layers of lateral cerebral cortex (scale bar, 50  $\mu$ m). Box plots (1<sup>st</sup>/3<sup>rd</sup> quartile, median, whiskers<sup>min,max</sup>) summarizing qRT-PCR in PFC of WT, TG (*CK-Setdb1*<sup>+</sup> transgenic line), KO and RC (*CK-Setdb1*<sup>+</sup> transgenic rescue of conditional *CK-Cre*<sup>+</sup>, *Setdb1*<sup>2flox/2lox</sup> mutants). N=6/group, \*\*\*P<0.001. *Pcdha8*, t<sub>(WT/KO)</sub> = 9.59, t<sub>(KO/RC)</sub>=8.33; *Pcdhb8*, t<sub>(WT/KO)</sub>=11.07, t<sub>(KO/RC)</sub>=10.03; *Pcdhga8*, t<sub>(WT/KO)</sub>=10.07, t<sub>(KO/RC)</sub>=7.39. One-way ANOVA, Bonferroni corrected. Additional ISH gene expression data are shown in Supplementary Figures 12–15.



**Figure 5. Epigenomic editing at the *cPcdh* locus**

(a) *cPcdh* locus and surrounding sequences ~2Mb of mouse chr. 18, including TADs called (TADtree) and H3K9me3 tracks for KO and WT. Notice ‘shrinkage’ of broadly (>100–200kb) stretched ‘R1’ and ‘R2’ blocks of H3K9me3-tagged chromatin in KO neurons. (b) Overview on cell-type specific 3C-PCR, cropped agarose gels showing specific loop products for *cPcdh* and *B2m* control. No lig=3C without DNA ligase, L=100bp DNA ladder. Dot graphs summarizing 3C-PCR (mean±S.E.M.; 1 dot=1 animal) *cPcdh* loop1,2,3 as indicated. All data normalized to *B2m* 3C,  $N_{(Loop1)}=3/\text{group}$ ,  $*P_{(Loop1)}=0.05$ , Mann Whitney, one-tailed;  $N_{(Loop2)}=4/\text{group}$ ,  $*P_{(Loop2)}=0.014$ , Mann Whitney, two-tailed. Loop defects in KO include A/R1 (de novo CTCF peak A in R1)-HS16 and A/R1-B/R2 (de novo

CTCF peak B in R2). In contrast, shorter-range *Pcdha8* promoter-HS5 enhancer loop is maintained in KO neurons. Complete gels shown in Supplementary Figure 16c. (c) Summary presentation of 3C-PCR. (d) dCas9-SunTag superactivation of HS16 *cPcdh* enhancer with U6-sgRNA cassette upstream of *CK-dCas9-10xGCN4<sup>epitope</sup>-BFP* cassette, and *CK-svFv-sGFP-VP64* cassette on separate vector. Representative FACS sort shows dually labeled BFP<sup>+</sup>GFP<sup>+</sup> NG108 cells. NC=negative control (e) RT-PCR quantification (mean±S.E.M.; 1dot=1cell culture or animal) of *Pcdha3*, *Pcdha8*, *Pcdhb16*, *Pcdhgb2* and *Pcdhgb8* transcripts (black arrows in panel d mark genomic positions), normalized to *Gapdh* RNA. (Top) BFP<sup>+</sup>GFP<sup>+</sup> NG108 cells with (HS16/VP64) and without (VP64) sgRNA<sup>HS16</sup> cassette. (Bottom) adult KO and WT PFC. N=4 VP64/3 hs16/vp64 (NG108 cells), \*P(*Pcdha8*)=0.0268, \*P(*Pcdha11*)=0.0437, \*P(*Pcdhgb8*)=0.0126, unpaired t test, one-tailed; N=6/group (mice), \*\*P(*Pcdha3*)=0.002, \*\*P(*Pcdha8*)=0.002, \*P(*Pcdha11*)=0.026, \*\*P(*Pcdhgb8*)=0.0022, Mann Whitney, two-tailed. See also Supplementary Figure 16A for additional 3C-PCR loop quantifications.



**Figure 6. Regulatory mechanisms at human and mouse TAD<sup>cPCDH</sup>**

(a) (Top) Neuronal *in situ Hi-C* interaction matrices, and H3K9me3 landscapes, for ~2Mb of mouse and human *cPCDH*, including superTAD spanning across  $\alpha, \beta, \gamma$  clusters. (Bottom left) Setdb1 peaks in mouse embryonic stem cell and lymphocytes match to *de novo* CTCF peak in *Setdb1* KO neurons. (Bottom right) ‘PGC’, Psychiatric Genomics Consortium risk haplotype *chr5:140,023,664-140,222,664* with lead polymorphism rs111896713 matching to Setdb1, KAP1 and ZNF143 peaks. Note epigenomic similarities of ‘R1’ (mouse) and ‘PGC’ (human). (b) Neural progenitor cell (NPC) differentiation into neurons and astrocytes, with phenotypic markers as indicated. Scale bar, NPC (neuron/astrocyte) 100 (50)  $\mu\text{m}$ .

Conformations for three representative 40kb bins from 200kb ‘PGC’ haplotype, with bin harboring the index polymorphism (‘PGC-3’) showing dramatically increased *cPCDH* contact. (c) Dot graphs show *cPCDH* gene expression in epigenomically edited NPC, with *PCDHGB6* (but not *PCDHGA8*) transcript decreased by sgRNA-guided dCas9-KRAB in 3/3 experiments. dCas9-VP64 elicits increased expression of a subset of *Protocadherin* transcripts. Scr, scrambled control. All data normalized to 18srRNA, shown as fold change. N=6–9/group,  $P < 0.05$ , Mann Whitney, two-tailed. (d) ZNF-specific motif enrichments in CTCF-up sequences. Dot graphs (1dot/cell culture) summarize expression of specific *Pcdha*,  $\beta$  and  $\gamma$  genes after *shRNA*-induced Zfp143 knock-down in NG108 neuroblastoma cells. Unpaired t test, two-tailed, N=3 per treatment. \* $P < 0.05$  (e) Schematic summary of TAD<sup>*cPcdh*</sup> epigenomic architectures in WT and KO neurons. Loss of repressive long-range contacts in KO shifts the balance towards facilitative shorter range promoter-enhancer loopings.