2. Materials and Methods

2.1 Materials

2.1.1 Chemicals

Standard chemicals were ordered from Amersham (Amersham Pharmacia Biotech Europe GmbH, Freiburg, Germany), Biochrom (Berlin, Germany), Calbiotech (Bad Soden, Germany), Invitrogen (Karlsruhe, Germany), Merck (Darmstadt, Germany), Roche Biochemicals (Basel, Switzerland), Roth (Karlsruhe, Germany), Sigma-Aldrich (Sigma-Aldrich Chemie, Munich, Germany) in the quality pro Analysis (p.a.). For special chemicals and solutions, the manufacturer's name is indicated in the individual methods section.
Buffers were prepared with millipore water. Aqua ad iniectabilia (Baxter, Unterschleissheim. Germany) was used to take up DNA pellets and prepare small amounts of aqueous solutions.

2.1.2 Labware

All reusable labware was autoclaved before use. Plasticware was ordered from BD Biosciences (Heidelberg, Germany) or Eppendorf (Hamburg, Germany).

2.1.3 Kits

Blood & Cell Culture DNA Maxi-kit (Qiagen, Hilden, Germany)
DNeasy tissue kit (Qiagen)
QIAquick PCR purification kit (Qiagen)
QIAGEN Plasmid Mini kit (Quiagen)
QIAquick gel extraction kit (Quiagen)
Chromatin Immunoprecipitation (ChIP) Assay Kit (Upstate/Biomol, Hamburg, Germany)

2.1.4 Vectors

pCR®2.1-TOPO® vectors from the TOPO TA cloning® system (Invitrogen) were used.

2.1.5 Bactrial strains

One Shot® TOP10F' competent cells were used for transformations.

2.1.6 Oligonucleotides

Oligonucleotides used were designed with the Primer3 program except for primers for bisulfite sequencing, that were designed manually and ordered from MWG Biotech (Ebersberg, Germany). Stocks were stored as 100 mM solutions in H_2O at -20°C.

2.1.7 Antibodies

Antibody	Species	Company	Catalogue number
anti-acetylated-histone H3	rabbit polyclonal IgG	Upstate/Biomol	06-599
anti-FKBP51 (FKBP5)	goat polyclonal IgG	Santa Cruz Biotechnology,	sc-11518
anti-glucocorticoid receptor (Ab-2)	mouse monoclonal IgG	Oncogene (San Diego, CA, USA)	GR32L
anti-MeCP2 C-terminus	rabbit polyclonal IgG	Upstate/Biomol	07-013
anti-mSin3A	rabbit polyclonal IgG	Santa Cruz Biotechnology	sc-944
anti-SGK1	goat polyclonal IgG	Santa Cruz Biotechnology	sc-15885

Table 3. Primary antibodies used for ChIP and immunohistochemistry.

Antibody	Species	Company	Catalogue number
FITC-anti-rabbit	donkey	Dianova (Hamburg, Germany)	111-095-152
Cy3-anti-goat	donkey	Dianova	705-165-147

Table 4. Secondary antibodies used for immunohistochemistry.

2.1.8 Animals

For preparation of histological sections, C57BL/6 strains mice were used. Primary neurons, RNA and genomic DNA from brain, was prepared from in house bred CD1 strain mice.

2.1.9 Cell culture

Cell and tissue culture flasks (γ-irradiated and cell culture treated to facilitate binding of the cells to the plastic surface) were ordered from TPP Biochrom (Berlin, Germany).

DPBS s/o Ca and Mg (BioWhittaker/Biozym, Hess Oldendorf, Germany) was used to wash cells.

Media	Supplier	Other material	Supplier
RPMI1640	Invitrogen	FBS	Biochrom
DMEM	BioWhittaker	P/S (penicillin/streptomycin)	Invitrogen
DMEM:F12	BioWhittaker	L-glutamine	BioWhittaker
		Fungizone	Invitrogen
		NON-essential amino acids	Invitrogen
		Trysin	Invitrogen
		Poly-L-lysine 0.1% in H_2O	Sigma
		Collagen S Type I	Roche
		DPBS s/o Ca and Mg	BioWhittaker
		B27 with antioxidants	Gibco/Invitrogen

Table 5. Media and other cell culture components.

2.1.10 Cell lines and primary cells

Cell lines were ordered from DSMZ (Braunschweig, Germany) and cultured according the manufacturer's instructions. Primary mouse cell lines were established from CD1 animals, the human lymphoblastoid cell line was established (in house) from a male control patient.

2.1.11 Bioinformatics databases and tools

Database name	Database type	Internet address
Pfam	Protein families	http://www.sanger.ac.uk/Pfam
Smart	Protein families	http://smart.embl-heidelberg.de
Prosite	Protein families and domains	http://www.expasy.org/prosite
BLink	Precalculated Blast searches	http://www.ncbi.nlm.nih.gov
SSDB	Sequence similarity database	http://ssdb.genome.jp
Celera	Sequence retrieval and analysis	http://www.celera.com
Ensembl	Sequence retrieveal and analyis	http://www.ensembl.org
Unigene	Gene-oriented sequence clusters	http://www.ncbi.nlm.nih.gov/UniGene
CDD	Conserved domain database	http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml
Expasy	Protein sequences and analysis tools	http://www.expasy.org
PDB	Biological macromolecular 3-D structures	http://www.pdb.org
HUGE	KIAA gene database	http://www.kazusa.or.jp/huge
HUGE PPI	KIAA protein interaction database	http://www.kazusa.or.jp/huge/ppi
Pubmed	Medline biomedical articles	http://www.pubmed.org
Transfac	Transcription factor binding sites	http://www.biobase.de
RettBASE	Mutation database for MECP2	http://www.mecp2.org.uk

Table 6. Databases used in the different projects of this thesis.

Tool name	Internet address	Used in this thesis for
Ensembl Blast	http://www.ensembl.org/Multi/blastview	Genomic blasts / batch blasts
NCBI Blast	http://www.ncbi.nlm.nih.gov/BLAST	Masked protein blast
NCBI HomoloGene	http://www.ncbi.nlm.nih.gov/HomoloGene	Homologous genes
EBI Blast (TBLASTX)	http://www.ebi.ac.uk/Blast2/index.html	Translated nt query blasted against translated nt database
ClustalW	http://clustalw.genome.jp/	Sequence alignments
ClustalX Windows version	ftp://ftp-igbmc.u-strasbg.fr/pub/ClustalX	Graphical representation of alignments
Plogo	http://www.cbs.dtu.dk/~gorodkin/appl/plogo.html	Creation and display of logos
LALIGN	http://www.ch.embnet.org/software/LALIGN_form.html	Calculation of identity scores
MATCH	http://www.biobase.de	Find transcription factor binding sites
GCG package	http://www.accelrys.com/products/gcg_wisconsin_package	Sequence comparison and
Primer3	http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi	Primer design

Table 7. Bioinformatics tools used in this thesis.

2.2 Methods

2.2.1 Bioinformatics methods

2.2.1.1 Database screens

MBD protein family:

Using "MBD" or the MBD of human MECP2 (PDB ID: 1QK9) as query, the NCBI (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Protein), Pfam (release 7), Prosite (release 17.17) and Smart (version 3.4) databases were screened for proteins with this domain. The resulting polypeptides were sorted according to their species of origin and additional identified domains within their sequence. SSDB at GenomeNet was searched with pfm:MBD as query motif.

MECP2 paralogues:

SSDB at GenomeNet was searched for paralogues with hsa:4204 (the accession number of MECP2 in GenomeNet) as query and a threshold of 200 to get only the most specific candidates. The resulting data was exported as a list and GenomeNet accession numbers were

converted into NCBI accession numbers to be comparable to the BLink output list.

For P51608 (the accession number of MECP2 in the Entrez protein database) the pre-calculated blast hits form the BLink database were sorted by taxonomy proximity and score cut-off of 150 was chosen. All entries for *homo sapiens* were exported as a list for further analysis with Perl scripts.

Mecp2 binding sites:

The genomic region of *FKBP5* and *Fkbp5* were compared in the Ensembl database using compara tool in the Contig View mode. Furthermore CpG islands, CpG content and Eponine regions were mapped to the *Fkbp5* locus.

The MATCH tool at the Transfac database to find all matches for Transfac matrices in the genomic region of *Fkbp5*. The results were compared to the findings in the Ensembl database and resulted in two regions that were considered as possible promoters and covered with primer pairs for PCR fragments of ~200 bp.

Glucocorticoid receptor response elements in the genomic region of FKBP5 were found by mapping the Transfac matrix for glucocorticoid binding sites in man, mouse and rat (Transfac accession number: M00921) to the genomic region of FKBP5. For the resulting 6 regions, primers were designed and binding of GR was confirmed by ChIP.

2.2.1.2 Blast searches

MBD protein family:

Non-redundant GenBank, high throughput genomic sequences and expressed sequence tag (EST) databases were searched using BLASTP (expect=10, word size=3, matix=BLOSUM62, gap costs=existence11, extension1) and TBLASTN (expect=10, word size=3, matix=BLOSUM62, gap costs=existence11, extension1) of the NCBI web tools.

The sequence of the human MECP2 MBD (chain A, NCBI accession number: 1QK9A) was used as a query input.

TBLASTX (matrix=default, DNA strand=both, exp.thr=default, filter=none, view filter=none, sensitivity=high) was carried out applying the web tools at the European Bioinformatics Institute (EBI).

BLASTP and BLASTN searches were carried out against the Celera database with settings corresponding to the ones used in the NCBI blast searches.

KIAA1887 was detected as an MBD protein due to a blast search with MBD of KIAA1461

(amino acids 11-81) as query for a BLASTP at NCBI using the parameters specified above.

MECP2 paralogues:

To find repetetive regions to be masked in the BLAST search, the sequence of MECP2 was compared to itself using the Compare program of the GCG Wisconsin package (Version 10.3-UNIX) (comparison window size=25, stringency=9) to calculate a matrix of points of similarity and the DotPlot program to visualize the output.

Of the MECP2 protein sequence (NCBI accession number: P51608) the region aa20 - aa90, aa160 - aa200 and aa340 - aa400 were masked in a BLASTP (standard settings as defined above) at NCBI by using the "mask lower case"-option.

2.2.1.3 Sequence comparison

MBD protein family:

Alignments and phylogenetic trees were computed using the ClustalW program at GenomeNet with standard settings and the ClustalX program (version 1.8) (Thompson *et al.*, 1997) for graphical representation. The sequence logo was constructed by means of the plogo script. File formats were converted with the GCG Wisconsin package when necessary. Sequence comparison between mouse and man was carried out using the LALIGN algorithm at EMBNET with default settings. Homologues of *KIAA1461, CLLL8* and *KIAA1887* were identified using the ortholog prediction in the Ensembl database.

Mecp2 target genes:

Sequence analysis was done using the reformat and pileup functions of the GCG Wisconsin package to get multiple sequence alignments that could be screened for the presence of cytosines not modified by the bisulfite treatment.

2.2.1.4 Perl scripts

Several small scripts were written using the script language Perl (version 5.8.0). File formats were converted to be suitable as input files for GCG. Accession numbers of GenomeNet derived from the SSDB searches for MECP2 paralogues were transformed into NCBI-GI accession numbers. With the help of a Perl script, sequences files formats of bisulfite treated and cloned DNA fragments were sorted and converted to be suitable as input files for the GCG PileUp program.

2.2.2 cDNA microarrays

2.2.2.1 Microarray production

Murine cDNA clones (n = 10,080; arrayTAG clone collection) were purchased from LION Bioscience (Heidelberg, Germany). These cDNA clones map to the 3´ end of the respective transcript and are sequence-verified and sequence-specific (i.e. do not contain poly-A tails or large repeats). An additional 3510 murine cDNA clones, representing brain-expressed transcripts were obtained from the resource center of the German Human Genome Project (RZPD, Berlin, Germany) and 3 murine cDNA sequences were amplified with specific primers. As negative controls 17 plant cDNA sequences, amplified with specific primers, were included as duplicates resulting in 34 plant derived cDNAs on the chip. This resulted in a set of 13,627 clones that were used for the array and could be kept as plasmid inserts, stored as *E. coli* glycerol stocks.

For the array production, clone inserts were amplified by PCR (Tables 8, 9). Primers Lion 3' and Lion 5' were used for clones from LION bioscience, and primers M13for and M13rev were used for RZPD clones, as well as for the 3 murine and the plant clones. All PCR products were evaluated by agarose gel electrophoresis (see section 2.2.4.5). These steps were automated with a robot (Tecan Genesis Workstation 200, Crailsheim, Germany) in combination with a temperature controlled hotel (automatic incubator cytomat 6002, Hereaus, Hanau, Germany).

2 µl of the bacterial template clones stored in 96-well microtiter plates were mixed with 50 µl PCR master mix in a new 96-well microtiter plate.

Primer name	Primer sequence	Annealing temperature	Annealing time
Lion 3'	5'-tcgagcggccgcccgggcaggt-3'	68 °C	30 sec
Lion 5'	5'-agcgtggtcgcggccgaggt-3'	68 °C	30 sec
M13for	5'-gtaaaacgacggccag-3'	54 °C	90 sec
M13rev	5'-caggaaacagctatgac-3'	54 °C	90 sec

Table 8. Pimer pairs used for the amplification of plasmid inserts for the array production.

LION Bioscience clones			RZPD and plant clones			
temperature	**time**	**cycle numbers**	**temperature**	**time**	**cycle numbers**	**PCR master mix (total volume = 50 µl)**
94°C	3 min	1	94°C	5 min	1	5 µl 10 x Perkin Elmer PCR buffer
94°C	30 sec		94°C	45 sec		10 µl dNTPs (1 mM each)
68°C	30 sec	35	54°C	90 sec	35	2 U MPI Taq (made in house) (10 U/µl)
72°C	50 sec		72°C	2 min		2 µl forward primer (10 µM)
72°C	10 min	1	72°C	10 min	1	2 µl reverse primer (10 µM)
4°C	infinite	1	4°C	infinite	1	29 µl nuclease free water

Table 9. PCR conditions used for the amplification of cDNAs for the microarray.

Finished plates were stored in the temperature controlled hotel until use for PCR amplification in a PrimusHT multiblock thermal cycler (MWG Biotech) or alternatively were frozen at -20° C for later amplification.

The same robot could be used to mix 5 µl of PCR product from readily amplified plates with 5 µl of gel-loading buffer in a new 96-well microtiter plate. This mixture was analyzed on agarose gels using the Roboseq 4204S system (MWG Biotech) for gel loading.

45 µl (remaining after the agarose gel analysis) of each PCR product were combined with 2.5 volumes EtOH and 0.1 volumes sodium acetate 3 M pH=5. DNA was precipitated by incubation at -80°C for 30 min, and centrifugation at 20,000g for 15 min at 4°C. The pellet was resuspended in 18 µl 3 x SSC and 8 µl of each product were transferred to a well in a 384-well plate with a Multimek 96/384 robot (Beckman Coulter, Krefeld, German). The resulting 384-well plates were stored at -20°C until further use.

PCR products purified and processed in such a manner were printed on Corning GAPS II slides by using a robotic spotting device (SDDC-2 MicroArrayer, ESI, Toronto, Canada/ChipWriter Pro, Bio-Rad, Munich, Germany) with SMP3 pins from TeleChem International (Sunnyvale, CA).

2.2.2.2 Microarray hybridization and washing

Total RNA from the brain of a 74 day old *Mecp2*$^{-/y}$ animal with late symptoms (gait ataxia, hind limb clasping, breathing irregularities, uneven teeth, small stature) and a *wt* male litter mate was sent by our collaborators in Edinburgh. Labeled target cDNA was generated by direct incorporation of fluorescent nucleotide analogs in a reverse transcription reaction. Total

RNA (75 µg of each sample) was used in an oligo dT primed reaction in the presence of 100 µM Cy3- or Cy5-dUTP (Amersham Pharmacia Biotech Europe GmbH, Freiburg, Germany), 200 µM dTTP and 500 µM dATP, dCTP, and dGTP. The labeled cDNA was purified using the Qiaquick PCR purification kit. Labeled cDNA targets were resuspended in hybridization solution (50% formamide, 6 x SSC, 0.5% SDS, 5 x Denhardt´s solution), to which 1 µl poly-dA (10 µg/µl) and 1 µl mouse Cot-1 DNA (20 µg/µl) were added. The resulting reaction solution was concentrated to 35 µl, denatured at 95°C for 3 min and snap-cooled on ice. Hybridization took place under a coverslip at 42°C for 16 hours. The slides were washed at room temperature in 0.2 x SSC and 0.01% SDS for 5 min, followed by two washing steps in 0.2 x SSC for 5 min each. In total, two co-hybridizations (*Mecp2*$^{-/y}$ versus *wt* brain cDNAs) were performed with dyes swapped in the repeat experiment.

2.2.2.3 Image aquisition and data analysis

Fluorescence intensities of Cy3 and Cy5 were measured separately at 532 and 635 nm with a laser scanner (Affymetrix 428 Array Scanner, Affymetrix). The resulting 16-bit data files were imported into Microarray Suite image analysis software (version 2.0), which runs as an extension of IPLab Spectrum software (Scanalytics, Fairfax, VA). Raw spot intensities of Cy3 and Cy5 were locally background subtracted by the MicroArray Suite software. Empty spots and spots carrying plant gene sequences were excluded from further analysis. Each dye swap experiment was normalized by applying variance stabilization (Huber *et al.*, Bioinformatics 2002) using the vsn package of bioconductor (http://www.bioconductor.org). Means of normalized log-products and log-ratios of each dye swap experiment pair were used for further analysis. Normalization procedures were performed using R (http://cran.R-project.org). The data sets of two co-hybridization experiments with dye swap were first quality checked for pin- and PCR plate-specific effects by local regression according to each pin or PCR plate. Furthermore, background intensities across the slide were checked for homogeneity. Spots with local contaminations were excluded from further analysis. Local background-subtracted raw intensities on a logarithmic scale were normalized by Analysis of variance between groups (ANOVA) using the MATLAB code, with slight modifications. The underlying ANOVA model we applied was:

$$\log(y_{ijkg}) = \mu + A_i + D_j + V_k + G_g + (AG)_{ig} + (VG)_{kg} + \varepsilon_{ijkg}$$

$$i=1,2;\ j=1,2;\ k=1,2;\ g=1,\dots,13627;$$

(where y is the log intensity of each channel, μ is the overall mean, A is the overall array effect, D is the overall dye effect, V is the overall variety effect, G is the overall gene effect, AG describes array gene interactions and VG describes variety-gene interactions). Genes were then sorted according to their variety-gene differences ((V G)k=sample, g - (V G)k=control , g), which is an estimate of the log ratio of the sample versus control normalized intensities. Clones showing more than 2-fold changes were considered relevant. All data analyses were performed using MATLAB, Version 6.0.0.88, Release 12, MathWorks Inc., MA, USA.

2.2.3 Cell culture

2.2.3.1 Coating of culture flasks

Poly-L-lysine 0.1% in H_2O was diluted 1:10 with sterile H_2O. This solution was applied to cell culture flasks and incubated at room temperature for 5min. After three washes with sterile H_2O, flasks were left to dry under sterile conditions. Poly-L-lysine coated flasks were incubated with collagen S Type I solution diluted 1:100 in Neurobasal medium for 2 h at 37°C followed by 3 washes with DBPS, just before use.

2.2.3.2 Primary fibroblasts

Primary fibroblasts were prepared from adult CD1 mice (in house breeding facility): Dermis from dorsal skin was dissected, washed in sterile DPBS s/o Ca and Mg several times, placed in trypsin and chopped into pieces of 1-2 mm diameter. The pieces were placed in a 25 cm^2 cell culture flask and left to attach for three to four hours. Once the cells had attached to the flask, 5 ml DMEM:F12 medium 1:1 (BioWittacker) supplemented with 20% FBS (Biochrom, Berlin, Germany), P/S (100 U/ml, 10 μg/ml respectively) (Invitrogen), L-glutamine (2 mM) (BioWhittaker) and Fungizone (2.5 μg/ml) (Invitrogen) were added carefully. Cells were grown for 1 week to a confluency of 95% before passaging. For passaging, medium was removed, cells were washed with sterile DPBS s/o Ca and Mg twice and separated by incubation in 2 ml trypsin (Invitrogen) for 5 min at 37°C. The trypsin solution containing the cells was mixed with 13 ml fresh medium and plated in a 75 cm^2 flask. This step was repeated after 5 days to get a 150 cm^2 cell culture flask with primary fibroblasts. Once the cells had reached 95% confluence, they were harvested for DNA isolation.

2.2.3.3 Lymphoblastoid cell line

Eppstein-BarrVirus (EBV) transformed primary lymphoblastoid cells were grown as suspension cultures in RPMI1640 medium supplemented with 10% FBS, P/S (100 U/ml, 10 µg/ml), and L-glutamine (2 mM) and passaged 1:2 every 3 days..

2.2.3.4 Primary neurons

Heads of mouse embryos from a litter between embryonic days 16 to 18 were carefully prepared and placed in DPBS s/o Ca and Mg + 6 g/l glucose (Sigma). After cutting off the snout, brains were squeezed out and placed on ice in DPBS s/o Ca and Mg with 10% FBS and 6 g/l glucose until further use. Single cells were isolated mechanically by pipetting, fragmented with a cell strainer (40 µm; BD Falcon, Heidelberg, Germany), and centrifuged at 700 rpm for 1 min with slow acceleration and brake. The cell pellet was resuspended in 2ml medium (see below). The cell concentration was determined with a CASY1 Cell Counter (Schärfe System, Reutlingen, Germany). 1.5 x 10^7 cells were plated per 150 cm^2 cell culture flask coated with collagen S Type I and poly-L-lysine. The medium was changed every 3-4 days. After 7-10 days, cells were harvested for ChIP or treated with glucocorticoids for ChIP.
For 500 ml medium, 485 ml Neurobasal Medium (Invitrogen), 10 ml B27 with antioxidants (Gibco; mit Antioxidantien), 5 ml P/S (100 U/ml, 10 µg/ml respectively), (Invitrogen) and 1.25 ml L-glutamin (2 mM) were used.

2.2.3.5 Glucocorticoid treatment of primary neurons

Stock solutions of dexamethasone (Sigma-Aldrich) 10 mM in EtOH and RU-486 (Mifepristone, Sigma-Aldrich) 100 mM in EtOH were prepared. Twenty hours before harvest, respective amounts of the stock solutions (Table 10) were added to the culture medium of primary neuron cultures. EtOH was added to negative control cultures to consider potential effects of EtOH on the primary neurons.

Culture	Dexamethasone (10mM in EtOH)	RU-486 (100mM in EtOH)	EtOH 100%
EtOH	-	-	2.5 µl
Ru	-	1.25 µl	1.25 µl
RU, Dex	1.25 µl	1.25 µl	-
Dex	1.25 µl	-	1.25 µl

Table 10. Scheme depicting the amounts and concentrations of chemicals used for glucocorticoid treatment experiments.

2.2.3.6 CCRF-CEM

CCRF-CEM cells were grown in RPMI1640 medium supplemented with 10% FBS, P/S (100 U/ml, 10 µg/ml), L-glutamine (2 mM) and Fungizone (2.5 µg/ml), according to manufacturer's instructions. Saturated cultures were split 1:2 every 2-4 days by detaching cells in with trypsin and reseeding in new cell culture flasks with fresh medium as described for primary fibroblasts. CCRF-CEM cells were harvested at 95% confluence for RNA isolation.

2.2.3.7 NEURO-2A

NEURO-2A cells were grown according to manufacturer's instructions in DMEM medium with 10% FBS, P/S (100 U/ml, 10 µg/ml), L-glutamine (2 mM), 1% NON-essential amino acids (NEAA) and Fungizone (2.5 µg/ml). Cells were grown to 85% confluence and split 1:4 once a week as described for primary fibroblasts. Cells were harvested at 85% for DNA isolation and ChIP.

2.2.4 Molecular biology techniques

2.2.4.1 DNA isolation

General:

Genomic DNA from CCRF-CEM cells and primary fibroblasts was isolated with the Qiagen Blood Maxi-KIT following the manufacturer´s instructions.

Genomic DNA from CD1 mouse brains was isolated DNeasy tissue kit following the manufacturer´s instructions.

Mecp2 binding sites:

After ChIP, DNA was isolated using the phenol/chloroform extraction followed by

precipitation of the DNA. DNA containing solutions were mixed 1 volume of phenol:chloroform 1:1, carefully inverted for 5 min, and centrifuged for 10 min at 4000rpm in a table centrifuge. The upper aqueous phase was transferred to a new tube, mixed with 1 volume of chloroform, carefully mixed for 5 min and centrifuged for 10 min at 4000rpm. The upper phase was then transferred to a new tube and combined with 2.5 volumes of ice cold EtOH, 1/10 volume sodium acetate and 1 µl glycogen. After inversion of the tube, the solution was cooled down for 30 min at -80°C (alternatively overnight at -20°C) and then centrifuged for 45 min at 4°C. The resulting DNA pellet was washed with 70% EtOH (in water) briefly and centrifuged for 45 min at 4°C. The supernatant was discarded and the pellet dried in an concentrator 5301 speedvac (Eppendorf) at 37°C after puncturing the lid of the tube with a needle (to prevent contaminations as far as possible). The dried pellet was redissolved in 50 µl ultra pure H_2O and subjected to SR1 and SR2 amplifications (see section 2.2.4.8.)

2.2.4.2 RNA isolation

Total RNA from CCRF-CEM cells was isolated using the Trizol reagent (Invitrogen). All plastic- and glassware used was rinsed with water containing 0.05% DEPC and autoclaved before use to inactivate RNAses. The cell pellet was dissolved carefully in 10 ml Trizol, transferred to and 30 ml tube and incubated at RT for 5 min 2 ml chloroform were added and the solution was mixed vigorously for 15 sec, incubated at RT for 2-3 min followed by a centrifugation step at 4°C and 5000rpm for 20 min. The supernatant was transferred to a new tube and well mixed with 5 ml isopropanol. After incubation at RT for 5 - 10 min the solution was centrifuged at 4°C and 10000rpm for 10 min. The supernatant was discarded and the pellet washed with 70% EtOH (prepared with RNAse-free water) followed by a centrifugation at 4°C at 10000rpm for 5 min. The resulting pellet was dried and dissolved in 500 µl RNAse-free water. RNA concentration was measured using a Ultrospec 3100pro UV/VIS photometer (Amersham) and RNA quality was checked on an 1% agarose gel.

2.2.4.3 Standard PCR

Standard PCR was performed in an GeneAmp PCR System 9700 cycler (Applied-Biosciences, Darmstadt, Germany) with Perkin-Elmer Taq polymerase (Applied-Biosystems). If not indicated otherwise, the following PCR conditions were used:

Step	Temperature	Time	Cycles	PCR mix
Initial denaturation	94°C	5 min	1	2 µl template
Denaturation	94°C	45 sec		5 µl 10x reaction buffer
Annealing	Primer-specific	1 min	35	1 µl dNTPs (10 mM each)
Elongation	72°C	1 min		1 µl forward primer (1 µM)
Final elongation	72°C	7 min	1	1 µl reverse primer (1 µM)
Storage	4°C	infinite	1	0.5 µl PE-Taq polymerase
				39.5 µl H_2O

Table 11. Standard PCR temperature profile and PCR mix.

Touchdown PCR was applied for products that proved difficult to amplify:

Step	Temperature	Time	Cycles	PCR mix
Initial denaturation	94°C	5 min	1	2 µl template
Denaturation	94°C	45 sec		5 µl 10x reaction buffer
Annealing	(T_A + 10°C)	1 min		1 µl dNTPs (10 mM each)
	- 1°C/cycle		10	
Elongation	72°C	1 min		1 µl forward primer (1 µM)
				1 µl reverse primer (1 µM)
Denaturation	94°C	45 sec		0.5 µl PE-Taq polymerase
Annealing	Primer-specific	1 min	30	39.5 µl H_2O
Elongation	72°C	1 min		
Final elongation	72°C	7 min	1	
Storage	4°C	infinite	1	

Table 12. Touchdown PCR temperature profile and PCR mix. T_A is the primer specific annealing temperature.

2.2.4.4 cDNA synthesis and subsequent gene-specific PCR (RT-PCR)

RNA (20 µg) isolated from total mouse brain, CCRF-CEM cells or lymphoblastoid cells using the Trizol reagent (Invitrogen) (see section 2.4.2) was subjected to a reverse transcriptase reaction in the presence of 25 ng/ml oligo(dT) and 2.5 mM dA/C/G/TTP with 10 U/ml SSII reverse transcriptase and without enzyme in negative control reactions. The RNA in 17 µl RNAse-free water was mixed with the oligo-dTs and the dNTPs to a final volume of 23 µl and incubated at 65°C for 5 min followed by 5 min on ice. After addition of 8 µl 5 x first-strand Buffer, 4 µl 0.1 M DDT , 1 µl RNAsin, and 4 µl SSII reverse transcriptase (200 U/µl), the mixture was incubated for 55 min at 42°C and for 10 min at 70°C.

2 µl of the mixture were used in gene-specific PCR reactions or alternatively, the resulting cDNA was purified with the QIAquick PCR purification kit and 200ng of the purified cDNA were subject to gene-specific PCRs.

Primer name	**Forward primer**	**Reverse primer**	**Annealing temperature**	**Annealing time**
mFkbp5_exon1_exp_for	5'-gcggcgacaggtcttcta-3'	5'-gctttgataacctggccttg-3'	56°C	1 min
KIAA1887_exon_6	5'-cagaccccctactgtatttc-3'	5'-caaaaggttaaagcttccat-3'	49°C	1 min
KIAA1461	5'-ctagaccatgggaaaaatgt-3'	5'-acttggagactgctcctcta-3'	51°C	1 min
β-actin	5'-tgaaccctaaggccaaccgtg-3	5'-gctcatagctcttctccaggg-3'	57°C	45 sec

Table 13. Primers used for the amplification of gene specific PCR products from cDNA templates.
Standard PCR conditions were applied except for β-actin where the annealing time was only 45 sec.

2.2.4.5 Agarose gel electrophoresis

1% agarose gels were used to separate DNA or RNA samples by gel electrophoresis.
Gel composition: 1% w/v ultra pure agarose in TBE buffer (0.1 M Tris, 0.1 M boric acid, 2 mM EDTA). 0.5 µl/ml EtBr was added to the gels to make visualization of the DNA via UV-light possible. Before loading, samples were supplemented with at least 0.5 volumes of gel loading buffer composed of 0.25 % bromphenol blue, 0.25 % xylene cyanol FF, and 30 % glycerol. 2 different DNA size markers were used depending on the size of the fragment sizes expected: pUC mix 8 (MBI Fermentas, St. Leon-Rot, Germany) with a range from 110 bp - 1116 bp and Lambda DNA / EcoR1+HindIII 3 (MBI Fermentas) with a range from 564 bp to 21226 bp. Voltage (80 -150 V) and running times (15 - 45 min) depended on the expected fragment sizes. For visualization, the E.A.S.Y Win32 gel documentation system (Herolab, Wiesloch, Germany) was used.

2.2.4.6 Sequencing

For sequencing of PCR products, the fragments were purified by gel extraction using the QIAquick gel extraction kit. Using the TOPO TA cloning kit, PCR products were then ligated into a pCR2.1-TOPO vector. For transformation, 'One shot competent *E. coli* cells' were used according to the manufacturer´s instructions. *E. coli* cells were grown overnight at 37°C on selective plates (containing 50 µg/ml ampicillin) and supplemented with 50 µl 4% X-Gal and 50 µl IPTG (0.1M). White clones were picked and grown in 2 ml LB medium (Tryptone 10

g/l, NaCl 10 g/l, yeast extract 5 g/l) with 50 µg/ml ampicillin overnight. Plasmid DNA for sequencing was isolated using the Qiagen plasmid mini kit according to the manufacturer´s instructions. Per 100bp to be sequenced 10 ng DNA were required and subject to sequencing PCR with M13rev primers:

Step	Temperature	Time	Cycles	PCR mix
Initial denaturation	94°C	2 min	1	2 µl Dye Terminator (made in house)
Denaturation	94°C	20 sec		10 pmol primer
Annealing	54°C	15 sec	30	7 µl DNA solution
Elongation	60°C	4 min		

Table 14. Temperature profile and PCR mix for sequencing reactions with M13 primers. After the final elongation step, the PCR plates are stored on ice.

The PCR-reaction product was precipitated by addition of 25 µl EtOH. The solutions were mixed gently and the incubated in the dark for 10 min at RT. After a centrifugation step for 45 min at 4000 rpm at RT the obtained pellet was washed with 100 µl 70% EtOH, mixed gently and centrifuged for 30 min at 4000 rpm at RT. The supernatant was discarded, the pellet dried and the obtained fragments separated and analyzed on an ABI377 DNA sequencer.

2.2.4.7 Northern blotting

A cDNA fragment of KIAA1461 was PCR-amplified with exon specific primers KIAA1461 (see section 2.2.4.4) and human genomic DNA from mouse primary fibroblasts as template using standard conditions. For KIAA1887 a cDNA fragment was PCR-amplified with exon 6 specific primers KIAA1887_exon_6 (see section 2.2.4.4) and cDNA from a lymphoblastoid cell line as template using standard conditions. A cDNA fragment of β-actin amplified with primers β-actin (see section 2.2.4.4) was used as a loading control. These probes were radioactively labeled with 32P-dCTP in a random prime reaction and hybridized in ExpressHyb solution (CLONTECH, Palo Alto, CA, USA) to a human multiple tissue Northern blot (CLONTECH, Palo Alto, CA, USA) for 16 h at 65°C. Washing was performed in 2 × SSC / 0.1% SDS at 65°C for 10 min. Signals were detected with a PhosphorImager (Amersham Biosciences, Freiburg, Germany).

2.2.4.8 Chromatin immunoprecipitation

Chromatin immunoprecipitation was performed with NEURO-2A cells to establish the procedure and test primers pairs. Final experiments were carried out with brain tissue and primary neurons.

NEURO-2A cells and primary neurons (generally one 150cm^2 flask was used per condition or treatment) were crosslinked by addition of 1% formaldehyde (Roth) and incubation at 37°C for 10 min to the culture medium. Subsequent quenching was achieved by adding glycine (Merck) to a final concentration of 125 mM.

Frozen total brain tissue was pulverized with a mortar, taken up in 10 ml DMEM (BioWhittaker) with protease inhibitors (1 tablet Complete Mini Cocktail (Roche) per 10 ml solution as well as 2 mM PMSF) and incubated for 10 min at 37°C in the presence of 1% formaldehyde. Formaldehyde treatment was quenched by addition of glycine to a final concentration of 125 mM.

The Chromatin immunoprecipitation protocol was based on the Upstate Biotechnology ChIP kit protocol with the following modifications:

Sonication was carried out with a Branson Sonifier 450 (Branson, Danbury, CT, USA) was used at output control level 5 with 100% duty and pulses of 30 sec. The number of pulses depended on the cell type and the amount of chromatin in the sample. After sonication, DNA fragment sizes 5 µl of the sheared material was loaded on a 1% agarose gel to check the fragment sizes. If the smear on the gel was > 200 - 1000 bp, additional shearing was necessary.

DNA concentration in the sheared chromatin was determined with an Ultrospec 3100pro UV/VIS photometer (Amersham) and samples were diluted to 100 µg/ml DNA and split into portions of 1 ml in 1.5 ml eppendorf tubes. The tubes were either subjected to immunoprecipitation or stored at -20°C until further use.

The chromatin was then subjected to immunoprecipitation using antibodies specific to acetylated histone H3, mSin3A, the MeCP2 C-terminus or the DNA binding domain of GR according to the Upstate ChIP kit protocol. To reduce unspecific binding in the immunoprecipitation, the pre-clearing was performed with 75 µl (instead of 60 µl) Protein A agarose / Salmon sperm DNA and all washing steps described in the Upstate protocol were carried out twice. After reverse crosslinking, protein digestion, and DNA isolation, fragments were dissolved in 50 µl aqua ad iniectabilia and subjected to a two-step DOP-PCR. In the first

step 20 µl template were ligated to degenerated SR1 primers in a PCR reaction. In the SR2 PCR the fragments ligated to the SR1 primer were amplified using the SR2 primer.

	SR1	**SR2**
Primer	5´-gccgtcgacgaattcnnnnnnnnn-3´ (where n can be any base a, g, t or c)	5´-gccgtcgacgaattc-3´
Template	20 µl purified ChIP product	2 µl SR1 -PCR product
PCR mix	20 µl template 1 µl dNTPs (10 mM each) 5 µl 10x reaction buffer (Perkin-Elmer) 2 µl SR1-primer (10pMole/ul) 2 µl PE-Taq (Perkin-Elmer) 20 µl ultra pure H_2O	2 µl template 5 µl 10 x reaction buffer (Perkin-Elmer) 1 µl dNTPs (10 mM each) 2 µl primer SR2 2 µl PE-Taq (Perkin-Elmer) 38 µl ultra pure H_2O

Table 15. PCR mix for SR1 and SR2 reaction.

	SR1			**SR2**		
	SR1 temperature profile			SR2 temperature profile		
Step	Temperature	Time	Cycles	Temperature	Time	Cycles
1	94°C	5 min	1	94°C	5 min	1
2	94°C	30 sec		94°C	30 sec	
3	15°C	60 min		50°C	45 sec	60
4	20°C	45 min		72°C	1 min	
5	25°C	30 min		72°C	4 min	1
6	30°C	30 min		4°C	infinite	1
7	35°C	20 min	5			
8	40°C	1 min				
9	45°C	30 sec				
10	50°C	30 sec				
11	55°C	20 sec				
12	60°C	20 sec				
13	60°C	7 min	1			
14	4 °C	forever	1			

Table 16. Temperature profile for SR1 and SR2 PCR reaction.

2 µl of the DNA samples amplified by SR1 and SR2 reactions were subject to standard PCR amplifications with primers specific for binding sites of MECP2 and GR with the following annealing temperatures and cycle numbers.

Primer name	Forward primer	Reverse primer	Template	Annealing temperature
m1_0	5‘-tgctcccttagattcatcccacac-3‘	5‘-ccactggctccgatacacattctc-3‘	2 µl	55°C
m1_4	5‘-agtacccaacagaggtcaga-3‘	5‘-tgtggatacaaaatattcca-3‘	2 µl	49°C
m2_1	5‘-tcttggccttacctttaat-3‘	5‘-agttctcagggacttttcag-3‘	2 µl	49°C
m2_2	5‘-acacagaaacaataacaaaagc-3‘	5‘-tcaacaatatggctgtagga-3‘	2 µl	49°C
m2_4	5‘-gcacaatgctggactagata-3‘	5‘-tttaatgtgctcagtgcttc-3‘	2 µl	49°C
Fkp	5‘-agccacggtcctagatgagagc-3‘	5‘-gtgtgtgaaggagagtggcagaac-3‘	2 µl	60°C
Fkp1	5‘-tgctcccttagattcatcccacac-3‘	5‘-ccactggctccgatacacattctc-3‘	2 µl	60°C
GRE_2	5‘-tccaaagtccctatgtaccc-3‘	5‘-tggtccacaaacatacacg-3‘	2 µl	53°C

Table 17. Primer used to amplify DNA fragments precipitated by ChIP.

Three buffers, not included the Upstate ChIP kit, were prepared in the lab:

	Nuclei extraction buffer	Nuclei wash buffer	Re-suspension buffer
HEPES	10 mM	10 mM	
Sucrose	320 mM	320 mM	
$MgCl_2$	5 mM	5 mM	
Tris pH= 7.5			15 mM
EDTA			1 mM
NaCl			150 mM
Triton X-100	1%		0.5%
Millipore H_2O	in millipore H_2O pH=7.4 adjusted with NaOH	in millipore H_2O pH=7.4 adjusted with NaOH	in millipore H_2O

Table 18. Composition of the ChIP buffers not included in the Upstate ChIP Kit.

2.2.4.9 Bisulfite sequencing

Genomic DNA from brain was isolated using the DNeasy tissue kit (see section 2.2.4.1). Conversion of genomic DNA by sodium bisulfite was performed as described in the CpGenome DNA Modification Kit manual (Chemicon, Temecula, USA). Regions of interest were amplified from the bisulfite treated genomic DNA with specific primers. Amplification was performed using standard PCR conditions or touchdown PCR (TD) and fragments were cloned and sequenced.

Primer name	Forward primer	Reverse primer	Template
FKB5musP1_4bis	5'-aatacccaacaaaaatcaaaaaaaa-3'	5'-tgtggttataaaatattttataggtaat-3'	3 µl
FKB5_2_1meth_new	5'-cacacttctataaccttacctttaat-3'	5'-gtttttagggatttttaggtgtgtt-3'	3 µl
FKB5_2_2meth_new	5'-aaattaaaacacaaaaacaataacaaaaac-3'	5'-ttgttttttttaataatatggttgtagga-3'	3 µl
mFKB5_2_4_me4for/ LH1rev	5'-gtataatgttggattagataattagggaattag-3'	5'-tttaatatactcaatacttgtatcctattata-3'	3 µl
mFKB5_2_4_me5for/ LH2rev	5'-atttagtttagtataatgttggattagata-3'	5'-cctaatacctttaatatactcaatacttc-3'	3 µl

Table 19. Primers used for bisulfite sequencing of the regions 1_4, 2_1, 2_2, and 2_4. The touchdown PCR protocol was applied with an annealing temperature of 53°C and an annealing time of 1 min and 15 sec.