Supplemental Material

Allele-specific DNA methylation in mouse strains is mainly determined by *cis*-acting sequences.

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Supplemental Methods

Bone marrow-derived macrophages

To obtain bone marrow-derived macrophages (BMM) of wild-type and F1 hybrids , bone marrow precursor cells were cultured in RPMI 1640, 10% FCS, and 200 ng/ml human rCSF-1 (Cetus) in bacteriological square plates (Sterilin; Barloworld scientific, UK) for 5 days. On day 5, the medium was replaced. Cells were harvested on day 6 and replated at a density of 10 x 10^6 cells/10-cm tissue culture dish (Falcon) at 1 x 10^6 cells/ml, with human rCSF-1 added back overnight. After 7 days in the presence of human rCSF-1, bone marrow cultures usually consist of >98% macrophages (Rehli et al. 2005). For mRNA expression analyses, BMM were either left untreated or treated with interleukin 4 (10 ng/ml, PeproTech Germany GmbH, Hamburg) for 4 or 18 h before harvest.

Whole genome transcriptome analysis of BMM

RNA preparations from BMM cultures from two independent experiments were analyzed using Whole Mouse Genome Oligo Microarrays (Agilent). Labelling and hybridization were performed using the Agilent Gene Expression system according to the manufacturer's instructions. In brief, 200 ng to 1000 ng of high-quality RNA were amplified and Cyanine 3-CTP labelled with the One Color Low RNA Input Linear Amplification Kit (Agilent). Labelling efficiency was controlled using the NanoDrop spectrophotometer, and 1.65 µg labelled cRNA were fragmented and hybridized on the Whole Mouse Genome Expression Array (4x44K, Agilent). Images were scanned using a DNA microarray scanner (Agilent), and processed with Feature Extraction Software 9.5.1 (Agilent) using default parameters (protocol GE1-v5_95_Feb 97 and Grid: 014868_D_20060807) to obtain background subtracted and spatially detrended, processed signal intensities. Features flagged as non-uniform outliers were excluded. Extracted data were further processed with GeneSpring GX 7.3.1 as follows: Data Values below 10.0 were set to 10.0. Each measurement was divided by the 50,0th percentile of all measurements in that sample. The percentile was calculated using only genes marked present. Each gene was divided by the median of its measurements in all samples. If the median of the raw values was below 0 then each measurement for that gene was divided by 0 if the numerator was above 0, otherwise the measurement was thrown out. Median polishing was done, where each chip was normalized to its median and each gene was normalized to its median. These normalizations were repeated until the medians converged. Samples were normalized to the

median of untreated C57BL/6 samples. Each measurement for each gene in those specific samples was divided by the median of that gene's measurements in the corresponding control samples. We initially extracted 311 probes that showed a statistically significant, at least five fold difference in C57BL/6-BALB/c comparisons (parametric test, variances not assumed equal (Welch t-test); p-value cut-off 0.05; multiple testing correction: Bonferroni.). For methylation analysis, Microarray data have been submitted and are available from the NCBI/GEO repository (accession number GSE14644). Median normalized expression ratios for genes associated with DMR are given in **Supplemental Table S2**.

Quantitative RT-PCR

To validate differential gene expression as determined by expression microarray analysis, a representative set of genes was selected for qRT-PCR. Primers were designed using PerlPrimer Software (http://perlprimer.sourceforge.net) and controlled using PCR and BLAT functions of the UCSC Genome Browser (http://genome.ucsc.edu/) and GeneRunner Software (http://www.generunner.net/) and are listed in **Supplemental Table S3.** One µg of total RNA of untreated C57BL/6 and BALB/c BMM was reverse transcribed using Superscript II MMLV-RT (Promega, Mannheim, Germany). Real-time PCR was performed on Realplex Mastercycler EP (Eppendorf) using the QuantiFast SYBR Green PCR Kit (Qiagen) according to the manufacturer's instructions. Melting curves were analyzed to control for specificity of the PCR reactions. Expression data were normalized for expression of *Hprt1*. The relative units were calculated from a standard curve plotting 3 different concentrations of log dilutions against the PCR cycle number (CP) at which the measured fluorescence intensity reached a fixed value. For each sample, data of at least three independent RNA preparations measured in duplicates were averaged.

Methyl-CpG immunoprecipitation.

Methyl-CpG immunoprecipitation was essentially performed as described with slight modifications (Gebhard et al. 2006; Schilling and Rehli 2007; Schmidl et al. 2009). In brief, genomic DNA was sonicated to a mean fragment size of 350-400 bp. Four µg of each sample were incubated with 200 µl Protein A-Sepharose 4 Fast Flow beads (GE Healthcare) coated with 65 µg purified MBD-Fc protein in 2 ml Ultrafree-MC centrifugal filter devices (Amicon/Millipore) for 3 h at 4°C in buffer containing 300 mM NaCl. Beads were centrifuged to recover unbound DNA fragments (300 mM fraction) and subsequently washed with buffers containing increasing NaCl concentrations (400,

500, 550, 600 mM). Densely CpG-methylated DNA was eluted with a high-salt buffer (1000 mM NaCl) and all fractions were desalted using the MinElute PCR purification kit (Qiagen). The separation of CpG methylation densities of individual MCIp fractions was controlled by qPCR using primers covering an imprinted regions of *Mest* (mest_s_5'-CAG ACG CCA CCT CCG ATC C-3'; mest_as_ 5'-GGC CGC ATT ATC CCA TGC C-3') and *Snrpn* (snrpn_s_5'- ACA TTC CGG TCA GAG GGA CGA AG-3'; snrpn_as_ 5'- CCG CAA TGG CTC AGG TTT GTC-3') and a genomic region lacking CpG's within a range of 1000 bp (empty_s_5'-GGT GAG TTG TAT GAC CTT GTT CAA TTC C-3'; empty_as_ 5'-AGT GCA GTG AGT CAG ACA TAA CCC-3'). Prior to labelling, fractions containing mainly unmethylated DNA (300-400 mM, CpG pool) or methylated DNA (≥500 mM, mCpG pool) were combined.

Oligonucleotide microarray design, handling and analysis.

Based on the expression analysis, the set of 311 differential probes was further reduced by removing redundant probes and probes covering invalidated transcripts, resulting in a final set of 165 known genes showing significantly different gene expression between C57BL/6 and BALB/c BMM. A set of 16 control genes was randomly picked from probes that were not differentially expressed. We next created a custom 240k tiling array (Agilent) for these 181 regions covering 28 Mb of the mouse genome. Regions and corresponding genes are given in **Supplemental Table S1**. To generate fluorescently labelled DNA for microarray hybridization the hypo- and hypermethylated DNA pools were labelled with Alexa Fluor 5 (C57BL/6) and Alexa Fluor 3 (BALB/c) using the BioPrime® Total Genomic Labelling System (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Two independent comparative hybridizations of hypo- and hypermethylated fractions of two independent MCIp preparations were performed using a stringent protocol. The corresponding differently labelled fractions (e.g. hypo-C57BL/6 vs. hypo-BALB/c) were combined to a final volume of 80 µl, supplemented with 50 µg mouse Cot-1 DNA (Invitrogen), 52 µl of Agilent blocking agent (10-fold) (Agilent Technologies, Böblingen, Germany), 15% deionized formamide (Sigma) and 250 µl Agilent hybridization buffer (2-fold) as supplied in the Agilent oligo aCGH Hybridization kit. The samples are heated to 95° for 3 min, mixed and subsequently incubated at 37°C for 30 min and spun down afterwards for 1 min. Hybridization was then carried out at 67°C for 40 h using an SureHyb chamber (Agilent) and an Agilent hybridization oven. Slides were washed in Wash I (6xSSPE, 0.005% N-lauroylsarcosine) at room temperature for 5 min and in pre-warmed Wash II (37°C, 0.06xSSPE) for additional 5 minutes. Afterwards, slides were dried using acetonitrile (J.T.Baker) within an ozone-free environment. Images were scanned immediately after washing using a DNA microarray scanner (Agilent) and processed using Feature

Extraction Software 9.5.1 (Agilent) and the standard CGH protocol. Processed signal intensities were imported into Microsoft Office Excel 2007 and further normalized. Array probes were binned according to their GC-content and for each resulting subarray $\log_{10}$ ratios were independently normalized to fit the baseline using a fitted linear regression function. Probes with signal intensities close to background (below 10^2) in both channels were removed from further analysis.

Virtual Comparative Genome Hybridization (vCGH)

To allow a 'virtual' comparison of mouse strain genomes, at each individual probe, signal intensities of both independent experiments and both genome pools were added up for each strain. Log_{10} ratios of cumulative signal intensities were plotted against the genomic location to identify copy number variations and deletions. Unbalanced hybridization behaviour in the vCGH with a $\log_{10}$ ratio of cumulative signal intensities >0.15 as compared to the surrounding probes was used as a threshold for detecting genetic differences between strains (32860 probes). Increased signal intensities in BALB/c may indicate technical artefacts. Only 1245 probes showed unbalanced hybridization behaviour with a $\log_{10}$ ratio of cumulative signal intensities >-0.15, suggesting that the majority of the C57BL/6 really detect genetic variability. All probes with underlying genetic differences were removed before microarray results were further analyzed for differentially methylated regions.

Microarray detection of differentially methylated regions (DMR)

The vCGH-corrected datasets were further analyzed to identify differentially methylated regions. First, hypomethylation scores were calculated by subtracting the $\log_{10}$ signal intensity ratios from the CpG pool hybridization from those of the mCpG pool hybridization of the same experiment. Hypomethylation scores were then analysed using a sliding window approach. The window included five probes with a maximal distance of 500 bp between two neighbouring probes. A cumulative hypomethylation score of the five probes >1.5 (in both replicate experiments) or >1.3 (if the value for each of the five probes was >0.15 in both replicate experiments) was used as a threshold for detecting hypomethylation in C57BL/6. A cumulative hypomethylation score of the five probes <-1.5 (in both replicate experiments) or <-1.3 (if the value for each of the five probes was <-0.15 in both replicate experiments) was used as a threshold for detecting hypomethylation in BALB/c.

Quantitative PCR validation of copy number variations (CNV)

Primer sequences for CNV candidate regions were designed using PerlPrimer Software (http://perlprimer.sourceforge.net) and controlled using PCR and BLAT functions of the UCSC Genome Browser (http://genome.ucsc.edu/) and are given in **Supplemental Table S3**. QPCRs were performed on a Mastercycler EP Realplex (Eppendorf; Hamburg, Germany) using the QuantiFast SYBR Green PCR Kit (Qiagen). Specificity of the PCR reactions was controlled by melting curve analysis. All measurements were performed in duplicates and repeated at least twice.

Sequencing of genomic DNA.

Primers for amplifying specific genomic regions of C57BL/6 and BALB/c gDNA were designed and controlled as described above. Primer sequences are given in **Supplemental Table S3**. PCR-reactions were carried out using Phusion™ Hot Start High Fidelity DNA Polymerase (Finnzymes, Espoo, Finland) according to the manufacturer's instructions and 100 ng gDNA as a template. The cycling parameters were as follows: initial denaturation 98°C for 30 s, amplification at 98°C for 10 s, 62°C for 20 s, 72°C for 30 s / 1 kb, for 35 cycles. PCR-reactions were either gel purified using agarose gel electrophoresis followed by purification with a PCR-Purification kit (Qiagen) or directly used for PEG precipitation. Purified PCR-products were sequenced using the PCR primers and additional internal sequencing primers by GENEART (Regensburg, Germany) and analysed using the UCSC Browser (http://genome.ucsc.edu/) together with GeneRunner (http://www.generunner.net/) and BioEdit Software (http://www.mbio.ncsu.edu/BioEdit/BioEdit.html).

Mass spectrometry analyses of bisulfite-converted DNA.

We initially chose a set of 22 regions based on the MCIp microarray results and sequenced underlying gDNA of both strains (for primer sequence see **Supplemental Table S3** and for sequence alignments see **Supplemental Figure S2**). Four regions were excluded (mainly because of large indels) and the residual 18 regions were analysed on the EpiTYPER platform (Sequenom, San Diego, CA). Primers for bisulfite converted DNA were designed based on the sequences of genomic PCR products using Methprimer (www.urogene.org/methprimer/). For each reverse primer, an additional T7 promoter tag for in vivo transcription was added, as well as a 10-mer tag on the forward primer to adjust for melting temperature differences. In some cases, sequence variability between strains required strain-specific amplicons as indicated in **Supplemental Table S4**. All

primers were purchased from Sigma (Taufkirchen, Germany) (for sequences see **Supplemental Table S4**). Sodium bisulfite conversion was performed using EZ DNA methylation kit (Zymo Research, California, USA) using 1 µg of genomic DNA and an alternative conversion protocol. The incubation parameters where changed as follows: 95°C for 30 sec, 50°C for 15 min (repeated for 20 cycles). Amplification of target regions was followed by SAP treatment, reverse transcription and subsequent RNA base-specific cleavage (MassCLEAVE, Sequenom, San Diego, CA) as previously described (Ehrich et al. 2005). Cleavage products were loaded onto silicon chips (spectroCHIP, Sequenom, San Diego, CA) and analysed by MALDI-TOF mass spectrometry (MassARRAY Compact MALDI-TOF, Sequenom, San Diego, CA). Methylation was quantified from mass spectra using the EpiTYPER software v1.0.5 (Sequenom, San Diego, CA).

Allele-specific analyses of bisulfite-converted DNA.

Sodium bisulfite conversion and amplification was performed as described above. Specific amplification products were directly cloned into a pCR®2.1-Topo vector using the TOPO TA Cloning kit (Invitrogen). DNA from insert-containing plasmids of single colonies was purified using the NucleoSpin® Plasmid QuickPure kit (Macherey-Nagel). Inserts were sequenced using the M13-R sequencing-primer at GENEART (Regensburg, Germany) and analysed using GeneRunner (http://www.generunner.net/) and BioEdit (http://www.mbio.ncsu.edu/BioEdit/BioEdit.html) software tools.

Supplemental Tables

Supplemental Table S1

Supplemental Table S1 lists all gene loci that were selected for the locus-wide tiling array. Genomic locations are based on the Build 36 assembly by NCBI and the Mouse Genome Sequencing Consortium (mm8).

Supplemental Table S2

Supplemental Table S2 contains the complete list of mouse strain-specific DMR discovered in this study. The genomic location (Build 36 assembly by NCBI, mm8) of the 'core' region of the DMR is given along with information on the associated (neighbouring) gene, its distance to the transcription start site (TSS), its methylation status and BMM expression data (if available) for the associated gene. Microarray expression data was median-normalized to the untreated C57BL/6 BMM (0 h) sample. The call status of individual probes in each sample is given in brackets behind the relative expression values (P, present; A, absent; M, marginal).

Supplemental Table S3

Supplemental Table S3 is a list of all oligonucleotides that were used to amplify genomic DNA fragments or to quantify transcription levels using qRT-PCR.

Supplemental Table S4

Supplemental Table S4 is a list of all oligonucleotides that were designed to generate amplicons from bisulfite-treated DNA for EpiTyper (MALDI-TOF MS) analysis. Amplicon-specific sequences are given in capital letters. Genomic locations are based on the Build 36 assembly by NCBI and the Mouse Genome Sequencing Consortium (mm8). The last column indicates whether the same amplicon was used to analyze both strains or whether an amplicon was specific for the indicated mouse strain.

Supplemental Table S5

Supplemental Table S5 contains EpiTyper (MALDI-TOF MS) results for the initial, large test set of 59 amplicons. Methylation ratios are given for BMM, spleen and testis that were measured from two individual animals of each mouse strain. Values are colour-coded to visualize methylation levels of individual CpGs. The scale ranges from white (0% methylation) to dark blue (100% methylation), strain-specifically absent CpGs are coloured black, non-detectable CpGs (NA) are marked in gray.

Supplemental Table S6

Supplemental Table S6 contains EpiTyper (MALDI-TOF MS) results of BMM samples for the reduced set of 24 amplicons. Values are colour-coded to visualize methylation levels of individual CpGs. The scale ranges from white (0% methylation) to dark blue (100% methylation), strain-specifically absent CpGs are coloured black, non-detectable CpGs (NA) are marked in gray.

Supplemental Table S7

Supplemental Table S7 contains EpiTyper (MALDI-TOF MS) results of spleen samples for the reduced set of 24 amplicons. Values are colour-coded to visualize methylation levels of individual CpGs. The scale ranges from white (0% methylation) to dark blue (100% methylation), strain-specifically absent CpGs are coloured black, non-detectable CpGs (NA) are marked in gray.

Supplemental Table S8

Supplemental Table S8 contains EpiTyper (MALDI-TOF MS) results of testis samples and control DNAs for the reduced set of 24 amplicons. Values are colour-coded to visualize methylation levels of individual CpGs. The scale ranges from white (0% methylation) to dark blue (100% methylation), strain-specifically absent CpGs are coloured black, non-detectable CpGs (NA) are marked in gray.

Supplemental UCSC Genome Browser Track File

This file contains microarray results and positional information that can be uploaded to the UCSC Genome Browser (http://genome.ucsc.edu/) for display. It contains the following tracks:

CpG-C57BL/6:	C57BL/6 hypomethylation scores for probes without detectable genetic variation.
CpG-BALB/c:	BALB/c hpomethylation scores for probes without detectable genetic variation.
CGH-C57BL/6:BALB/c:	vCGH signals for all probes.
EpiTyper mm8	Genomic location of amplicons used for EpiTYPER analysis.
DMR mm8	Genomic location of DMR core regions.

Supplemental Figures

Supplemental Figure S1:

Expression profiling of bone marrow-derived macrophages (BMM) from two inbred mouse strains. (A) BMM from BALB/c and C57BL/6 strains were left untreated or treated with IL-4 for 4 h or 18 h. Expression analysis was performed using Mouse Whole Genome arrays (Agilent) to identify genes that are differentially expressed in either strain. Shown is a hierarchical clustering of 311 probes that show at least five-fold differences in median normalized signals between untreated (0 h) or IL-4 treated (4 h, 18 h) BMM. With few exceptions, differential gene expression was already detected in untreated BMM. Gene ontology terms enriched in either of the two gene clusters are shown on the right. Numbers in brackets indicate: genes in cluster/genes in cluster with specific GO term/total genes/total genes with specific GO term. (B) Validation of differential gene expression in untreated macrophages using qRT-PCR. Results for all genes (GOI, genes of interest) were normalized for *Hprt1* expression. Data represent mean values ± SD of at least four independent qPCR analyses from at least two independent experiments.

Supplemental Figure S2:

DNA sequences of 34 selected regions in C57BL/6 and BALB/c. Regions were randomly selected from the vCGH data (A-I), or as possible targets for methylation analysis using MALDI-TOF MS (J-AH). Genomic DNA was PCR amplified and directly sequenced. All sequences were deposited with GenBank (accession numbers: FJ751937-FJ752004). Differences between BALB/c and the reference strain C57BL/6 are marked as follows: deletions or insertions (relative to C57BL/6) are in red lettering; single nucleotide polymorphisms are boxed in blue (BALB/c). Variations between the published reference sequence and the C57BL/6 sequence used in this study were only observed in a fragment that represents a pseudogene or duplication of the *Sfi1* locus (in AC).

Supplemental Figure S3:

Correlation of DMR and genetic variation. (A) The bar graph shows the distribution of SNP-densities across all genomic loci on the microarray (bin size 10 SNP/100000 bp). (B) The origin of each DMR was determined using the Mouse Ancestry Mapper and Mouse Haplotype Block Viewer

provided by Perlegen Sciences (mouse.perlegen.com). The first column indicates classifications for individual DMR. For regions that were either not classified or predicted to be of the same haplotype the number of DMR showing unbalanced hybridisation behaviour are given in brackets. The second column indicates how many loci were associated with either type of classification. (C) Numbers of DMR/100kb were plotted against SNP counts per 100 kb for all loci present on the array. (D) The box plot shows the distribution of DMR counts conditional on the SNP counts at corresponding loci. The red lines denote medians, boxes the interquartile ranges, and whiskers the 5th and 95th percentiles. The total numbers of loci within each SNP-density group are given in brackets.

Supplemental Figure S4:

Validation of strain-specific CpG methylation by MALDI-TOF MS of bisulfite treated DNA. Three additional examples of DMR detected by the MCIp-microarray approach and validation using MALDI-TOF MS of bisulfite treated DNA. MCIp results are presented in the upper panels. Shown are the following tracks (from top to bottom) that were generated using the UCSC Genome Browser (http://genome.ucsc.edu/): repetitive regions as identified by the RepeatMasker program, single nucleotide polymorphisms from the dbSNP (NCBI database for genomic variation) build 126 (both in black), hypomethylation scores for BMM of both mouse strains (defined as the difference product of $\log_{10}$ signal intensity ratios of both hybridizations; in green), vCGH signals indicating the presence of genetic variation at probe level (in brown) as well as gene structures (in purple) and the position of amplicons (Epityper Ampl.; in blue) that were designed for MALDI-TOF MS analysis of bisulfite treated DNA. The relative position of CpGs within amplicons is indicated below by small lollipops (with the upward orientation representing C57BL/6, and the downward orientation representing BALB/c). Sequence variations are highlighted in red and blue, black bars mark the position of exons and gray lollipops are not analysed by the MS. Methylation levels of individual CpGs in the indicated cell types (two individuals for each strain) are shown colour-coded. The scale ranges from pale yellow (0% methylation) to dark blue (100% methylation), strain-specifically absent CpGs are coloured black, non-detectable CpGs are marked in gray.

Supplemental Figure S5:

Correlation matrix of all MALDI-TOF MS detected methylation ratios. The complete dataset was reduced to all autosomal CpG units that were measured in all samples. Correlation coefficients were calculated from mean methylation ratios of the indicated sample.

Supplemental Figure S6:

Inheritance of stain-specific methylation patterns in F1 hybrids. Three examples (*Slc27a6, Zfp568,* and X-linked *3110007F17Rik*) for allelic inheritance of stain-specific methylation patterns are shown. In the top panels, hypomethylation scores for BMM of both strains are displayed as described in **Figure 3**. Averaged methylation levels of individual CpGs were determined by MALDI-TOF analysis at the indicated DMR in BMM, spleen, and testis and are shown colour coded (as in **Figure 3**) for parental strains and F1 hybrids.

Supplemental Figure S7:

Methylation ratios of wildtype mice versus F1 hybrids. Averaged CpG methylation ratios of parental spleen (n=3 for each strain) are plotted against averaged CpG methylation ratios of F1 hybrids derived from C57BL/6 (top, n=5) or BALB/c (bottom, n=4) sires. In eight out of eleven DMR analyzed by MALDI-TOF (marked in black), methylation patterns in F1 hybrids are almost identical to the average methylation level in parental strains (r^2>0.97). Three DMR (*Sfi1 pseudogene, Isoc2b, and Eps8l1,* marked in red, green and blue, respectively) either acquire (*Sfi1 pseudogene*) or loose methylation (*Isoc2b, Eps8l1*) in F1 hybrids relative to parental strains.

Supplemental Figure S8:

Allele-specific bisulfite sequencing of DMRs in *1699921P15Rik*, *Asb4*, and *Slc27a6*. The genomic position of CpGs within each amplicon is shown at the top. Sequence variations used to distinguish the different parental alleles are shown blue for C57BL/6 and red for BALB/c. Individual CpGs are represented by either white (unmethylated) or black (methylated) squares. Lines of squares represent independently sequenced clones derived from two independent sample preparations derived from reciprocal crosses.

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