Methyl-CpG binding domain 2 (Mbd2) deficiency causes cognitive, social and emotional deficits and down-regulation of ortholog Autism risk genes

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Abstract

Methyl-CpG-binding domain 2 (Mbd2) binds methylated DNA and was shown to play an important role in cancer and immunity but its role in brain function is mostly unknown. Here we show that *Mbd2*-deficiency in mice results in deficits in cognitive, social and emotional functions. Chromatin immunoprecipitation (ChIP-seq) revealed that Mbd2 binds to regulatory DNA regions of neuronal genes in the hippocampus. Loss of *mbd2* alters the expression of hundreds of genes with a robust down-regulation of neuronal gene pathways. Genes down-regulated by loss of *mbd2* are highly enriched for ortholog Autism Spectrum Disorder (ASD) risk genes. Further, a genome-wide DNA methylation analysis found an altered DNA methylation pattern in regulatory DNA regions of neuronal specific genes in *mbd2* deficient mice. Intra-hippocampal viral down-regulation of *mbd2* recapitulate most of the behavioral phenotypes observed in knockout mice. Taken together, our data suggest that Mbd2 regulates hippocampal DNA methylation landscape to control neuronal gene expression and ASD-like phenotypic behaviors.

Introduction

Epigenetic modifications of the genome are long known to play a crucial role in normal brain function and a wide range of neuropsychological functions and neuropsychological disorders[1]. The most studied epigenetic modification is DNA methylation, the addition of a methyl group to the DNA on a cytosine in a CpG dinucleotide context. DNA methylation in promoters and other regulatory regions suppress gene expression by interfering with transcription-factors and transcription machinery binding[2,3]. An additional mechanism involves recruitment of members of a methylated-DNA binding proteins family (MECP2 and MBD1-6) which share a Methyl-CpG Binding Domain (MBD) [4,5]. MECP2 and MBD2 were shown to recruit chromatin repressive complexes to genes and thus cause alterations in histone modification and silencing of gene-expression[6-8]. These proteins are highly expressed in brain tissues[9,10].

The most extensively studied MBD protein is MeCP2 since mutations and duplications of this gene cause Rett syndrome[11,12]. Some studies on the role of MBD1 suggest it has a role in neurodevelopment and neurodevelopmental disorders like autism[13]. In contrast, little is known about the roles of other MBD proteins in the brain.

Several studies associated MBD2 with neuropsychiatric disorders. Polymorphisms in *MBD2* genes were associated with schizophrenia[14]. An increased MBD2 DNA binding on the promoters of *BDNF*, *GAD1* and *RELN* genes was observed in post-mortem brains of schizophrenia and bipolar disorder patients[15]. One study associated polymorphism of *MBD2* gene to rare cases of autism[16]. More importantly, several studies found Copy Number Variants (CNV) at and around the genomic position of the *MBD2* gene (18q21.2) in ASD patients (see SFARI-CNV database:

https://gene.sfari.org/database/cnv/ and Fig4E). Recently, we found MBD2-bound DNA regions to become hypo-methylated in the prefrontal cortex of depressed suicide-completers[17]. Further, in an animal model for maternal-care, we found low maternal care rat pups to show reduced Mbd2 expression in the hippocampus which led to reduced glucocorticoid receptor expression and elevated stress[18]. However, to date the mechanisms by which MBD2 affect gene-expression and ultimately brain function and behaviors are unknown.

In the present study, we directly assessed the role of Mbd2 in behavior using a knockout mouse model (*mbd2-/-*). A comprehensive behavioral battery found cognitive, social and emotional deficits in mbd2-/- mice. Since the behavioral abnormalities pointed to hippocampal functions we examined the molecular footprints of Mbd2 in the hippocampus. We applied unbiased genome-wide approaches. Using ChIP-seq with an Mbd2-specific antibody we mapped the genome binding sites of Mbd2 and found that it binds methylated and unmethylated CpGS on and around many neuronal genes in the hippocampus as well as other genomic locations. Loss of Mbd2 binding in *mbd2-/-* mice led to down-regulation of neuronal-genes. In contrast, genes that are not specific to brain were activated by *mbd2*-deficiency. *mbd2*-deficiency led to increased methylation on promoters and enhancers of neuronal genes, suggesting that Mbd2 maintains DNA hypomethylation status on neuronal genes and hence hippocampal genome functions. Furthermore, we found that genes down-regulated by loss of *mbd2* are highly enriched for ortholog ASD risk-genes implying an important role for Mbd2 in neurodevelopment and neuropsychiatric disorders.

Methods

Full details of all methods can be found in the supplementary file. All procedures were carried out in accordance with the guidelines set down by the Canadian Council of Animal Care and were approved by the Animal Care Committee of McGill University.

Results

Mbd2 is required for a range of cognitive, social and emotional behaviors

We investigated whether Mbd2 plays a role in behavioral phenotypes using a battery of behavioral test in *mbd2-/-* mice and wild-type mice. There was no change in general locomotion in an open-field box (Fig 1A) or in other exploratory behaviors (speed, entries to the center of arena, time near walls; Fig S1). Several forms of memory were explored as well. Short-term working-memory was assessed by spontaneous alternations in Y-Maze and was found to be intact in *mbd2-/-* mice (Fig 1E). In contrast, *mbd2-/-* mice showed impaired memory-retention in the long-term object-location memory test compared to wild-type mice and failed to explore the object in the novel location for more than chance (Fig1B). *mbd2-/-* mice also exhibited reduced social-interaction time and increased anxiety in the dark-light box (Fig 1C-D). Taken together, these findings suggest that Mbd2 is involved in regulating a range of cognitive, social and emotional behaviors.

Landscape of Mbd2 binding in the hippocampus

We focused our analysis on the hippocampus since the behaviors affected are known to involve hippocampal functions[19-23]. In addition, other members of the MBD protein family were shown to have an important role in hippocampal function[13,24-26]. Mbd2 ChIP-seq experiment from hippocampi of wild-type mice revealed 2436 Mbd2 peaks annotated to 1222 genes. As

expected, Mbd2 binds mostly to CpG-containing and GC-rich DNA regions (X^2=43.364; df=1, p=4.54E-11 and X^2=-1129.7; df=1, p=2.2E-16, respectively). Highly-enriched peaks were observed in proximity to transcription-start-sites (Fig2A and FigS2A, Spearman rho=-0.13, p=2.4E-11, for absolute distance from TSS). Highly-enriched peaks were also correlated with peak length (Spearman rho=-0.489, p=2.2E-16, data not shown). Interestingly, de-novo motif discovery found the transcription-factors E2F7 (p=1E-197) and Zic3 (p=1E-143) to be highly enriched in hippocampal Mbd2 peaks (FigS2B). The E2F family and Zic family have been found before to have a role in neurogenesis and brain development[27-29]. We also compared our data to publicly available ChIP-Seq data of the mouse hippocampus histone-marks[30]. As expected, Mbd2 bound overall to more repressive histone-marks (mostly H3K9me3) than to active histone-marks. However, a subset of the peaks was bound to both active and repressive histone-marks implying Mbd2 binds the same DNA regions in different cell populations within the hippocampus potentially participating in chromatin remodeling (Fig2B-C). Mbd2 co-occupancy with histone-marks on promoters, 5' UTRs and gene-bodies (mostly exons) was enriched for active histone-mark and depleted from repressive histone-marks (Fig2D). Pathway-analysis by GO-enrichment found that Mbd2 binds many neuronal- and brain-related pathways such as: trans-synaptic signaling, synapse organization and behavior (Fig2E). These pathways also showed good clustering as GO-networks (FigS3) and protein-protein interactions analysis found forebrain-development and axon-guidance as the top 2 annotations (log10p= -6.3 and -5.6, respectively) further supporting a role for Mbd2 in neuronal function.

We selected 15 peaks of Mbd2 that were associated with promoters or enhancers in neuronal-related genes for further validation and for determining the impact of Mbd2 binding loss on

transcription-initiation (see Table S1 for genomic coordinates of validated peaks). Quantitative-ChIP confirmed binding of Mbd2 to these regions and loss of binding in *mbd2-/-* mice (Fig2F). We then determined whether loss of Mbd2 binding in promoters or enhancers in *mbd2-/-* mice affects transcription onset. *mbd2*-deficiency resulted in reduced transcription onset since occupancy of RNA-polymerase phosphorylated on Serine5; (RNApolII(SP5)) the form found on promoters upon transcription-initiation[31,32] was reduced in *mbd2-/-* mice. Interestingly, this was associated with increased abundance of histone-mark (H3K4me1) marking enhancers in *mbd2-/-* (Fig2G-H). H3K4me1 modification marks active as well as inactive enhancers[33,34]. H3K4me1 peaks that are flanking the TSS were shown to exhibit a peak-valley-peak pattern. The valley usually overlaps with transcription-initiation and RNApolII(SP5) peaks[35] reflecting a nucleosome-free zone and thus reduced histone presence and reduced signal for the histone-marks, which is prerequisite for transcription-initiation[36]. In transcriptionally inactive genes H3K4me1 peaks cover the entire TSS forming one continuous peak centered at the TSS and overlapping with the RNApolII(SP5) peaks. The increase of H3K4me1 concurrently with reduction of RNApolII(PS5) binding at the same position in *mbd2-/-* mice is consistent with inhibition of transcription onset in response to *mbd2-/-*. These data suggest that Mbd2 is involved in activation of transcription turn on in these neuronal-specific promoters.

Mbd2 is required for expression of neuronal specific genes

To further elucidate the role of Mbd2 in hippocampal gene-expression we delineated the transcriptomes of wild-type and *mbd2-/-* mice using RNA-Seq. We found 2907 genes to be differentially-expressed (FDR<0.05), of which 1590 genes were up-regulated and 1,317 gene were down-regulated in *mbd2-/-* mice (Fig3A, and FigS4 for validations). Pathway-analysis by

GO-enrichment found a robust down-regulation of neuronal-related pathways such as neuron-projection development, trans-synaptic signaling and behaviors (Fig3B) which were highly organized in clusters based on clustering analysis of the GO-networks (Fig3C). In strike contrast, the same analyses applied to up-regulated genes revealed mostly homeostasis and metabolism related pathways with no enrichment of neuronal-related pathways. GO-network analysis revealed poor clustering of the differentially-expressed genes in these pathways (Fig3D-E).

Next, we determined whether the differentially-expressed genes were interacting with Mbd2 by overlapping the Mbd2 ChIPseq and RNAseq results. A significant fraction of the down-regulated genes had Mbd2 peaks annotated to them (85 genes; p=1.8E-9; hypergeometric-test) while fewer up-regulated genes (55) had annotated Mbd2 peaks (p=0.31; hypergeometric-test). Meta-analysis of the overlapping ChIP-seq and RNA-seq enriched pathways by unsupervised clustering found that the top downregulated pathways in *mbd2-/-* mice were trans-synaptic signaling, behavior and synaptic organization (Fig3F-G). These findings are consistent with a role for Mbd2 in regulating neuronal-specific gene-expression.

The impact of *mbd2* depletion on the DNA methylation landscape

MBD proteins are "readers" of DNA methylation and bind specifically to methylated DNA[4,37]. However, it is possible that they also play role in maintaining DNA methylation/demethylation landscapes as has been previously proposed. Previous studies in cancer cells and in T-regulatory cells have suggested that depletion of *mbd2* causes hypermethylation of regulatory regions which in turn results in downregulation of gene-expression[38]. Therefore, we tested whether *mbd2*-deficiency would alter the landscape of DNA methylation in the hippocampus. We

mapped with targeted-bisulfite-sequencing the methylation state of regulatory DNA regions (promoters and enhancers, see methods) in the hippocampi of *mbd2-/-* and wild-type mice at a single-base resolution. A global methylation analysis revealed that Mbd2 binding regions defined in wild-type mice by ChIP-seq were hypermethylated in *mbd2-/-* mice (Fig 3H). Hypermethylation in response to *mbd2* depletion was observed also in a genome-wide scale analysis which examined DNA outside the regions that bind Mbd2 ($p<2.2E-16$, K-S test, data not shown). The fact that changes in DNA methylation occur in regions that do not bind Mbd2 suggests *mbd2*-deficiency could affect DNA methylation indirectly. Taken together these data suggest that Mbd2 protects certain sequences from hypermethylation and maintains them in a hypomethylated state. However, loss of *mbd2* affects DNA methylation in both directions. An analysis of the sequence properties of Mbd2-dependent DNA methylation suggests that *mbd2*-deficiency affects low to intermediate methylated CpGs (10-40% methylation) but highly methylated CpGs (>40% methylation) are unaffected as expected if Mbd2 function is to prevent hypermethylation. Partially methylated genes represent a group of genes that are heterogeneously methylated and possibly heterogeneously expressed across hippocampal nuclei. Mbd2 might be regulating the state of methylation of these genes.

We also analyzed differential-methylation at single CpG resolution. At this resolution differential-methylation analysis (at least10% difference) revealed 323 differentially-methylated CpGs, with 133 hypo-methylated CpGs (annotated to 103 genes) and 190 hyper-methylated CpGs (annotated to 119 genes) (differential-methylation data ($p<0.001$) is detailed at Table S2). This finding also supports a significant overall hypermethylation in *Mbd2-/-* mice hippocampus ($p=0.0018$, binomial-test). For pathway-analysis, we applied a more lenient significance cut-off

for our data ($p<0.001$) resulting in 3005 differentially-methylated CpGS (1519 hypo-methylated and 1486 hyper-methylated). Next, we focused the analysis on CpGs located on promoters (±1000bp from TSS). We found 494 hypo-methylated CpGs located in 460 gene-promoters and 476 hyper-methylated CpGs located in 434 gene-promoters (FigS5A). Pathway-analysis for differentially-methylated gene-promoters revealed adrenergic receptor signaling and sodium- and metal- ion transports as the top three hyper-methylated pathways (-log10p-values: 4.86, 4.16 and 3.42, respectively). In contrast, the top hypo-methylated pathways were not specifically related to neurons or brain functions, although neuronal ion-channel clustering and positive regulation of sodium ion-transport pathways were enriched (-log10p-values: 3.289 and 3.280, respectively) (FigS5C-D). Overall, this analysis suggests an enrichment of hyper-methylated genes related to neuronal system in *mbd2-/-* mice hippocampi.

To assess the relation between promoter DNA methylation and gene-expression we determined the correlation between methylation and expression. We found a significant linear inverse correlation between promoter DNA methylation and gene-expression (Fig3I), supporting a role for DNA methylation in gene-repression in the hioppocampus. Unexpectedly, a subset of differentially-methylated CpGs in promoters showed positive correlation between expression and methylation. We explored this finding by analyzing DNA motifs around these CpGs. While CpGs with inverse correlation (n=66) had no significantly enriched motifs; CpGs with positive correlation (n=40) had 3 enriched motifs (p-values= 0.015-0.025). These enriched motifs were highly similar to known motifs of the KLF and SP transcription-factor families with top similarities to: KLF12, KLF14, SP3 and SP8 (FigS5B). The KLF and SP transcription-factors are known to prefer binding to methylated-DNA (KLF) or to be unaffected by DNA methylation (SP)

[39]. This finding suggests that genes whose methylation status does not correlate inversely with their expression, are regulated by transcription-factors that are not inhibited by DNA methylation.

We also validated our DNA methylation analysis results by targeted-sequencing of bisulfite-converted PCR amplicons of nine Mbd2 binding regions. Methylation levels in this cohort strongly correlated with the levels obtained in the genome-wide capture sequencing analysis (r>0.91). Loss of *mbd2* promoted bi-directional alterations in DNA methylation status with more robust hyper-methylation, as observed before (FigS6A-B). Overall there was a significant hyper-methylation in *mbd2-/-* samples (Fig3J and FigS6C) as was observed in the genome-wide capture-sequencing.

Genes whose expression is reduced in *mbd2-/-* are associated with ASD risk genes

Other MBD proteins were associated with ASD[12,13,24,26,40]. The presentation of behavioral abnormalities in *mbd2-/-* mice described above resembles some of the observed symptoms of ASD. CNVs that were shown to associate with ASD include those that map onto genomic regions of the *Mbd2* gene. We further explored whether differentially-expressed genes due to loss of *mbd2* in the mouse hippocampus are associated with ortholog human "risk genes". Comparative analyses of our RNA-seq data with that of two human ASD databases ([41]) and SFARI-gene (see methods), found a robust overlap between ASD-risk genes and down-regulated genes in *mbd2-/-* mice. Eight (24.24%) out of 33 top loss-of-function ASD risk-genes (FDR<0.1) and 17 genes (15.88%) out of 107 ASD risk-genes (FDR<0.3) found by De Rubeis et al.[41] were down-regulated in *mbd2-/-* mice (p=0.0014 and p=0.00095 respectively, hypergeometric-test,

Fig4A-B and tableS3). In contrast, only 1 gene from the 33 gene-list and 5 genes from the 107 gene-list were up-regulated in *mbd2-/-* mice (p=0.942 and p=0.947 respectively, hypergeometric-test). Next, we compared our RNA-seq results to the SFARI-gene list of ASD-associated genes which contains all known human genes associated with ASD. Here again, out of 951 human genes associated with ASD, 125 (13.14%) were down-regulated (p=8.8E-13, hypergeometric-test, Fig4C) while only 44 were up-regulated in *mbd2-/-* mice (4.62%, p=0.99, hypergeometric-test, Fig4C and table S3). These cross-species comparative analyses suggest that Mbd2 might serve as an up-stream activator to many ASD-associated genes as has previously been shown for liver, breast and prostate cancer genes in cancer cell lines[42-44] NGFIA in hippocampal cells[18] and *Foxp3* gene[38] in regulatory T-cells. A protein-protein association network analyses of Mbd2-regulated ASD-associated gene-lists found significant protein-protein interactions enrichments. The 125 down-regulated genes which appears also in the SFARI ASD-associated gene-database projected into a significantly enriched protein-protein network (p<1E-16; Fig 4D) with "nervous-system development" and "behavior" as the most enriched GO-pathways within the network (p=5.84E-15 and p=2.14E-13 respectively, table S4). All other differentially-expressed ASD-associated genes analyzed have significant protein-protein interactions with top GO-pathway annotations related to nervous-system development and learning (see table S4 and figS7). The human *MBD2* gene locus and its surroundings (18q21.2) has known CNVs that were previously associated with ASD (Fig4E). We further analyzed the protein levels of few members of the protein-protein network of ASD-risk genes and found reduced protein levels in *mbd2-/-* mice (Fig 4F) which agrees with the mRNA reduction we observed before.

Morphological changes in the hippocampus were observed as well. We found increased neuron count in the CA1 and CA2 regions of the hippocampus (Fig4G-K and FigS8), reduced CA1 thickness and a negative correlation between neuron-size and neuron count (CA1) and between neuron-size and thickness (CA2 and total hippocampus). These changes indicate an increased neuronal density and reduced thickness in the hippocampus of *mbd2-/-* mice, phenomena which were previously reported in ASD rodent models[45,46]. Increased neuronal density was also observed in the cortex of children with autism[47].

Hippocampal Mbd2 down-regulation impaired cognitive and emotional behaviors

Next, we asked whether hippocampal Mbd2 is casually involved in regulation of behaviors which are impaired in *mbd2-/-* mice. We therefore examined whether Mbd2 down-regulation in adult mice results in the same phenotypes as those seen in *mbd2-/-* mice which lacked the gene during embryogenesis and post-natal development. To this end, we constructed several shRNA lentiviruses which targeted *mbd2* mRNA (sh-*mbd2*; Fig5A-D). The construct which was the most active in *Mbd2* depletion was infused *in-vivo* into the hippocampus of adult wild-type mice. Following the infusion and recovery we tested the mice in the same behavioral tests that were found to be abnormal in *mbd2-/-* mice. Down-regulation of Mbd2 did not affect locomotion in an open-field (Fig5E) or interaction time in the social-interaction test (Fig5G). However, Mbd2 down-regulation resulted in failure of treated mice to remember location of objects in the object-location-memory test. While control mice explore the object in the novel-location significantly more than the one in the familiar location, sh-*mbd2*-treated mice did not explore the object in the novel location any longer beyond chance (Fig5F). In the Dark-Light-Box test, sh-*mbd2*-treated mice showed increased anxiety expressed as reduced time in the light

compartment (Fig5H). Taken together, these results show that knockdown of hippocampal Mbd2 in adult mice recapitulate most of the behavioral abnormalities found in *mbd2-/-* mice.

Discussion

There is growing evidence for the crucial role of epigenetic mechanisms in neuropsychological disorders and CNS function. In this study, we explore the role of Mbd2, a methylated-CpG binding protein, in gene-expression and brain function. Mbd2, like other MBD proteins, serves as a "reader" of the epigenome[4] translating DNA methylation marks into gene-expression regulation mechanisms. We found that loss of Mbd2 resulted in several forms of behavioral deficits: cognitive, social and emotional, implying a rather broad role for this protein in normal brain function. Most of the phenotypic findings were replicated in hippocampus-specific down-regulation of Mbd2 in wild-type mice, providing further evidence for the casual role of hippocampal Mbd2 in regulating these phenotypes.

Mbd2 regulates gene-expression by binding to methylated CpGs in DNA[48]. Mbd2 was previously shown to be involved in both suppression of promoters through recruitment of transcriptional-repressors, activation of promoters in cancer cells and the hippocampus through recruitment of transcriptional-activators[18,42-44] such as CBP and NGFIA[18,44,49] as well as targeting DNA demethylation[50,51] possibly through recruitment of demethylating activities such as Tet2[38] . Since the effects of *mbd2*-deficiency on behavior were broad and relating to hippocampal function[19-23], we used genome-wide approaches to define the landscape of Mbd2 binding in normal animals and then determined how its deficiency affects the landscapes of DNA-methylation and gene-expression using *mbd2-/-* mice. Consistent with an important role

in brain function detected by the behavioral assays presented here, pathway-analyses revealed a highly clustered and networked enrichment of genes relating to cognitive functions and brain development such as trans-synaptic signaling, synapse organization and behavior. Consistent with previous data we found evidence for the bimodal function of Mbd2. *mbd2*-deficiency resulted in both gene activation and repression as well as hypermethylation and hypomethylation. Interestingly, the neuronal specific functions seem to be mainly repressed and hypermethylated by *mbd2*-deficiency, suggesting a mostly activating role for Mbd2 on neuronal-specific genes. This is supported by the observation that Mbd2 localizes to promoters.

To further examine the role of Mbd2 in transcriptional activation/repression, we examined several promoters and enhancers that bind Mbd2 and examined the consequences of *mbd2*-deficiency on transcription-initiation. Most of the genes examined had reduced RNApolII(PS5) occupancy and increased H3K4me1 coverage of the transcription-initiation region indicating reduced transcription-initiation upon *mbd2*-deficiency.

While previous studies suggested that MBD2 might be involved in psychiatric disorders[14-16], our data offers a possible mechanism. Interestingly, several of the genes that we found here to be Mbd2-dependent were associated with ASD in GWAS studies. Indeed, there is a significant enrichment of ASD-associated genes in the list of genes that are repressed by *mbd2*-deficiency and several CNVs were discovered in and around the *MBD2* locus in ASD patients[52-54]. The behavioral phenotypes that we characterize here for *mbd2-/-* mice resemble several of the phenotypes frequently observed in ASD patients such as social-avoidance, anxiety and difficulties in learning and memory. This is accompanied by morphological changes in the

hippocampus similar to those previously reported in ASD patients and ASD rodent models. Therefore, our data points to a potential role for Mbd2 in ASD.

MBD proteins are generally considered to have a repressive role on gene-expression by interacting with chromatin modification inactivating complexes[55]. Therefore, it is somewhat surprising that we found an overall repressive effect on neuronal specific genes upon *mbd2-/-* deficiency. However, more recent evidence suggest other MBD proteins are also involved in gene-expression in more than one way. For example, hypothalamic Mecp2 dysfunction led to robust changes in gene-expression with 85% of the genes were activated by Mecp2, possibly mediated by interaction of the transcriptional-activator Creb1 and Mecp2 in binding gene-promoters and regulatory regions[56]. Similarly, others showed that in regulatory T-cells (Treg) Mecp2 promotes the expression of *Fopx3*, a key regulator of Treg function, by collaborating with Creb1[57]. Neuronal Mbd1 was also found to have a mixed effect on gene-expression in the hippocampus with more than half of the genes down-regulated in *mbd1-/-* mice, many of them associated with neurogenesis[58].

Taken together, our findings are in line with evidence from other studies showing the importance of MBD proteins in neuropsychological disorders. Our findings provide evidence that Mbd2 is a regulator of neuronal gene-expression, brain physiology and behavior.

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Conflict of Interest

All others declare they have no conflict of interest.

Supplementary information is available at MP's website

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Figure Legends

Figure 1. Behavioral analysis of *mbd2-/-* mice A. Locomotion in Open-field box was assessed for 5 min. n=12-14/group. B. Exploration time during object-location memory training (left) and discrimination ratio in object-location memory test (right). n=11-13/group. C. Social interaction, mice were introduced to a novel mouse for 5 min and interaction time was recorded. n=12-17/group. D. Time in the light compartment of the Dark-Light Box (left) and number of entries to the light side (right). n=7-8/group. E. Y-maze spontaneous alteration were not affected by *mbd2-/-* (right). SAP-Spontaneous Alteration Performance, AAR-Alternate Arm Return, SAR-Same Arm Return, exploration is expressed as number of arm entries (left). n=13-17/group. Data are presented as mean ±SEM *p<0.05, **p<0.01 for wild-type vs *mbd2-/-*. ### p<0.005 for wild-type over chance (50%) exploration (T-test).

Figure 2. A. Position of Mbd2 binding peaks Vis-a Vis distance to TSS and their peak fold enrichment. B. Heatmap of the overlap between Mbd2 peaks and the following histone-marks in the hippocampus: H3K4me1, H3K4me3, H3k2me3, H3K27ac, H39Kme3, H3K36me3 and

H4K20me1. C. Analysis of the number of Mbd2 peaks located on histone-marks. D. Co-occupancy enrichment analysis of Mbd2 and histone-mark. E. Pathway analysis enrichment of Mbd2 binding peaks F. Q-ChIP validation of Mbd2 peaks, $p<0.001$. G-H. Q-ChIP for RNApolII(SP5) (G) and H3K4me1 (H). For Q-ChIP of Mbd2, RNApolII(SP5) and H3K4me1 pool of 5-6 mice analyzed by triplicate/group). Data are presented as mean ±SEM *$p<0.05$, ***$p<0.001$ (T-test). TTS-Transcription Termination Site.

Figure 3. A. An unsupervised clustered heatmap of differentially-expressed genes in *mbd2-/-* and wild-type mice. B-E. Pathway analysis enrichment (B, D) and gene-network (C, E) of down-regulated (B, C) and up-regulated (D, E) genes. F. Circus plot depicting the relation between gene represented in CHIP-seq and RNA-Seq experiments, showing stronger connection between Mbd2 peaks and down-regulated genes in comparison with up-regulated genes. Purple lines link shared genes across gene-lists, blue lines link different genes which fall into the same ontology term G. Unsupervised clustering of gene pathways of Mbd2 binding and differentially-expressed genes. H. A cumulative distribution of methylation levels of regulatory DNA regions bound by Mbd2 demonstrating a global hyper-methylation in *mbd2-/-* hippocampus (K-S test). I. Correlation between differential promoter DNA-methylation and gene-expression in *mbd2-/-* hippocampi. J. A capture from IGV genomic browser depicting Mbd2 binding peak around Sfi1 transcription start site (TSS) with methylation levels and differential-methylation between wild-type and *mbd2-/-* mice hippocampus.

Figure 4. A. An overlap analysis revealed a significant intersection between human ASD high risk genes (Loss of Function; FDR<0.1) and down-regulated (Top) but not up-regulated (Bottom) genes in *mbd2-/-* hippocampi. B. Similar results were obtained for the expanded ASD risk gene

list (Loss of Function; FDR<0.3) and down-regulated (Top) but not up-regulated (Bottom) genes. C. An overlap analysis revealed a significant intersection between human ASD high risk genes based on the SFARI Gene Project and down-regulated (Top) but not up-regulated (Bottom) genes. (Hypergeomtric tests) D. STRING protein-protein association network analysis for 125 ASD-risk genes which are also down-regulated by *mbd2* loss (disconnected nodes are omitted from the graph for clarity). E. A heatmap of CNVs in the Human chromosome 18. Region 18q21.2 is flanked by lines and the *MBD2* gene is marked by an arrow. F. Western blot analysis showing reduced protein levels of Drd2, Pogz and Zbtb16 in *mbd2*-/- mice hippocampi. Proteins weight as determined using molecular weight markers is indicated. G. Representative immunofluorescence images of the hippocampus (CA1 layer) in wild-type and *mbd2*-/- mice showing neuronal nuclei immunoreactivity (NeuN, green immunoreactivity). Scale bar: 100µm. H. Quantification of average neuronal soma size of the CA1 layer. I. Quantification of neuronal cell count in CA1 layer. J. Reverse correlation between average neuronal soma size and neuronal cell count (Pearson's r=-0.7265, p=0.0266). K. Average thickness of CA1 layer. Data are presented as mean ±SEM. *p<0.05, **p<0.01 (t-tests and U-test for (K)); n=4-5/group.

Figure 5. A. Schematic illustration of the sh-*mbd2*/scramble constructs. RRE- Rev Response Element, hCMV-human Cytomegalovirus promoter, tGFP-turbo GFP reporter, IRES-Internal Ribosomal Entry Site, PURO^{R}-Puromycin Resistance, WPRE-Woodchuck hepatitis Posttranslational Regulatory Element. B. In-vitro validation of sh-*mbd2* construct in NIH3T3 cells by qPCR (fold change over wild-type). C. Western blot for Mbd2 protein levels from lentivirus-treated NIH3T3 cells (fold change over wild-type). D. Illustration of infusion site adapted from the Paxinos and Franklin mouse brain atlas (left) and fluorescence microscope image showing

viral-GFP expression in the hippocampus (right). E. Locomotion in Open-field box. F. discrimination ratio in object-location memory test. G. Social interaction, mice were introduced to a novel mouse for 5 min and interaction time was recorded. H. Time in the light compartment of the Dark-Light Box (left) and number of entries to the light side (right). Data are presented as mean ±SEM *p<0.05, ***p<0.001 for wild-type vs *mbd2-/-*. # p<0.05 for wild-type over chance (50%) exploration (T-test).

Figure S1. Average speed (left), entries to center (middle) and time near walls (right) in the open-field box tests. p>0.05 in all cases.

Figure S2. A. Histogram of Mbd2 binding peaks around Transcription start sites (TSS) B. Top De-novo motif discovery sequences found in HOMER analysis.

Figure S3. Gene-network analysis of Mbd2 binding peaks.

Figure S4. A. A capture from genome browser showing RNA-seq read alignments for the first 3 exons of the *mbd2* gene. *mbd2-/-* mice show expression of the first exon only, as expected by the design of this mouse line[59]. B. QPCR of 9 neuronal genes (*grin2b, dclk2, chl1, apba2, kcnd2, clstn1, gria3a, jarid2, gria1*) shows reduced expression in *mbd2-/-* mice in agreement with the RNA-Seq (n=5-9/group; *p<0.05).

Figure S5. A. A table summarizing the number of differentially-methylated CpGs and genes under the different analytical conditions. B. Significant motifs discovered for promoter CpG with positive correlation between DNA methylation and gene-expression are depicted. No significant motifs were found for CpGs with negative correlation between DNA methylation and gene-expression. C-D. Pathway analysis of hypo- (C) and hyper- (D) methylated gene promoters.

Figure S6. A. Average change in methylation levels across all CpGs (with at least 1% change in methylation) show larger changes towards hyper-methylation (n=114) over hypo-methylation (n=80) (t-test, **p<0.01) B. Number of differentially-methylated CpGs (with at least 1% change in methylation) shows more CpGs became hyper-methylated then hypo-methylated (binomial test, *p<0.05). C. A cumulative distribution of methylation levels within Mbd2 peak regions in a validation cohort (K-S test).

Figure S7. A-B. Protein-Protein Interaction Networks for down-regulated genes overlapped with De Rubeis et al.[41] top ASD risk genes at FDR<0.1 (A) and FDR<0.3 (B). C. Protein-Protein Interaction Networks for up-regulated genes overlapped with SFARI-GENE dataset.

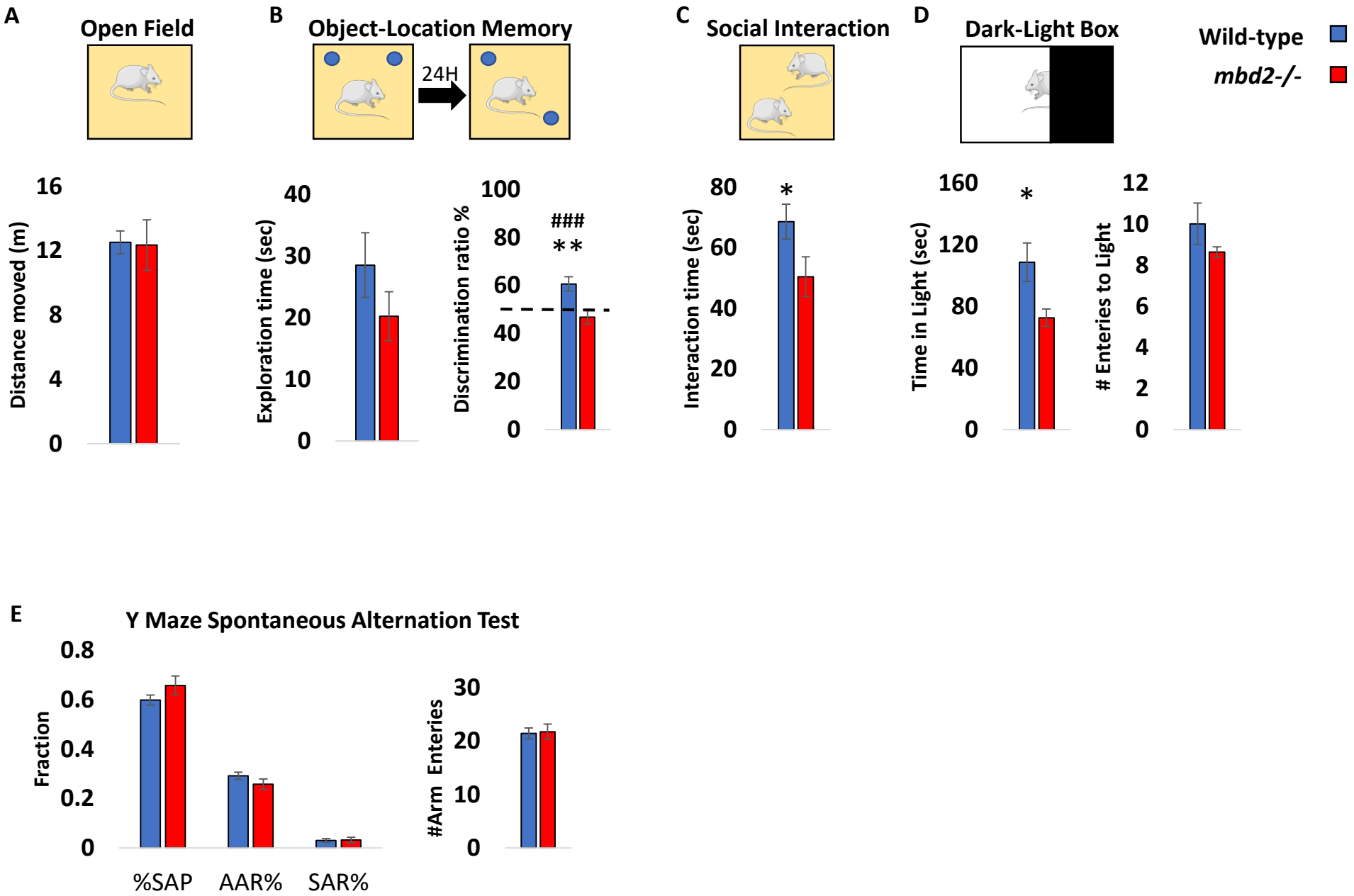
Figure S8. A. Neuron count (left), average neuron size (middle) and layer thickness (right) of the CA2 layer in wild-type and *mbd*2-/- mice. B. Neuron count (left), average neuron size (middle) and layer thickness (right) of the CA3 layer C. Correlation analyses between neuronal size and layer thickness (left) and between neuron count and thickness (right) of the CA1 layer. D. Correlation analyses between neuronal size and layer thickness (left), between neuron count and average neuronal size (middle) and between neuron count and thickness (right) of the CA2 layer. E. Correlation analyses between neuronal size and layer thickness (left), between neuron count and average neuronal size (middle) and between neuron count and thickness (right) of the CA3 layer. F. Correlation analyses between neuronal size and layer thickness (left), between neuron count and average neuronal size (middle) and between neuron count and thickness (right) of the total hippocampus (collapsing CA1, CA2 and CA3 data). *p<0.05, t-tests for between group comparisons. Pearson's Rs for linear correlation analyses, with exact p-values indicated within each graph.

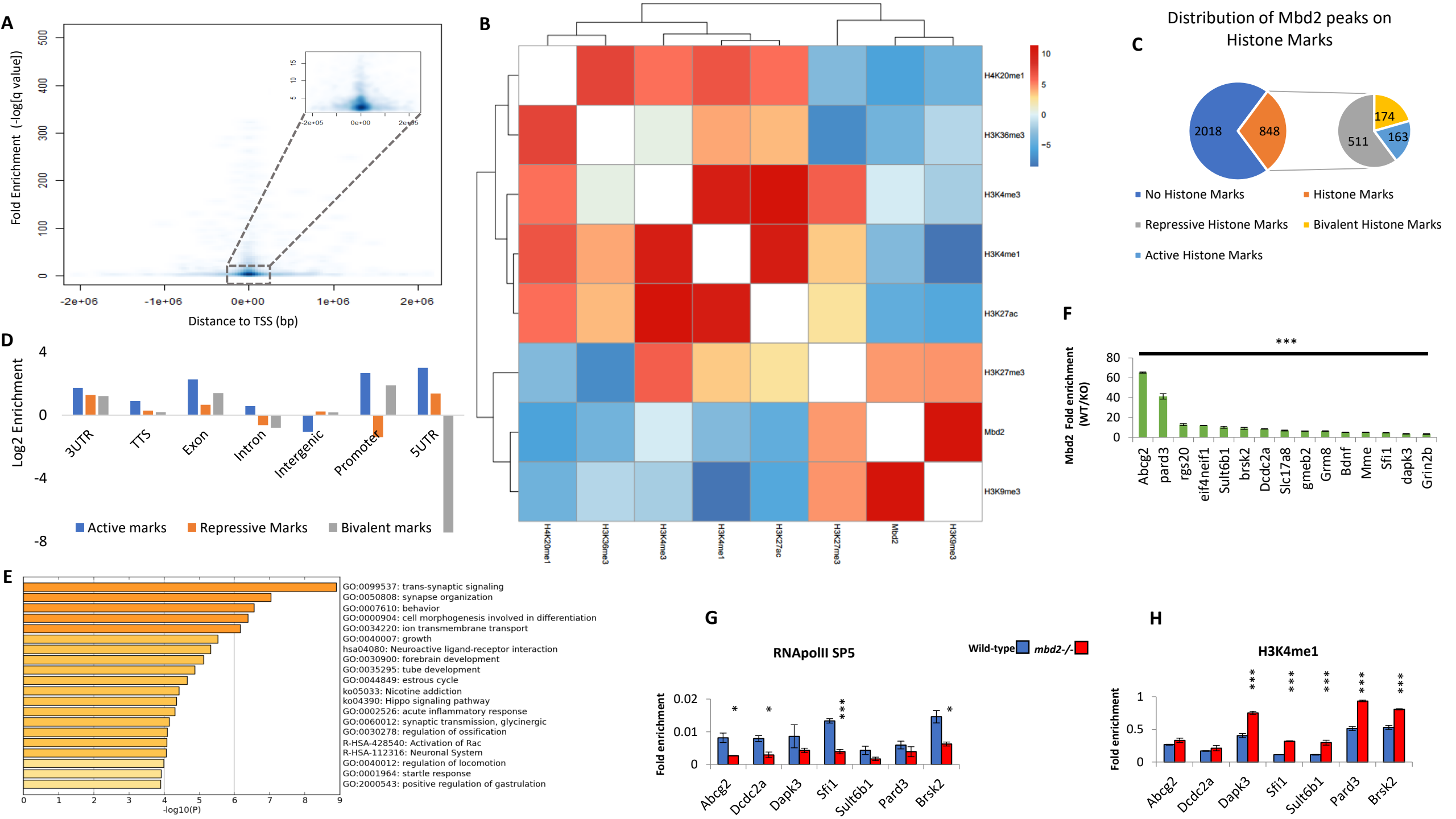
Table S1. Genomic coordinates of validated peaks.

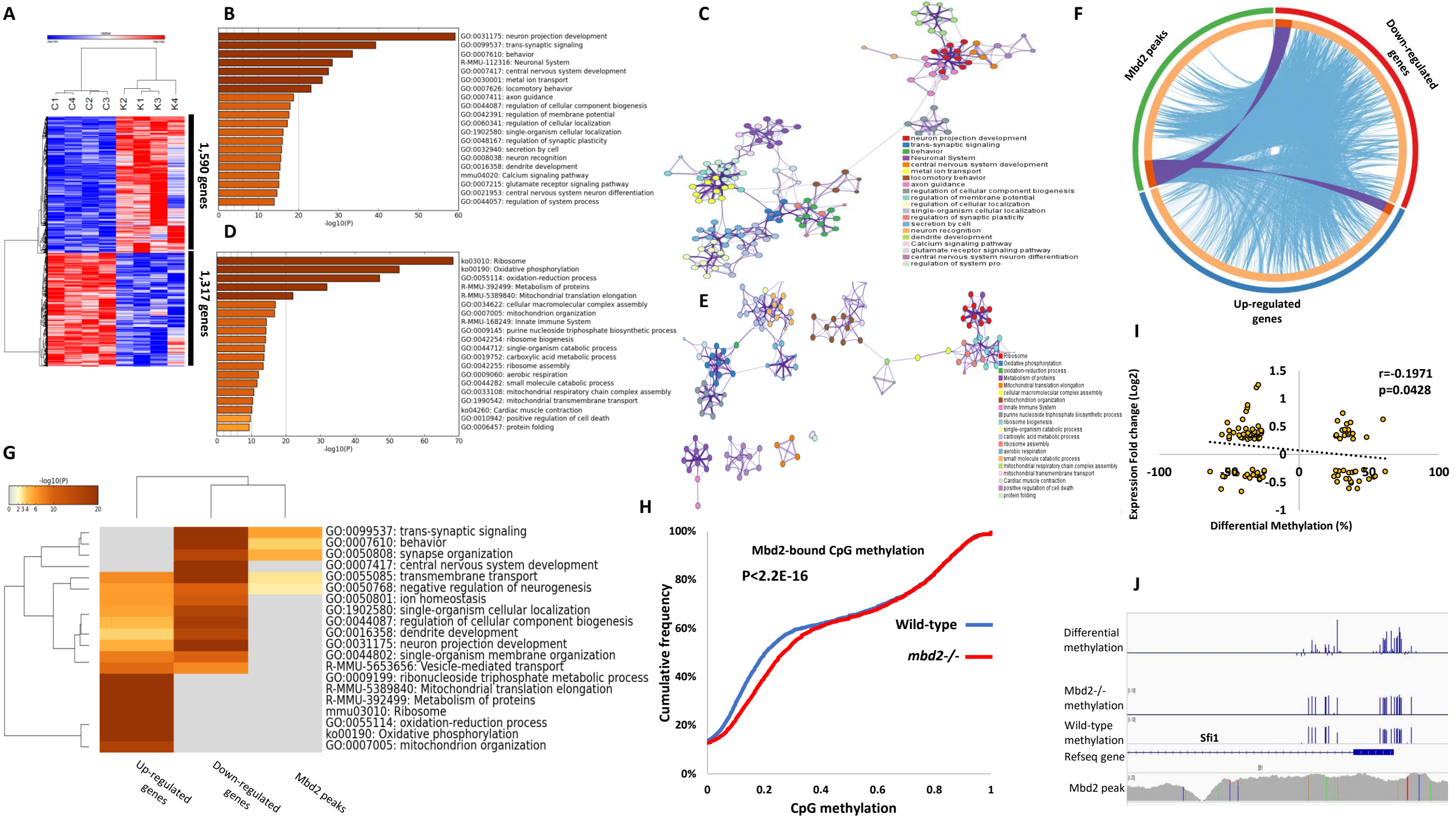
Table S2. Differential-methylation data ($p<0.001$).

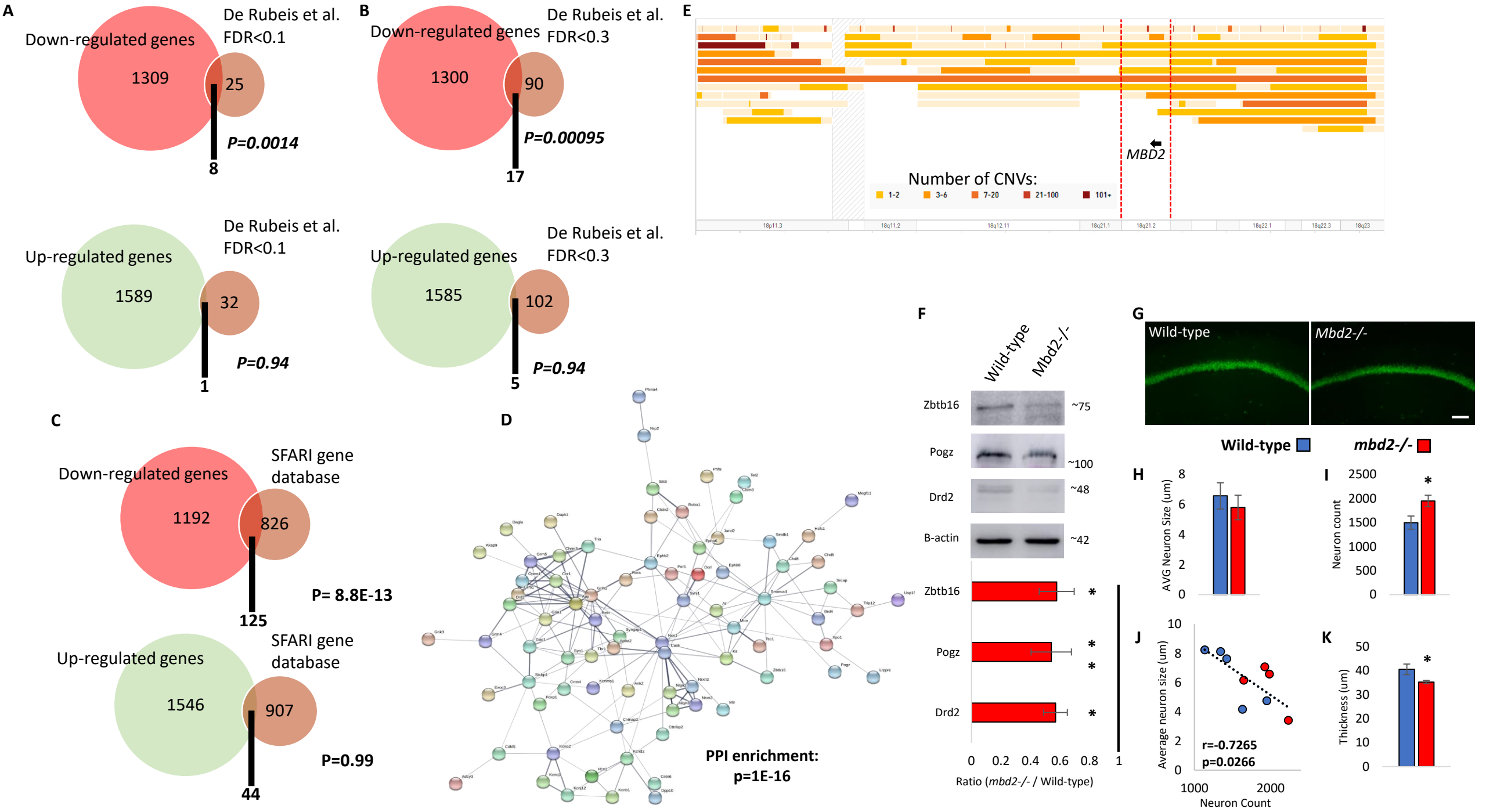
Table S3. Common gene-lists of Mbd2-regulated and ASD-risk genes.

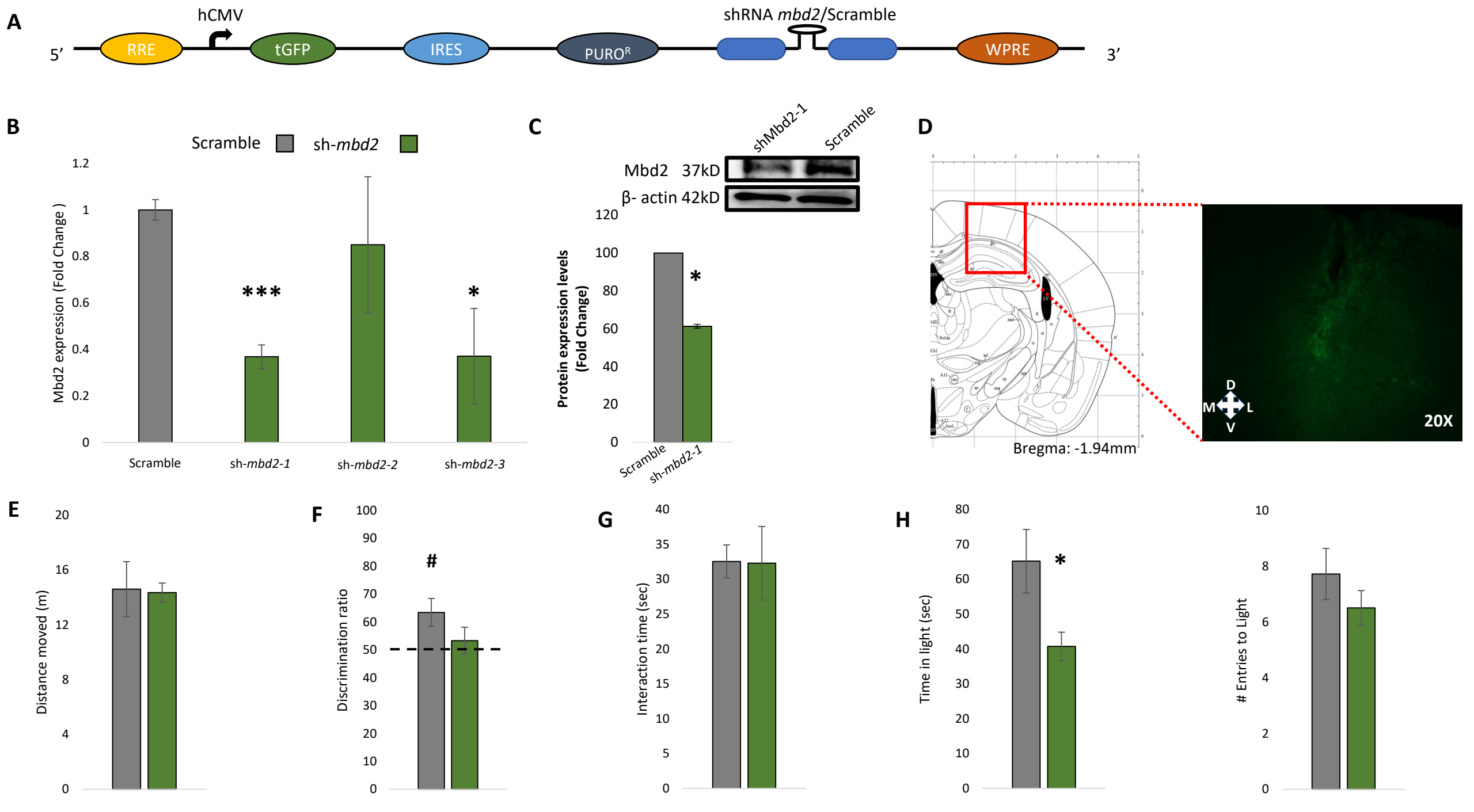
Table S4. STRING protein-protein association-networks statistics and functional enrichments.


A
Open Field
B
Object-Location Memory
24H
C
Social Interaction
D
Dark-Light Box
Wild-type
mbd2-/-
Distance moved (m)
16
12
8
4
0
Exploration time (sec)
40
30
20
10
0
Discrimination ratio %
100
80
60
40
20
0
###
**
Interaction time (sec)
80
60
40
20
0
*
Time in Light (sec)
160
120
80
40
0
*
Enteries to Light
12
10
8
6
4
2
0
E
Y Maze Spontaneous Alternation Test
Fraction
0.8
0.6
0.4
0.2
0
%SAP
AAR%
SAR%
#Arm Enteries
30
20
10
0


A
Fold Enrichment (-log[q value])
Distance to TSS (bp)
B
H4K20me1
H3K36me3
H3K4me3
H3K4me1
H3K27ac
H3K27me3
Mbd2
H3K9me3
C
Distribution of Mbd2 peaks on Histone Marks
2018
848
511
174
163
No Histone Marks
Histone Marks
Repressive Histone Marks
Bivalent Histone Marks
Active Histone Marks
D
Log2 Enrichment
3UTR
TTS
Exon
Intron
Intergenic
Promoter
5UTR
Active marks
Repressive Marks
Bivalent marks
E
GO:0099537: trans-synaptic signaling
GO:0050808: synapse organization
GO:0007610: behavior
GO:0000904: cell morphogenesis involved in differentiation
GO:0034220: ion transmembrane transport
GO:0040007: growth
hsa04080: Neuroactive ligand-receptor interaction
GO:0030900: forebrain development
GO:0035295: tube development
GO:0044849: estrous cycle
ko05033: Nicotine addiction
ko04390: Hippo signaling pathway
GO:0002526: acute inflammatory response
GO:0060012: synaptic transmission, glycinergic
GO:0030278: regulation of ossification
R-HSA-428540: Activation of Rac
R-HSA-112316: Neuronal System
GO:0040012: regulation of locomotion
GO:0001964: startle response
GO:2000543: positive regulation of gastrulation
-log10(P)
F
Mbd2 Fold enrichment (WT/KO)
Abcg2
pard3
rgs20
eif4neif1
Sult6b1
brsk2
Dcdc2a
Slc17a8
gmeb2
Grm8
Bdnf
Mme
Sfi1
dapk3
Grin2b
G
RNApolII SP5
Wild-type
mbd2-/-
Fold enrichment
Abcg2
Dcdc2a
Dapk3
Sfi1
Sult6b1
Pard3
Brsk2
H
H3K4me1
Fold enrichment
Abcg2
Dcdc2a
Dapk3
Sfi1
Sult6b1
Pard3
Brsk2


A
C1
C4
C2
C3
K2
K1
K3
K4
1,590 genes
1,317 genes
B
GO:0031175: neuron projection development
GO:0099537: trans-synaptic signaling
GO:0007610: behavior
R-MMU-112316: Neuronal System
GO:0007417: central nervous system development
GO:0030001: metal ion transport
GO:0007626: locomotory behavior
GO:0007411: axon guidance
GO:0044087: regulation of cellular component biogenesis
GO:0042391: regulation of membrane potential
GO:0060341: regulation of cellular localization
GO:1902580: single-organism cellular localization
GO:0048167: regulation of synaptic plasticity
GO:0032940: secretion by cell
GO:0008038: neuron recognition
GO:0016358: dendrite development
mmu04020: Calcium signaling pathway
GO:0007215: glutamate receptor signaling pathway
GO:0021953: central nervous system neuron differentiation
GO:0044057: regulation of system process
-log10(P)
C
neuron projection development
trans-synaptic signaling
behavior
Neuronal System
central nervous system development
metal ion transport
locomotory behavior
axon guidance
regulation of cellular component biogenesis
regulation of membrane potential
regulation of cellular localization
single-organism cellular localization
regulation of synaptic plasticity
secretion by cell
neuron recognition
dendrite development
Calcium signaling pathway
glutamate receptor signaling pathway
central nervous system neuron differentiation
regulation of system pro
D
ko03010: Ribosome
ko00190: Oxidative phosphorylation
GO:0055114: oxidation-reduction process
R-MMU-392499: Metabolism of proteins
R-MMU-5389840: Mitochondrial translation elongation
GO:0034622: cellular macromolecular complex assembly
GO:0007005: mitochondrion organization
R-MMU-168249: Innate Immune System
GO:0009145: purine nucleoside triphosphate biosynthetic process
GO:0042254: ribosome biogenesis
GO:0044712: single-organism catabolic process
GO:0019752: carboxylic acid metabolic process
GO:0042255: ribosome assembly
GO:0009060: aerobic respiration
GO:0044282: small molecule catabolic process
GO:0033108: mitochondrial respiratory chain complex assembly
GO:1990542: mitochondrial transmembrane transport
ko04260: Cardiac muscle contraction
GO:0010942: positive regulation of cell death
GO:0006457: protein folding
-log10(P)
E
Ribosome
Oxidative phosphorylation
oxidation-reduction process
Metabolism of proteins
Mitochondrial translation elongation
cellular macromolecular complex assembly
mitochondrion organization
Innate Immune System
purine nucleoside triphosphate biosynthetic process
ribosome biogenesis
single-organism catabolic process
carboxylic acid metabolic process
ribosome assembly
aerobic respiration
small molecule catabolic process
mitochondrial respiratory chain complex assembly
mitochondrial transmembrane transport
Cardiac muscle contraction
positive regulation of cell death
protein folding
F
Mbd2 peaks
Down-regulated genes
Up-regulated genes
G
-log10(P)
GO:0099537: trans-synaptic signaling
GO:0007610: behavior
GO:0050808: synapse organization
GO:0007417: central nervous system development
GO:0055085: transmembrane transport
GO:0050768: negative regulation of neurogenesis
GO:0050801: ion homeostasis
GO:1902580: single-organism cellular localization
GO:0044087: regulation of cellular component biogenesis
GO:0016358: dendrite development
GO:0031175: neuron projection development
GO:0044802: single-organism membrane organization
R-MMU-5653656: Vesicle-mediated transport
GO:0009199: ribonucleoside triphosphate metabolic process
R-MMU-5389840: Mitochondrial translation elongation
R-MMU-392499: Metabolism of proteins
mmu03010: Ribosome
GO:0055114: oxidation-reduction process
ko00190: Oxidative phosphorylation
GO:0007005: mitochondrion organization
Up-regulated genes
Down-regulated genes
Mbd2 peaks
H
Mbd2-bound CpG methylation
P<2.2E-16
Wild-type
mbd2-/-
Cumulative frequency
CpG methylation
I
r=-0.1971
p=0.0428
Expression Fold change (Log2)
Differential Methylation (%)
J
Differential methylation
Mbd2-/- methylation
Wild-type methylation
Sfi1
Refseq gene
Mbd2 peak


A
Down-regulated genes
1309
De Rubeis et al.
FDR<0.1
25
P=0.0014
8
Up-regulated genes
1589
De Rubeis et al.
FDR<0.1
32
P=0.94
1
B
Down-regulated genes
1300
De Rubeis et al.
FDR<0.3
90
P=0.00095
17
Up-regulated genes
1585
De Rubeis et al.
FDR<0.3
102
P=0.94
5
C
Down-regulated genes
1192
SFARI gene
database
826
P= 8.8E-13
125
Up-regulated genes
1546
SFARI gene
database
907
P=0.99
44
D
PPI enrichment:
p=1E-16
E
MBD2
Number of CNVs:
1-2
3-6
7-20
21-100
101+
18p11.3
18q11.2
18q12.11
18q21.1
18q21.2
18q22.1
18q22.3
18q23
F
Wild-type
Mbd2-/-
Zbtb16
~75
Pogz
~100
Drd2
~48
B-actin
~42
Zbtb16
Pogz
Drd2
0
0.2
0.4
0.6
0.8
1
Ratio (mbd2-/- / Wild-type)
G
Wild-type
Mbd2-/-
Wild-type
mbd2-/-
H
AVG Neuron Size (um)
0
2
4
6
8
I
Neuron count
0
500
1000
1500
2000
2500
J
Average neuron size (um)
r=-0.7265
p=0.0266
1000
2000
Neuron Count
K
Thickness (um)
0
10
20
30
40
50


A
hCMV
shRNA mbd2/Scramble
5'
RRE
tGFP
IRES
PURO^R
WPRE
3'
B
Scramble
sh-mbd2
Mbd2 expression (Fold Change)
1.2
1
0.8
0.6
0.4
0.2
0
***
*
Scramble
sh-mbd2-1
sh-mbd2-2
sh-mbd2-3
C
shMbd2-1
Scramble
Mbd2 37kD
β- actin 42kD
Protein expression levels (Fold Change)
120
100
80
60
40
20
0
*
Scramble
sh-mbd2-1
D
Bregma: -1.94mm
D
M
L
V
20X
E
Distance moved (m)
20
16
12
8
4
0
F
Discrimination ratio
100
90
80
70
60
50
40
30
20
10
0
#
G
Interaction time (sec)
40
35
30
25
20
15
10
5
0
H
Time in light (sec)
80
70
60
50
40
30
20
10
0
*
Entries to Light
10
8
6
4
2
0