Mol. Cells *34*, 433-437, November 30, 2012
DOI/10.1007/s10059-012-0182-3
pISSN: 1016-8478 eISSN: 0219-1032

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Differential Promoter Methylation and Histone Modification Contribute to the Brain Specific Expression of the Mouse Mbu-1 Gene

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Mbu-1 (Csrnp-3) is a mouse gene that was identified in our previous study as showing highly restricted expression to the central nervous system. In this study, to elucidate the regulatory mechanism for tissue specificity of the gene, epigenetic approaches that identify the profiles of CpG methylation, as well as histone modifications at the promoter region were conducted. Methylation-specific PCR revealed that the CpG sites in brain tissues from embryo to adult stages showed virtually no methylation (0.052-0.67%). Lung (9.0%) and pancreas (3.0%) also showed lower levels. Other tissues such as liver, kidney, and heart showed much higher methylation levels ranging from approximately 39-93%. Treatment of 5-aza-2′-deoxycytidine (5-Aza-dC) significantly decreased promoter methylation, reactivating Mbu-1 expression in NG108-15 and Neuro-2a neuronal cells. Chromatin immunoprecipitation assay revealed that 5-Aza-dC decreased levels of acetylated H3K9 and methylated H3K4, and increased methylated H3K9. This result indicates that CpG methylation converses with histone modifications in an opposing sense of regulating Mbu-1 expression.

INTRODUCTION

Mbu-1 (also known as Csrnp-3; GenBank accession no., NM-178634) is a mouse gene that was first identified as a brain-specific unigene by using the differential digital display program (DDD) (Yang et al., 2007). The expression of Mbu-1 is strictly confined to the brain and spinal cord during development from embryo to adult. Mbu-1 (Csrnp-3) is a member of cysteine-serine-rich nuclear protein family that also includes Csrnp-1 and Csrnp-2. Deletion of the individual genes had no obvious consequences on normal mouse development. However, combined deficiencies caused partial neonatal lethality suggesting that the genes have redundant functions (Jensen Pena et al., 2012). The 480 bp of the 5'-flanking sequence was enough to show the highest expression in neuronal cell cultures. To identify transcriptional factors acting on the promoter and leading to the neuron-specific expression, an extensive search within databases of transcriptional factors was carried out on the 480 bp as well as upstream sequences extending up to 2 kb. However the work has failed to identify any neuron-specific activators or silencers, such as REST, that are known to suppress expression of neuron-specific genes in non-neuronal cells (Chong et al., 1995; Nakatani et al., 2005).

Methylation of the CpG sites at the promoter has emerged as an alternative explanation for the neuron-specific expression of brain genes (Davies et al., 2012; Furuya et al., 2012; Jensen Pena et al., 2012). In a comprehensive DNA analysis of neuronal and non-neuronal nuclei obtained from the human prefrontal cortex, neuronal nuclei manifested qualitatively and quantitatively distinctive DNA methylation patterns, including relative global hypomethylation, differential enrichment of transcription-factor binding sites, and higher methylation of genes expressed in astrocytes (Iwamoto et al., 2011). In a further study, DNA methylation was found to be dynamically regulated in the human cerebral cortex throughout the entire lifespan, involving differentiated neurons, and affecting a substantial portion of genes, increasing with age, predominantly (Siegmund et al., 2007).

Epigenetic modification of genomic DNA and histones has been tightly linked to chromatic organization and transcriptional regulation (Fuks, 2005; Lee and Lee, 2012; Majid et al., 2009). Histone acetylation in gene promoter/enhancer regions is generally correlated with transcriptional activation (Hattori et al., 2004; Tomikawa et al., 2006). Methylation of DNA is essential for mammalian development and is associated with gene silencing in conjunction with histone core modifications, probably through chromatin remodeling (Jones et al., 1998; Li et al., 1992). For example, histone acetylation in the Kiss1 gene, that is upregulated in the anteroventra periventricular nucleus of brain, enhanced chromatic loop formation of Kiss1 promoter and Kiss1 gene enhancer, resulting in an increase in Kiss1 gene-specific expression (Tomikawa et al., 2012).

The present study aims to determine the epigenetic regulatory mechanism underlying the neuron-specific expression of Mbu-1. We first identified the methylation level at the Mbu-1 promoter in various mouse tissues. Methylation-specific PCR (MSP) and sequence analysis of the bisulfite-treated DNA were

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Received July 17, 2012; revised September 4, 2012; accepted September 12, 2012; published online October 16, 2012

Keywords: 5-aza-2'-deoxycytidine, chromatin immunoprecipitation, histone modification, mouse brain unigene, promoter methylation

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carried out for DNAs from various mouse tissues. Next, a chromatin immunoprecipitation (ChIP) assay with histone antibodies was performed to determine whether the methylation is linked to histone modifications such as acetylation and methylation for the CNS-specific expression of Mbu-1.

MATERIALS AND METHODS

Cell culture and 5-Aza-2′-deoxycytidine treatment

A mouse neuroblastoma, Neuro-2a and a mouse neuroblastoma × rat glioma hybrid cell line, NG108-15, were purchased from the American Type Culture Collection (ATCC; Manassas, USA), and grown in Dulbecco's modified Eagle's medium (DMEM) 10% fetal bovine serum (FBS). The 1× HAT was supplemented (Invitrogen, USA) to the NG108-15 cell. Demethylation of the cytosine residues was achieved by exposing the cells to culture media containing a methyltransferase inhibitor, 5-Aza-dC (Sigma, USA), at 5 μM for 48 h and 72 h. Culture medium with or without treatment was changed every 24 h.

Bisulfite sequencing

Chromosomal DNA extracted from the cultured cell was subjected to bisulfite treatment using an EpiTect Bisulfite kit (Qiagen, USA). The bisulfite-treated DNA was subjected to polymerase chain reaction (PCR) to amplify the 174-bp promoter region of *Mbu-1* (nucleotides -402 to -228 of GenBank accession no. EF210820; transcriptional start site, +1) containing four CpG sites. The PCR conditions were as follows: 94°C for 2 min, followed by 30 cycles of 94°C for 20 s, 55°C for 20 s and 72°C for 30 s, with a final extension at 72°C for 5 min. The resulting products were purified using a Qiaex II gel extraction kit (Qiagen) and were then sub-cloned into the pGEM-T vector. The DNA sequences were confirmed by analyzing each plasmid clone in both directions.

Methylation-specific PCR (MSP)

Chromosomal DNA was isolated from mouse tissues and cell culture in a 75 cm^2 culture flask using a genomic DNA purification kit (Promega, USA) according to the manufacturer's protocol. The extracted DNA was then eluted with 250 μl of distilled water. Sodium bisulfite modification of genomic DNA was conducted using an EpiTect Bisulfite kit (Qiagen) according to the manufacturer's protocol using 0.1 mg of purified DNA. Design of PCR primers (sequences are in Supplementary Table S1) and reaction conditions were described previously (Kim et al., 2011). To assign a quantitative measure to the level of methylation, a methylation index was calculated for each sample using the following formula: methylation index = $[1 / (1 + 2^{-(CTu - CTme)})] \times 100\%$, as previously described (Kron et al., 2009), where CTu is the cycle threshold (CT) obtained from quantitative PCR analyses using the unmethylated primer set, and CTme is the average CT obtained using the methylated primer pair.

Quantitative real-time RT-PCR analysis

Total RNA from cell culture was prepared using Trizol reagent according to the manufacturer's protocols (GibcoBRL, USA). Reverse transcription was conducted using 10 μg of total RNA with a reverse transcription kit (Promega). One microliter of cDNA was used for the PCR, and duplicate reactions were performed for each sample using a Kapa SYBR Fast qPCR Kit (Kapa Biosystems, USA) with Mbu-1-specific primers (Supplementary Table S1) on an ABI 7300 instrument (Applied Biosystems). RNA quantity was normalized to GAPDH content, and gene expression was quantified according to the $2^{-\Delta Ct}$ method.

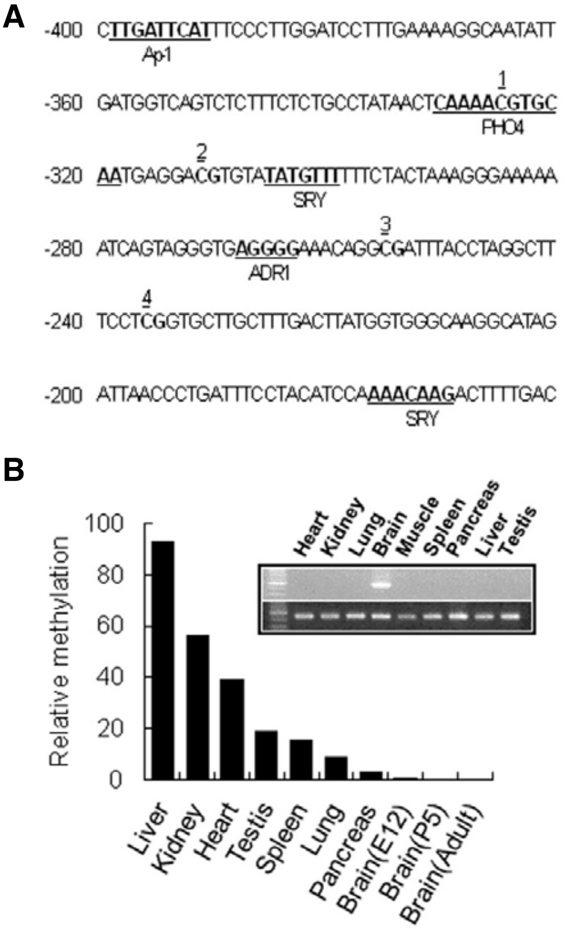

Fig. 1. Hypomethylation of Mbu-1 in the mouse brain. Methylation level of the Mbu-1 promoter was examined in various mouse tissues by real-time MSP analysis. A. Nucleotide sequence of the Mbu-1 promoter. Nucleotide sequence spanning from -400 to -161 (relative to the transcription start site) is shown. The four CpG sites are numbered on its sequence. The potential cis-regulatory elements in the promoter region are underlined and labeled. B. Relative methylation level of Mbu-1 in tissues. E12 and P5 are brain at embryo day12 and postnatal day5, respectively. Each sample was examined in duplicate and the average relative methylation level is presented. The inset is a result of RT-PCR for mouse tissues referred in the study by Yang et al. (2007), indicating brain-specific expression of Mbu-1.

Chromatin immunoprecipitation-PCR (ChIP-PCR)

ChIP assays were performed using the EZ ChIP™ Chromatin Immunoprecipitation kit (Millipore, USA) as described in the supplier's protocol. Briefly, after cell lysis, the cross-linked chromatin was sonicated and was then incubated with antibodies against modified histones at 4°C overnight. Antibodies against methylated H3 at K4 (ab8580), dimethylated H3 at K9 (ab8898), and acetylated H3 at K9 (ab10812) were purchased from Abcam (UK). The immunocomplex was precipitated with protein A-agarose (Abcam) and the beads were washed and sequentially treated with 10 μl of RNase A (37°C for 30 min) and 75 μl of proteinase K (45°C for 4 h), after which they were incubated at 65°C overnight to reverse the cross-link of the chromatin. The DNA was recovered by phenol-chloroform extractions and

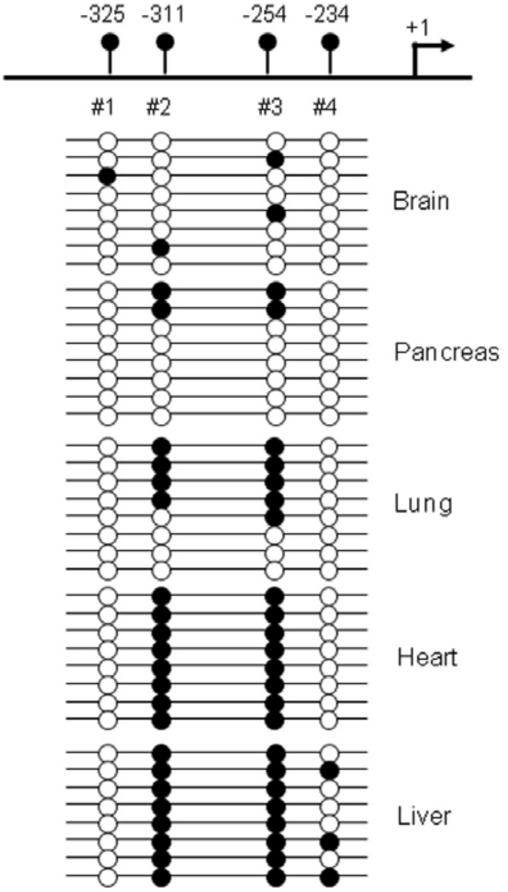

Fig. 2. Methylation status of CpGs at the Mbu-1 promoter. PCR-amplified product from bisulfite-treated DNA was sequenced for eight clones of each tissue, after subcloning into the pGEM-T plasmid. Open circle and black circle indicate no methylation and methylation, respectively. Location of CpG sites were indicated in the upper panel.

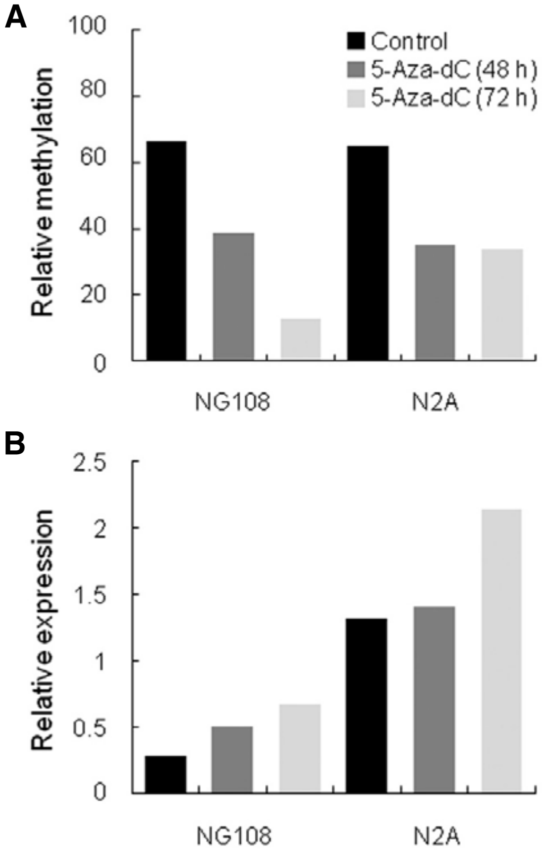

Fig. 3. Induction of Mbu-1 expression by demethylation. Cell lines were treated with 5-Aza-dC, and methylation (A) and expression level (B) were examined by real-time MSP and real-time RT-PCR. Black and gray bars represent before and after treatment of 5-Aza-dC, respectively. Each sample was examined in duplicate, and the average relative level is presented. Ct (cycle threshold) value of each sample in duplicate showed difference less than 0.2.

coprecipitation with glycogen, and was then dissolved in 50 μl of Tris-EDTA (TE) buffer. The DNA associated with modified histones was amplified by PCR using 1 μl of the precipitated DNA. PCR primers (sequences are in Supplementary Table S1) were designed to amplify 101 bp at the promoter. PCR condition was 30 cycles at 94°C for 40 s, 57°C for 1 min, and then 72°C for 40 s.

RESULTS

Promoter of Mbu-1 is hypomethylated in brain

In our previous study, the proximal promoter activity was shown to be located within -481 to -48, by using a luciferase reporter system in the NG108-15 brain cell line (Yang et al., 2007). Searching for potential transcriptional factor binding sites through the region identified only a few, including AP-1, SRY and ADR1 (Fig. 1A). Binding sites for brain-specific factors, such as REST, were not found. These results prompted us to speculate about other mechanisms that might determine the tissue specificity of Mbu-1, which involved an epigenetic approach. Because it has been known that promoter methylation could contribute to the brain-specific expression of genes such as HSD11B2, SNAP25, and PCDH9, we also examined the methylation status of the CpGs in the promoter. Real-time MSP revealed that the methylation level was variable in different mouse tissues (Fig. 1B). Interestingly all the brains from embryo, neonatal, and adult stage showed virtually no methylation, whereas other tissues showed variable methylation levels: liver, kidney, and heart showed high levels (39-93%); testis, spleen, lung, and pancreas showed intermediate or low levels (3.0-19%).

It is remarkable that the pancreas, in addition to the brain, showed a low methylation level, even though it did not express the Mbu-1 gene. To examine whether there might be a difference of methylation between levels of individual CpG sites between brain and pancreas, their methylation was determined for eight bisulfite-PCR clones from each tissue (Fig. 2). Interestingly, of the four CpGs, the first and forth CpGs were almost totally unmethylated, and only the two at the center represented a methylation difference among tissues showing a similar methylation pattern to the real-time MSP, with low levels in brain and pancreas, and with high levels in heart and liver. These results imply that even though hypomethylation of the Mbu-1 gene is possibly needed for the gene's brain-specific expression, it alone is not sufficient for that expression.

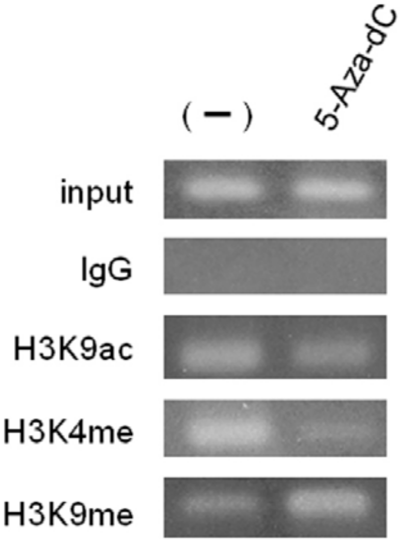

Fig. 4. Histone modification by 5-Aza-dC. ChIP was conducted using antibodies against H3K4me, H3K9me, and H3K9Ac for 5-Aza-dC-treated NG108-15 cell and control. The immunoprecipitated DNA was amplified by PCR using Mbu-1 promoter-specific primers. IgG is a negative control antibody.

5-Aza-dC reactivated Mbu-1 expression

To test the hypothesis that low methylation is needed for Mbu-1 expression, it was measured after inducing demethylation of the CpGs by 5-Aza-dC in cultured cell lines. At first, the methylation level was monitored in brain cell lines NG108-15 and Neuro-2a, which were treated with the methyltransferase inhibitor 5-Aza-dC. MSP was then performed to measure the demethylation-inducing efficacy of the inhibitor. Methylation in the treated cells was reduced to 15% and 32% after 72 h treatment of 5-Aza-dC, > 60% decrease in both NG108-15 and Neuro-2a (Fig. 3A). Alteration of expression by demethylation was monitored by real-time RT-PCR analysis (Fig. 3B). The gene expression was elevated to 2.2 and 1.6 times that of NG108-15 and Neuro-2a after 72 h treatment of 5-Aza-dC. Recovery of gene expression upon demethylation of the CpG sites implies that hypomethylation at the promoter was, at least in part, responsible for upregulation of the cultured cells.

5-Aza-dC induced histone modification in a way to gene silencing

Although the hypomethylation of the CpGs at the Mbu-1 promoter is responsible for gene activation, it does not completely explain the brain-specific expression because pancreas, for example, also showed lower methylation levels without any accompanying expression. To further identify the mechanism of tissue specificity, profiles of histone modification were examined. As the three modifications of acetylation at H3K9 and methylation at H3K4 and H3K9 have been known to be involved in gene activation or inactivation, their profiles were monitored at the four CpG-harboring promoter regions by ChIP-PCR. For the acetylation at H3K9, less acetylated histones were found at the 5-Aza-dC treated cells (Fig. 4). For the methylation of H3, less methylated histones at K4 and more methylated histones at K9 were found at the 5-Aza-dC treated cells. All these histone modifications at the 5-Aza-dC treated cells have been known to appear at inactivated genes (Fuks, 2005; Yan and Boyd, 2006). Therefore, at the hypomethylated promoter, the hypomethylation and histone modifications may have opposing influences on the regulation of Mbu-1 gene expression. This epigenetic combination may, in part, explain why the gene is expressed only in brain.

DISCUSSION

This study was carried out in order to identify molecular factors that contribute to the brain-specific expression of Mbu-1. So far, studies to elucidate the mechanism of tissue specificity for brain-expressed genes have been approached through identifying transcriptional factors that acted on the promoter. As examples, neuron-restrictive silencer elements (NRSE) play a role in specifying neuronal expression of a large number of genes in vertebrates, by binding a transcription factor (REST) and silencing neuronal genes in non-neuronal tissues. Within the developing nervous system, REST transiently repressed the synaptotagmin 4 brain-derived neurotrophic factor and calbindin genes in dividing progenitors, and its loss at terminal differentiation allowed their expression in mature neurons (Ballas et al., 2005; Tan et al., 2010). In contrast, outside of the nervous system, REST normally mediates long-term silencing of these genes (Lunyak et al., 2002; Roopra et al., 2004).

Even though REST is the predominant factor that suppresses neuron-specific genes in non-neuronal cells, no NRSE has been found in the majority of genes, including Mbu-1. This fact prompted us to consider other regulatory mechanisms for the neuron-specific expression. As alternative explanations to the brain-specific expression, epigenetic approaches, including promoter methylation and histone modifications, have newly emerged. In the mammalian brain, CpG promoter methylation is a postreplicative process, mediated by a group of DNA methyltransferases (DNMT), such as DNMT1, DNMT3a and DNMT3b. DNMTs played an important role in the initiation and maintenance of methylation of the promoter of estrogen receptor, levels of which are high in early postnatal development, but virtually absent in the adult cortex (Westberry et al., 2010). In glioma, DNMTs were upregulated, which inhibited tumor suppressors such as PTEN and p21 that are epigenetically silenced in the tumor (Rajendran et al., 2011). Several studies demonstrate that in addition to DNMTs, promoter methylation in the brain can be regulated by a putative DNA demethylation process that specifically removes the methyl group from the carbon-5 of cytosines (Dong et al., 2010). Reelin is an example of this category that was shown to be demethylated by nuclear extract from the adult mouse brain. A further study could reveal the mechanism of how methylation at the CpG sites in Mbu-1 is regulated by DNMT and/or demethylation.

It is interesting to note that Mbu-1 in mouse tissues showed various ranges of methylation, from non-methylation, as in brain, to nearly complete methylation, as in liver. Although a few tissues in addition to those of the brain showed lower methylation levels, hypomethylation is thought to be needed to maintain the expression status of the gene in the brain. This fact was proven by the discovery that the demethylation agent, 5-Aza-dC, induced an increase of mbu-1 expression. A limitation of our study design is seen in the cultured cell lines, NG108-15 and N2A that displayed higher levels of methylation than mouse brain. The aberrant methylation in the cells may imply that the cells lost epigenetic characteristics of neuronal cells in the course of establishment. However, 5-Aza-dC was successfully used in the study because the high methylation was rather utilized to look into the effect of demethylation induced by the reagent. In addition, as histones undergo multiple modifications other than H3K9ac, H3K4me, and H3K9me, this study is limited through overlooking the effect of other modification. Nonetheless, the three types of histone modification adopted in this study can explain the neuron-specific expression of Mbu-1 together with CpG methylation. Several lines of evidence suggest that DNA methylation and histone tail acetylation and methylation are closely linked to control chromatin structure and function in postmitotic mammalian neurons (Dong et al., 2007). In the Reelin promoter, for example, histone acetylation modulated DNA methylation in terminally differentiated neurons.

In conclusion, the present study demonstrates an epigenetic mechanism underlying the central nervous system-specific expression of Mbu-1. Our results indicate that the Mbu-1 prompter is hypomethylated in brain tissues, and that the methylation status is affected by the acetylation and methylation of histones.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

ACKNOWLEDGMENTS

This study was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2011-0004157).

REFERENCES

Ballas, N., Grunseich, C., Lu, D.D., Speh, J.C., and Mandel, G. (2005). REST and its corepressors mediate plasticity of neuronal gene chromatin throughout neurogenesis. Cell *121*, 645-657.

Chong, J.A., Tapia-Ramirez, J., Kim, S., Toledo-Aral, J.J., Zheng, Y., Boutros, M.C., Altshuller, Y.M., Frohman, M.A., Kraner, S.D., and Mandel, G. (1995). REST: a mammalian silencer protein that restricts sodium channel gene expression to neurons. Cell *80*, 949-957.

Davies, M.N., Volta, M., Pidsley, R., Lunnon, K., Dixit, A., Lovestone, S., Coarfa, C., Harris, R.A., Milosavljevic, A., Troakes, C., et al. (2012). Functional annotation of the human brain methylome identifies tissue-specific epigenetic variation across brain and blood. Genome Biol. *13*, R43.

Dong, E., Guidotti, A., Grayson, D.R., and Costa, E. (2007). Histone hyperacetylation induces demethylation of reelin and 67-kDa glutamic acid decarboxylase promoters. Proc. Natl. Acad. Sci. USA *104*, 4676-4681.

Dong, E., Chen, Y., Gavin, D.P., Grayson, D.R., and Guidotti, A. (2010). Valproate induces DNA demethylation in nuclear extracts from adult mouse brain. Epigenetics-Us *5*, 730-735.

Fuks, F. (2005). DNA methylation and histone modifications: teaming up to silence genes. Curr. Opin. Genet. Dev. *15*, 490-495.

Furuya, T.K., Silva, P.N., Payao, S.L., Bertolucci, P.H., Rasmussen, L.T., Labio, R.W., Braga, I.L., Chen, E.S., Turecki, G., Mechawar, N., et al. (2012). Analysis of SNAP25 mRNA expression and promoter DNA methylation in brain areas of Alzheimer's Disease patients. Neuroscience *220*, 41-46.

Hattori, N., Nishino, K., Ko, Y.G., Ohgane, J., Tanaka, S., and Shiota, K. (2004). Epigenetic control of mouse Oct-4 gene expression in embryonic stem cells and trophoblast stem cells. J. Biol. Chem. *279*, 17063-17069.

Iwamoto, K., Bundo, M., Ueda, J., Oldham, M.C., Ukai, W., Hashimoto, E., Saito, T., Geschwind, D.H., and Kato, T. (2011). Neurons show distinctive DNA methylation profile and higher interindividual variations compared with non-neurons. Genome Res. *21*, 688-696.

Jensen Pena, C., Monk, C., and Champagne, F.A. (2012). Epigenetic effects of prenatal stress on 11beta-hydroxysteroid dehydrogenase-2 in the placenta and fetal brain. PLoS One *7*, e39791.

Jones, P.L., Veenstra, G.J., Wade, P.A., Vermaak, D., Kass, S.U., Landsberger, N., Strouboulis, J., and Wolffe, A.P. (1998). Methylated DNA and MeCP2 recruit histone deacetylase to repress transcription. Nat. Genet. *19*, 187-191.

Kim, S.J., Kelly, W.K., Fu, A., Haines, K., Hoffman, A., Zheng, T., and Zhu, Y. (2011). Genome-wide methylation analysis iden-tifies involvement of TNF-alpha mediated cancer pathways in prostate cancer. Cancer Lett. *302*, 47-53.

Kron, K., Pethe, V., Briollais, L., Sadikovic, B., Ozcelik, H., Sunderji, A., Venkateswaran, V., Pinthus, J., Fleshner, N., van der Kwast, T., et al. (2009). Discovery of novel hypermethylated genes in prostate cancer using genomic CpG island microarrays. PLoS One *4*, e4830.

Lee, J.Y., and Lee, T.H. (2012). Effects of histone acetylation and CpG methylation on the structure of nucleosomes. Biochim. Biophys. Acta *1824*, 974-982.

Li, E., Bestor, T.H., and Jaenisch, R. (1992). Targeted mutation of the DNA methyltransferase gene results in embryonic lethality. Cell *69*, 915-926.

Lunyak, V.V., Burgess, R., Prefontaine, G.G., Nelson, C., Sze, S.H., Chenoweth, J., Schwartz, P., Pevzner, P.A., Glass, C., Mandel, G., et al. (2002). Corepressor-dependent silencing of chromo-somal regions encoding neuronal genes. Science *298*, 1747-1752.

Majid, S., Dar, A.A., Ahmad, A.E., Hirata, H., Kawakami, K., Shahryari, V., Saini, S., Tanaka, Y., Dahiya, A.V., Khatri, G., et al. (2009). BTG3 tumor suppressor gene promoter demethy-lation, histone modification and cell cycle arrest by genistein in renal cancer. Carcinogenesis *30*, 662-670.

Nakatani, T., Ueno, S., Mori, N., and Matsuoka, I. (2005) Role of NRSF/REST in the molecular mechanisms regulating neural-specific expression of trkC/neurotrophin-3 receptor gene. Brain Res. Mol. Brain Res. *135*, 249-259.

Rajendran, G., Shanmuganandam, K., Bendre, A., Muzumdar, D., Goel, A., and Shiras, A. (2011). Epigenetic regulation of DNA methyltransferases: DNMT1 and DNMT3B in gliomas. J. Neurooncol. *104*, 483-494.

Roopra, A., Qazi, R., Schoenike, B., Daley, T.J., and Morrison, J.F. (2004). Localized domains of G9a-mediated histone methylation are required for silencing of neuronal genes. Mol. Cell *14*, 727-738.

Siegmund, K.D., Connor, C.M., Campan, M., Long, T.I., Weisenberger, D.J., Biniszkiewicz, D., Jaenisch, R., Laird, P.W., and Akbarian, S. (2007). DNA methylation in the human cerebral cortex is dynamically regulated throughout the life span and involves differentiated neurons. PLoS One *2*, e895.

Tan, Y.P., Li, S., Jiang, X.J., Loh, W., Foo, Y.K., Loh, C.B., Xu, Q., Yuen, W.H., Jones, M., Fu, J., et al. (2010). Regulation of protocadherin gene expression by multiple neuron-restrictive silencer elements scattered in the gene cluster. Nucleic Acids Res. *38*, 4985-4997.

Tomikawa, J., Fukatsu, K., Tanaka, S., and Shiota, K. (2006). DNA methylation-dependent epigenetic regulation of dimethylarginine dimethylaminohydrolase 2 gene in trophoblast cell lineage. J. Biol. Chem. *281*, 12163-12169.

Tomikawa, J., Uenoyama, Y., Ozawa, M., Fukanuma, T., Takase, K., Goto, T., Abe, H., Ieda, N., Minabe, S., Deura, C., et al. (2012). Epigenetic regulation of Kiss1 gene expression mediating estrogen-positive feedback action in the mouse brain. Proc. Natl. Acad. Sci. USA *109*, E1294-1301.

Westberry, J.M., Trout, A.L., and Wilson, M.E. (2010). Epigenetic regulation of estrogen receptor alpha gene expression in the mouse cortex during early postnatal development. Endocrinology *151*, 731-740.

Yan, C., and Boyd, D.D. (2006). Histone H3 acetylation and H3 K4 methylation define distinct chromatin regions permissive for transgene expression. Mol. Cell. Biol. *26*, 6357-6371.

Yang, H.L., Cho, E.Y., Han, K.H., Kim, H., and Kim, S.J. (2007). Characterization of a novel mouse brain gene (mbu-1) identified by digital differential display. Gene *395*, 144-150.