Supplementary Methods

Nuclei preparation from postmortem brains

About 0.1 g fresh-frozen brain tissue was minced with razors and homogenized using a

Potter-Elvehjem homogenizer with a loose-fitting Teflon pestle. Homogenization was

performed at 1000 rpm by 10 strokes in 10 times volume of an ice-cold STKM buffer

(250 mM sucrose, 50 mM Tris-HCl pH 7.4, 25 mM KCl, 5 mM $MgCl_2$) containing an

EDTA-free protease inhibitor cocktail (Complete Mini, Roche, Basel, Switzerland).

Homogenates were filtered with a prewetted 40-μm cell strainer (BD, Franklin Lakes,

NJ, USA), and mixed with NP40 at a final concentration of 0.1%. Mixtures of

homogenates were incubated on ice for 15 min and were prepared in 3 mL of 19%

Percoll solution in a STKM buffer. The discontinuous density gradient was prepared in

an Optiseal tube (Beckman, Fullerton, CA, USA) by layering fractions of 0.8 mL of

26% Percoll/STKM buffer, 3 mL of 19% Percoll/sample solution, and 0.8 mL of 12%

Percoll/STKM buffer. The tube was centrifuged at 24,000 rpm for 10 min at 4°C by a

TLA 100.4 rotor (Beckman). Nuclei fractions floating in the lowest layer were

collected.

Illumina Golden Gate assay

A total of 250 ng bisulfite-modified DNA was used for the Golden Gate Cancer Panel I

set (Illumina) (Bibikova et al. 2006). All procedures are performed strictly according to the manufacturer's protocol. We confirmed that all control parameters provided by the manufacturer were within a satisfactory range, including first and second hybridization, allele-specific extension, assay intensity, extension gap, contamination check, gender call, and bisulfite conversion test. We identified CpG sites that showed a significant difference by Student two-tailed t test ($P < 0.05$) and an average beta value difference >0.18, according to a previous report (Ladd-Acosta et al. 2007). In identifying the differentially methylated CpG sites between human and chimpanzee cortex, we used an average beta value difference >0.20. Clustering was performed using GeneSpring 7.3.1 software (Agilent).

Pyrosequencing

Briefly, 4 µL of streptavidin-Sepharose beads (Amersham Biosciences) and binding buffer (5 mM Tris-HCl, 0.5 mM EDTA, 1 M NaCl, 0.05% Tween 20 pH 7.6) were mixed with PCR product for 10 min at room temperature. The reaction mixture was placed onto a MultiScreen-HV Clear Plate (Millipore). After applying a vacuum, the beads were treated with a denaturation solution (0.2 N NaOH) for 1 min at room temperature and washed twice with washing buffer (10 mM Tris-acetate pH 7.6). The

beads were then suspended with 50 µL annealing buffer (20 mM Tris-acetate, 2 mM

Mg-acetate pH7.6) containing 10 pmol of sequencing primer. The template-sequencing

primer mixture was transferred onto a PSQ95SNP Reagent Kit (Biotage) using a

PSQ96MA (Biotage), according to the manufacturer's instructions. The experiment was

performed in duplicate, according to standard pyrosequencing methods.

Enrichment of methylated fraction

Genomic DNA was digested with *Mse*I, *Tsp509*I, *Bfa*I, and *Rsa*I, which do not cut CpG

sites, to shear genomic DNA. After digestion, the reaction mixture was purified with a

MinElute Reaction Cleanup kit (Qiagen). Enrichment of methylated DNA was

performed using MethylCollector (Active Motif, Carlsbad, CA, USA). A total of 200 ng

DNA was used for one reaction. The His-tagged recombinant MBD2b protein and

digested genomic DNA were mixed. The MBD2b-methylated DNA complex was then

captured with nickel-coated magnetic beads. The beads were washed with

high-stringency salt buffer to remove DNA fragments with little methylation

(methylated CpGs is <6). Methylated DNA was retrieved by the 100 µL of elution

buffer including proteinase K. The reaction mixture was purified with a MinElute PCR

purification kit. Elution was performed with 10 µL of 10 nM TE (pH 8.0) twice. In each

experiment, genomic DNA of A431 and HeLa cells were used for positive and negative controls, respectively. PCR amplification of the MCJ locus (methylated in A431 but not in HeLa cells) was performed using these positive and negative controls as a template, according to the manufacturer's protocol.

Tiling array probe preparation

Briefly, a total of 10 µL of purified elutant was subjected to linear amplification with random primer having a primer annealing site (5′-GTTTCCCAGTCACGGTC(N)$_9$-3′, HPLC-purified) using Sequenase. Four cycles of denaturing (95°C for 4 min) and annealing/amplification (ramp from 10°C to 37°C over 9 min) were performed. In each cycle, Sequenase was freshly added after the denature step. Amplified products were purified with two rounds of Microspin S300HR column (GE Healthcare). Then, 20 µL of purified product was used for PCR amplification with a primer (5′-GTTTCCCAGTCACGGTC-3′) using Taq polymerase and dUTP containing dNTPs. PCR cycles consisted of an initial 15 cycles of 95°C for 30 s, 45°C for 30 s, 55°C for 30 s, and 72°C for 1 min, and then 15 cycles adding 5 more seconds to every 72°C step. After checking products of target and negative controls by agarose gel-electrophoresis, products were purified using a MinElute PCR purification kit. Elution was performed

with 20 μL of 10 nM TE (pH 8.0) twice. DNA concentration was measured using a

NanoDrop 1000 (Thermo Scientific, Wilmington, DE). Fragmentation and labeling

reaction were performed using a GeneChip WT Terminal Labeling Kit (Affymetrix).

Efficient and uniform fragmentation was confirmed by agarose gel-electrophoresis.

Tiling array data analysis

  Either *Mcr*BC-treated unmethylated DNA or nonspecific binding fraction of

MBD2-beads was used for reference. Each reference was prepared from the same brain

source from the same individual. We obtained similar and consistent results in the MAT

analysis, independent of the type of reference (data not shown).

  To consider the effect of restriction enzyme digestion and the number of CpGs

included in the resultant fragment on data analysis, we introduced a filtering procedure.

We performed *in silico* digestion by the four restriction enzymes around the obtained

peaks by MAT analysis, considering 1 kb upstream and downstream regions per peak.

We then selected the peaks containing *in silico* digested fragment, fulfilling the

following criteria: fragment length was >100 bp and number of CpGs included was ≥5.

Fragment length was determined based on the results of gel-electrophoresis of the probe

linear amplification step. Number of CpGs (≥5) was defined based on the characteristics

of MBD2-beads, which can effectively bind DNA fragments containing 6 or more

methylated CpGs. To estimate interindividual variations in DNA methylation, we used

tiling array suite (TAS) software (Affymetrix) to extract the signal intensities of all

probes on the array.

In the case of Nimblegen arrays, significant peaks were determined according to the

method described elsewhere (Scacheri et al. 2006), using SignalMap software (Roche).

In brief, after the calculation of the $\log_2$ ratio and scaling, the one-sided

Kolmogorov-Smirnov test is applied to determine whether the probes are drawn from a

significantly different distribution of intensities than those in the rest of the array. The

peaks are then detected by searching for at least 2 probes above a *P* value minimum

cutoff ($-\log_{10}$) of 2.

Bioinformatic analyses

For ontology analysis, we used PANTHER software (Thomas et al. 2003) with the lists

of RefSeq associated with MRs. The lists were compared to the reference list (NCBI,

human genome build 36) using the binomial test for each PANTHER term in biological

process and molecular function.

For transcription factor-binding site analysis, we used the RegionMiner function of the

Genomatix software package (Genomatix Software, Germany) using matrix library 7.1.

We used promoter sequences of human genome (about 500 bp upstream and 100 bp

downstream from the transcription start sites) as background. Input sequences were

common MRs (660 sequences with a total of 592,915 bases), neuronal-specific MRs

(676 sequences with a total of 196,454 bases), and nonneuronal-specific MRs (742

sequences with a total of 387,518 bases). Overrepresentation and Z-values were

calculated against the background. A TF matrix showing two or more

overrepresentations was considered significant.

  For module membership analysis, we used gene symbols as a unique identifier. Each

list of genes was cross-referenced with modules from the CTX (cortex) gene

coexpression network (Oldham et al. 2008). Instead of using the module definitions

from the previous paper, the kme (i.e., module memberships) values were used to

"redefine" the modules at various levels of kme significance. A one-sided Fisher's exact

test was used to determine whether genes in the modules were significantly enriched

with genes from the lists. Alternatively, average correlation was calculated between

each module eigengene (Oldham et al. 2008) and each gene. In the case of redundant

expression probe sets, the probe set showing the highest correlation was used. One-way

ANOVA followed by the Dunnet test was performed to find a significant differences. *P*

< 0.05 was considered significant.

Characterization and validation of variably methylated regions in neuronal nuclei

We calculated average and SD of signal intensities of each probe on the array and then

identified the variably methylated region (VMR). The VMR included the consecutive (>

1 Kb) probes whose average intensities and SD are greater than 20 and 10, respectively.

To rule out the effect of copy number variation, we determined copy number variation

in each subject using Affymetrix 500K SNP arrays with CNAT software (Iwamoto et al.,

submitted). Among the identified 28 VMRs, 7 VMRs overlapped with CNVs and were

omitted from analysis. We performed validation analysis by Q-PCR about three

arbitrarily chosen VMRs. Primers are listed in Supplementary Table 10. Q-PCR was

performed using SYBRGREEN chemistry with methylated DNA fragment eluted from

MBD2b beads as template. Due to limitation in availability of DNA extracted from

sorted brain samples, we amplified eluted DNA by the method similar to tiling array

probe preparation. Quantification was performed in triplicate. In addition, using

previously profiled DNA microarray data (Iwamoto et al. 2005), we compared the

relationship between gene expression and methylation level of VMR, if an appropriate

probe is available.

References

Bibikova, M., Z. Lin, L. Zhou, E. Chudin, E.W. Garcia, B. Wu, D. Doucet, N.J. Thomas, Y. Wang, E. Vollmer et al. 2006. High-throughput DNA methylation profiling using universal bead arrays. *Genome Res* **16**: 383-393.

Iwamoto, K., M. Bundo, and T. Kato. 2005. Altered expression of mitochondria-related genes in postmortem brains of patients with bipolar disorder or schizophrenia, as revealed by large-scale DNA microarray analysis. *Hum Mol Genet* **14**: 241-253.

Ladd-Acosta, C., J. Pevsner, S. Sabunciyan, R.H. Yolken, M.J. Webster, T. Dinkins, P.A. Callinan, J.B. Fan, J.B. Potash, and A.P. Feinberg. 2007. DNA methylation signatures within the human brain. *Am J Hum Genet* **81**: 1304-1315.

Oldham, M.C., G. Konopka, K. Iwamoto, P. Langfelder, T. Kato, S. Horvath, and D.H. Geschwind. 2008. Functional organization of the transcriptome in human brain. *Nat Neurosci* **11**: 1271-1282.

Scacheri, P.C., G.E. Crawford, and S. Davis. 2006. Statistics for ChIP-chip and DNase hypersensitivity experiments on NimbleGen arrays. *Methods Enzymol* **411**: 270-282.

Thomas, P.D., M.J. Campbell, A. Kejariwal, H. Mi, B. Karlak, R. Daverman, K. Diemer, A. Muruganujan, and A. Narechania. 2003. PANTHER: a library of protein families and subfamilies indexed by function. *Genome Res* **13**: 2129-2141.