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Setdb1-mediated histone H3K9 hypermethylation in neurons worsens the neurological phenotype of *Mecp2*-deficient mice

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SUMMARY

Rett syndrome (RTT, OMIM # 312750), a neurodevelopmental disorder of early childhood, is primarily caused by mutations in the gene encoding methyl-CpG-binding protein 2 (*MECP2*). Various molecular functions have been ascribed to *MECP2*, including the regulation of histone modifications associated with repressive chromatin remodeling, but the role of these mechanism for the pathophysiology of RTT remains unclear. Here, we explore whether or not neuronal expression of the histone H3-lysine 9 specific methyltransferase, Setdb1 (Set domain, bifurcated 1)/Eset/Kmt1e, which is normally present only at low levels in differentiated neurons, rescues the RTT-like phenotype of *Mecp2* deficient mice. A myc-tagged *Setdb1* cDNA was expressed through the *tau* locus for ubiquitous expression in CNS neurons, or under control of the calcium/calmodulin-dependent protein kinase II (CK) promoter to selectively target postmitotic neurons in forebrain. However, the *CK-Setdb1* transgene lead to an enhanced neurological deficit, and the *tauSetdb1* allele further shortened life span of mice with a brain-wide deletion of *Mecp2* during prenatal development. In contrast, no neurological deficits or premature death was observed in *CK-Setdb1* and *tauSetdb1* mice expressing wildtype *Mecp2*. However, levels of trimethylated H3K9 at pericentromeric repeats were fully maintained in differentiated neurons from symptomatic *Mecp2* null mutant mice. Based on these results, we draw two conclusions: First, neuronal chromatin in RTT brain is not affected by a generalized deficit in H3K9 trimethylation. Second, artificial up-regulation of this repressive chromatin mark via *Setdb1* gene delivery specifically to neurons is harmful for the *Mecp2* deficient brain.

Keywords

epigenetic; neurodevelopment; histone methylation; nucleosome; major satellite; repeat DNA; heterochromatin

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INTRODUCTION

RTT (MIM 312750) is a neurodevelopmental disorder in which affected girls show developmental regression often after a 6–18 month symptom-free period, while boys typically do not survive beyond early postnatal life (Chahrour and Zoghbi, 2007). The majority of cases are due to mutations in the X-linked gene, methyl-CpG-binding protein 2 (*MECP2*). Presently, treatment options for RTT patients address only the symptoms (Percy, 2002), while effective prevention and cure will require a better understanding of the underlying molecular pathology. Important clues have been provided by studies on genetically engineered mice. A Rett-like phenotype is induced by conditional ablation of *Mecp2* in neurons (Chen et al., 2001; Guy et al., 2001) and rescued with pan-neuronal expression of a *Mecp2* cDNA (Luikenhuis et al., 2004). These findings underscore that RTT is a neuronal disease. Importantly, activation of *Mecp2* expression—even after mutant mice became symptomatic—resulted in a robust phenotypic reversal, which suggested that the neurological deficits in this disorder may not be irreversible (Guy et al., 2007). It remains to be determined, however, which of the various molecular actions ascribed to MeCP2—including remodeling of facultative or constitutive heterochromatin, as well as a more complex role in the regulation of gene transcription, mRNA splicing, and structuring of higher order chromatin (Chahrour et al., 2008; Chahrour and Zoghbi, 2007; Jordan et al., 2007; Kumar et al., 2008; Skene et al., 2010; Yasui et al., 2007)—are critical for the maintenance of neuronal health and the development of RTT.

Here, we test the hypothesis whether repressive chromatin modifications, specifically, tri-methylation of histone H3-lysine 9 (H3K9me3), could rescue the phenotype of *Mecp2* deficient mice. To investigate this, we engineered mice with neuron-specific expression of the H3K9 specific methyltransferase, SET domain, bifurcated 1 (Setdb1, also known as Eset, Kmt1e) (Schultz et al., 2002; Yang et al., 2002). Setdb1 contains, in addition to a SET and a Tudor domain, a methyl-CpG-binding domain (Blackburn et al., 2003; Schultz et al., 2002; Yang et al., 2002), and, like MeCP2 (Jones et al., 1998; Nan et al., 1998), interacts with the mSin3-histone deacetylase (HDAC) co-repressor complex (Yang et al., 2003). Although MeCP2, in contrast to Setdb1, has no methyltransferase activity, it regulates H3K9 methylation via its interaction with Suppressor of variegation 3–9 homolog 1 (Suv39H1) methyl-transferase (Lunyak et al., 2002). Therefore, both Setdb1 and MeCP2 are implicated in repressive chromatin remodeling, but potentially involve different protein complexes.

In the present study, we observed that expression of MeCP2 and Setdb1 undergo dynamic changes during brain development, but in a strikingly opposite manner. Thus, Setdb1 levels became down-regulated, while *Mecp2* was up-regulated in mature neurons, consistent with earlier reports (Akbarian et al., 2001; Balmer et al., 2003; Jung et al., 2003; Shahbazian et al., 2002b). Given that (as discussed above) both *Mecp2* and Setdb1 contain a mCpG binding domain and are involved in repressive chromatin remodeling, suggesting a possible overlap in function, we therefore asked whether a persistent up-regulation of Setdb1 could rescue *Mecp2* deficient mice that typically develop symptoms several weeks after birth (Chen et al., 2001; Guy et al., 2001; Shahbazian et al., 2002a). To address this question, we used two independent mouse lines to express Setdb1 in differentiated neurons: either by a transgene driven by the calcium/calmodulin-dependent protein kinase II (CK) promoter, or through the endogenous *tau* locus.

METHODS

Animals

All experimental procedures were approved by the Institutional Animal Care and Use Committee of the University of Massachusetts Medical School (UMASSMED). Animals

were housed in groups of 2–4 per cage with food and water ad libitum under 12-hour light/dark cycle.

Generation of *tauSetdb1* knock-in mice

The PGKNeo resistant selectable marker from plasmid pK-11 (Frt-PGKNeo-Frt-Loxp-pBSSK) (Meyers et al., 1998) was inserted into the modified pCS2-MT-ESET (a gift from Dr. Liu Yang, University of Arkansas for Medical Sciences) (Yang et al., 2003). Then, the *myc-Setdb1* cDNA was fused in-frame into the exon 2 of mouse *Mapt* (*tau*) locus, using pTau-KR plasmid containing 3.8 kb of *tau* genomic sequence (a gift from Dr. Rudolf Jaenisch, Whitehead Institute, Cambridge, MA) (Luikenhuis et al., 2004). For in-frame fusion, an adaptor (5'-ATCGATGGATATC-3') with *Clal* and *EcoRV* sites was introduced into pTau-KR, replacing a 14 bp fragment and ATG start codon of wildtype *tau* exon 2 (5'-ATGGCTGACCCTCG-3'). The targeting vector, pTau-MT-ESET, was linearized with *SacII* and electroporated into 129Sv-strain AB2.2 embryonic stem (ES) cell lines at the Transgenic Animal Modeling Core. Neomycin-resistant clones were first pre-screened by PCR for correct insertion and further assayed by Southern blot with internal and external probes (see Results). The 5' external probe was comprised of 447 bp fragment (subcloned from PCR product using wildtype AB2.2 ES cell DNA) located 2 kb upstream from the ATG codon of wildtype *tau* locus. The 3' external probe was comprised of a subcloned, 982 bp fragment from wildtype ES cell DNA, located 4.7 kb downstream from the ATG codon of wildtype *tau*. The internal probe was a 1247 bp *EcoRI/BamHI* fragment from the 5'-end of *Setdb1* cDNA. Suitable clones (63F1, 63D3, 63E12) were used to generate chimeras by injection into C57BL/6 blastocysts at the Transgenic Animal Modeling Core. Chimeras were mated to C57BL/6J females; germline transmission was achieved with clone #63D3. *tauSetdb1* was then crossed into the conditional *Mecp2* knockout for the rescue experiments.

Mecp2 deficient mice with neuron-specific *myc-Setdb1* expression

For the rescue experiments, only male mice were included in this study. *CK-Setdb1* transgene: To obtain *CK-Setdb1/Mecp2*^{-/-} rescue mice, *CK-Setdb1* transgenic males (Jiang et al., 2010) were bred with *Mecp2*^{2lox/2lox} females. Then, *CK-Setdb1*^{+/-}/*Mecp2*^{2lox/2lox} females were crossed with *Nestin-Cre*^{+/-} transgenic males, and the following 4 genotypes were obtained at the expected frequencies: (1.) *Mecp2*^{2lox/y} (for simplicity, termed “wildtype” in the following), (2.) *CK-Setdb1*^{+/-}/*Mecp2*^{2lox/y} (for simplicity, termed “*CK-Setdb1*” in the following), (3.) *Nestin-Cre*^{+/-}/*Mecp2*^{2lox/y} (for simplicity, termed “*Mecp2*^{-/-}” in the following), and (4.) *CK-Setdb1*^{+/-}/*Nestin-Cre*^{+/-}/*Mecp2*^{2lox/y} (for simplicity, termed “*CK-Setdb1/Mecp2*^{-/-}” in the following). After Cre-mediated ablation of *Mecp2* coding sequences in this line of mutant mice, MeCP2 immunoreactivity becomes non-detectable, as previously described (Chen et al., 2001) (Fig. 6).

tauSetdb1 knock-in allele

Previous studies found that *tau* knockout mice, or animals homozygous for various *tau* knock-in alleles, which effectively do not express wildtype tau protein, appear in good health and without overt neurological defects (Dawson et al., 2001; Denk and Wade-Martins, 2007; Tucker et al., 2001). Therefore, both heterozygous and homozygous *tauSetdb1* mice were included in this study. *TauSetdb1*^{+/-}/*Mecp2*^{2lox/2lox} females were crossed with *tauSetdb1*^{+/-}/*Nestin-Cre*^{+/-} males, and the following genotypes were obtained at the expected frequencies: (1.) *Mecp2*^{2lox/y} (for simplicity, termed “wildtype” in the following), (2.) *tauSetdb1/Mecp2*^{2lox/y} (for simplicity, “*tauSetdb1*” in the following refers to both hetero- and homozygous knock-in animals), (3.) *Nestin-Cre*^{+/-}/*Mecp2*^{2lox/y} (for simplicity, termed “*Mecp2*^{-/-}” in the following), and (4.) *tauSetdb1/Nestin-Cre*^{+/-}/*Mecp2*^{2lox/y} (for simplicity, termed “*tauSetdb1/Mecp2*^{-/-}” in the following).

Novelty-induced locomotion (ambulatory beam breaks)

Locomotion was monitored in a rectangular test chamber (19.1×29.2×12.7cm) with clean bedding, using a photocell beam based computer detecting system (San Diego Instruments). Mice were placed into the test chamber for 90 minutes under standard room lighting conditions. The beam breaks were collected every 5 minutes to evaluate the spontaneous locomotor activity.

Rotarod

Rota-Rod (UGO BASILE 47600) was used to measure motor coordination in mice. The rotarod was set up with a slow acceleration mode, in which the rotation speed is accelerated from 2 to 40 rpm in 5 minutes. During the training session, mice were subjected to 10 consecutive 5-min trials with a 5-min intertrial interval. The latency (sec) to falling off or, alternatively, making a full-turn rotation was recorded. 24 hours after training, mice were tested by receiving 3 consecutive trials (at 5 min intervals), and the mean latency was used to present the performance.

In situ hybridization

Entire heads (Embryonic day (E) 12.5), or whole brains (E15.5) were fixed with 4% phosphate-buffered paraformaldehyde (4% PFA) at 4°C overnight, then frozen or paraffin-embedded for subsequent *in situ* hybridization. 18 µm sections from frozen samples, or 4 µm from paraffin-embedded samples were hybridized to 50 ng/50 µl of DIG-11-UTP labeled sense or antisense *Setdb1* RNA probes at 60°C overnight, and then washed and digested with RNase A, and developed with the DIG Nuclei Acid detection kit (Roche, Indianapolis, IN), according to the manufacturer's instructions. The *Setdb1* probe was *in vitro* transcribed and was corresponding to a 347 bp fragment in 3'-end of *Setdb1* cDNA. Similar studies were conducted on brains sections prepared from perfusion-fixed adult animals.

Primary culture of mouse cortical neurons

Cerebral cortex was dissected from C57Bl6/J mice at E15.5 and dissociated by trypsin digestion and trituration as described (Lesuisse and Martin, 2002). Cells (1.5×10^6 cells/well) were plated in poly-L-lysine coated 6-well plates, cultured in Neurobasal medium (Life Technologies) supplemented with B27 (GIBCO, #17504-044), 200 mM L-glutamine, and 1% v/v penicillin and streptomycin (GIBCO, #15140). Antimitotic reagents, Uridine 5'-Triphosphate and 5-Fluoro-2-deoxy Uridine (10 mg/ml each) (Sigma) were added to limit glia proliferation. Cells were harvested after 2, 7, or 14 days cultured *in vitro* (DIV), and then prepared for Western blot or immunofluorescence staining.

Immunohistochemistry

Cultured cells were fixed with phosphate-buffered 4% PFA, permeabilized with 0.2% Triton X-100, blocked with 10% goat serum, and then incubated with primary antibody (anti-TuJ1, -GFAP) overnight at 4 °C. The signal was detected by Alexa 488- or 594-conjugated secondary antibodies. Nuclei isolated from tissue homogenates in hypotonic solution (see below) were fixed in phosphate-buffered 4% PFA for 5 min, spread on slides and airdried, and then stained for anti-MeCP2 (Sigma, mouse monoclonal, clone men-8, directed against the N-terminus of MeCP2) and anti-NeuN immunofluorescence (Chemicon).

Immunoblotting

Samples were homogenized directly in 1x Laemmli buffer with 1x Complete Proteinase Inhibitor (Roche Applied Science, #11697498001), incubated at 37°C for 10 min, and centrifuged at 13,500 g for 5 min at 4°C. The supernatant was denatured at 95°C for 5 min, electrophoresed on a 4%–20% linear gradient Tris-HCl gel (Bio-Rad, Hercules, California),

and then transferred to nitrocellulose membrane (Bio-Rad, 0.4 μ m pore size). Immunostainings were performed with anti-c-myc (A-14) (Santa Cruz, #sc-789), anti-Setdb1 antibody (Upstate, #07-378), and anti-Mecp2 (Upstate #07-013, rabbit polyclonal, against Mecp2 C-terminus) and, for loading controls, anti-GADPH (Abcam, #ab9485) and anti-histone H4 antibody (Upstate, #07-108). Immunoreactivity was detected with peroxidase-conjugated secondary antibody in conjunction with chemiluminescence-based film autoradiography (Super Signal West Dura Extend Reagent; Pierce, Rockford, Illinois). For quantification, Quantity-One (Biorad) software was used.

Real-time RT-PCR

Total RNA was extracted from cerebral cortex, striatum, or forebrain of mice brains using RNeasy Lipid Tissue Mini kit (QIAGEN, #74804) according to manufacturer's protocol, and then subjected to SYBR green based one-step RT-PCR using Taqman One-Step RT-PCR Mix Reagents (Roche, #4309169). Two different sets of primers were designed to amplify approx. 100 bp intron-spanning fragments in the 3'-end of *Setdb1* cDNA. The primers were, 18-2: left 5'-CTTCGCCGACCAGCTAGTAA-3', right 5'-GTTCCACTTTCCCCCTCACT-3'; 19-2: left 5'-CTTCTGGCTCTGACGGTGAT-3', right 5'-GGAAGCCATGTTGGTTGATT-3'. The *Bdnf* primers used were: left 5'-GCGCCCATGAAAGAAGTAAA-3', right 5'-TCGTCAGACCTCTCGAACCT-3'. Quantifications were done with 18S rRNA as an internal control (Schroeder et al., 2007).

FACS-sorting of nuclei

Nuclei were extracted from adult mouse forebrain, immunotagged with anti-NeuN, and sorted as described (Jiang et al., 2008). Using this procedure, background immunofluorescence was minimal, and all sorted samples had a purity of 95% (NeuN+ nuclei) or more.

Chromatin immunoprecipitation

Samples were prepared either by micrococcal nuclease (MNase) digestion for native chromatin immunoprecipitation (NChIP) as described (Huang et al., 2006), or fixed by 1% formalin, sheared by sonication (Branson Sonifier 250) for XChIP. *NChIP*: In brief, MNase digestion was performed with a working concentration of 4U/mL at 37°C for 5 min. The resulting mono-nucleosomal preparation (approx. 146 bp) was precleaned by incubating with protein G agarose (which should also remove the antibodies used for NeuN labeling of nuclei), and then subjected to immunoprecipitation using anti-trimethyl-H3K9 (H3K9me3) antibody (Upstate, #07-442). Typically, we used $6 - 8 \times 10^6$ FACS-sorted nuclei as input. *XChIP*: Samples were fixed in 1% formalin for 5 min at room temperature, sonicated at power level 6 (Branson, Danbury CT) in ice water (10 runs of 30 sec impulse with 1 min resting interval), pre-cleaned, and then subjected to H3K9me3 immunoprecipitation. Control samples included samples processed with normal rabbit IgG, in parallel to samples with specific antibodies. Input DNA and immunoprecipitated DNA were subjected to slot-blot hybridization using a 32p labeled oligonucleotide probe 5' GGACC TGGAA TATGGC GAGAA A 3' to label mouse major satellite DNA.

RESULTS

Developmental expression profile of Setdb1 is opposite to Mecp2

To examine whether Setdb1 is developmentally regulated in mouse brain, we monitored Setdb1 expression in embryonic, postnatal and adult cerebral cortex (Fig. 1A–C). Levels of Setdb1 protein (Fig. 1A, C) and mRNA (Fig. 1B) were highest at the earliest stage examined (E12.5), but then showed a progressive and significant decline, resulting in 8-fold lower

Setdb1 immunoreactivity in 8–12 week old (adult) cortex (Fig. 1C). Although Setdb1 was subject to developmental downregulation, the protein was readily detectable in immunoblots from mature brain (Fig. 1C). To confirm that Setdb1 is expressed throughout all regions of the developing brain, we conducted *in situ* hybridization on E12.5 and E15.5 brains. The *Setdb1* antisense probe resulted in robust labeling throughout the developing brain (Fig. 2A, B), including mitotic cells within proliferative layers, such as the ventricular zone, as well as postmitotic neurons residing in the cortical plate and other territories (Fig. 2B, C). In contrast, labeling of sections from adult brain was weak and often not distinguishable from background (data not shown).

To further test whether the observed downregulation of Setdb1 protein during development (Fig. 1A, C) occurs within differentiating neurons, we monitored the temporal pattern of Setdb1 expression in primary neuronal cultures derived from E15.5 mouse cortical plate (Fig. 1D). After 14 days of culture *in vitro*, Setdb1 immunoreactivity was significantly decreased by more than 2-fold (Fig. 1E). In contrast, during the same time period, levels of MeCP2 immunoreactivity showed a 14-fold, significant increase (Fig. 1F). The latter finding is in good agreement with earlier studies in rodent (Jung et al., 2003; Shahbazian et al., 2002b), monkey (Akbarian et al., 2001) and human brain (Shahbazian et al., 2002b) reporting that MeCP2 levels are up-regulated during the course of neuronal maturation. We conclude that expression of both Setdb1 and MeCP2 undergo dynamic changes in differentiating neurons of developing brain, though in a strikingly opposite manner. This observation, in view of the potential overlap in MeCP2 and Setdb1 function, led us to speculate that the RTT phenotype in adult *Mecp2* deficient mice could be ameliorated by artificial expression of Setdb1 in mature neurons.

CK-Setdb1 transgene further impairs the neurological phenotype of *Mecp2* deficient mice

The calcium/calmodulin-dependent protein kinase II (CK) promoter typically drives transgene expression in a substantial portion of neurons, primarily those located in the forebrain (Mayford et al., 1996). The CK promoter-regulated, conditional or inducible expression of MeCP2 transgene significantly delays the onset of neurological symptoms and the premature death of *Mecp2* deficient mice (Giacometti et al., 2007; Jugloff et al., 2008), although negative findings have also been reported (Alvarez-Saavedra et al., 2007). Therefore, we wanted to find out if CK-driven Setdb1 expression would affect the phenotype of *Mecp2* deficient mice. To this end, we generated *CK-Setdb1* transgenic mice, in which a myc-tagged full length *Setdb1* cDNA was under the control of the CK promoter. Significant increases of Setdb1 expression were detected in most regions of adult *CK-Setdb1* transgenic forebrain. This was associated with increased of H3K9 methyl-transferase activity, because there was a significant, approximately 60% increase in major satellite DNA-associated trimethyl-H3K9 (H3K9me3) in *CK-Setdb1* forebrain (Jiang et al., 2010). The increase in satellite repeat-associated H3K9 methylation was specific because global tissue levels of H3K9 methylation, when measured in brain homogenates by immunoblot, remained unchanged in *CK-Setdb1* forebrain (Jiang et al., 2010). The observation that changes in Setdb1 levels are not associated with altered H3K9 methylation overall is not too surprising, however, given that there are genome-wide at least 6 or 7 H3K9 specific methyltransferase enzymes (Kouzarides, 2007), and many of these are robustly expressed in adult brain (Lein et al., 2007).

Because the disease progression and neuronal alterations of *Mecp2* deficient mice are known to be significantly affected by levels of brain-derived neurotrophic factor (*Bdnf*) (Chang et al., 2006; Wang et al., 2006; Zhou et al., 2006), we examined if expression of this neurotrophin was altered in the forebrain of adult *CK-Setdb1* transgenic mice. Levels of *Bdnf* mRNA, when normalized to 18S rRNA transcript, were not significantly different between transgenic and littermate controls (mean $\pm$ S.E.M., wildtype: 5.69 ± 0.59 ; CK-

Setdb1: 5.37 ± 0.39 , $N = 6/\text{group}$). Next, we crossed the *CK-Setdb1* transgenic line with *Mecp2* conditional mutant mice for a Cre/loxP mediated deletion of the methyl-CpG binding domain (MBD) (Chen et al., 2001), which is encoded by exon 2 of *Mecp2* isoform 1, or exon 3 of isoform 2, respectively (Kriaucionis and Bird, 2004; Mnatzakanian et al., 2004). As expected (Chen et al., 2001), conditional, Nestin-Cre mediated deletion of *Mecp2* during prenatal brain development resulted in premature death beginning at 2 months of age, impaired motor coordination, and decreased locomotor activity (Fig. 3B–D). Furthermore, the *Mecp2* deficient mice, which are of a mixed genetic background that includes C57Bl6/J, were significantly underweight at 4 weeks of age (Fig. 3A), as previously reported for the mutants of that inbred line (Guy et al., 2001). At later ages, mutant weight became more variable (data not shown). Survival, motor activity and coordination, and body weight of *CK-Setdb1* transgenic mice expressing wildtype MeCP2 were not significantly different from controls (Fig. 3). However, the transgene did not rescue the decrease in body weight, nor did it prevent neurological symptoms and premature death of *Mecp2* mutant mice (Fig. 3). Instead, a pronounced deficit in novelty-induced locomotion was observed in *CK-Setdb1/Mecp2*^{−/y} mice, which showed a significant 40%, or 20% decrease in activity scores compared to wildtype controls, or *Mecp2*^{−/y} animals, respectively (Fig. 3B).

Expression of *Setdb1* through the tau locus results in accelerated premature death of *Mecp2* deficient mice

As described above, neuronal expression of *Setdb1* in adult forebrain does not rescue the Rett-like phenotype of *Mecp2* mutant mice. Of note, previous studies that utilized the CK promoter to rescue *Mecp2* deficient mice yielded variable results (Alvarez-Saavedra et al., 2007; Giacometti et al., 2007; Jugloff et al., 2008). In contrast, when *Mecp2* cDNA is expressed under control of the promoter of the *microtubule-binding protein tau*, complete rescue is achieved (Luikenhuis et al., 2004). This may be due to the fact that expression through the endogenous *tau* locus is pan-neuronal, starting during prenatal development and continues throughout all postnatal stages and adulthood (Luikenhuis et al., 2004). Another advantage of the *tau* locus is that mice not expressing wildtype tau protein appear in good health and without overt neurological defects (Dawson et al., 2001; Denk and Wade-Martins, 2007; Harada et al., 1994; Tucker et al., 2001) other than subtle defects in motor coordination and fear conditioning in some studies (Denk and Wade-Martins, 2007).

To express *Setdb1* through the endogenous *tau* locus, we fused the myc-tagged *Setdb1* cDNA first with a neomycin resistance cassette, and then ligated it into a *tau* targeting vector as previously described (Luikenhuis et al., 2004), replacing the ATG start codon of wildtype *tau* and surrounding sequences (Fig. 4A). The targeting construct was electroporated into AB2.2 embryonic stem cells. From 384 neomycin-resistant clones, 13 were targeted correctly, including clone #63D3 (Fig. 4B) that was passed through the germline to generate *tauSetdb1* mice. As expected, expression of 180 kDa myc-tagged *Setdb1* in these knock-in mice was neuron-specific and present in all brain regions examined (Fig. 4C–E). As mentioned above, double-banding of the 180kDa immunoreactivity was observed at shorter exposure times (Fig. 4E).

Next, we crossed the *tauSetdb1* knock-in with *Mecp2* conditional mutant mice. Similar to the experiments described above, the *Mecp2* deficient mice of this outbred colony also showed significant deficits in motor coordination assayed by rotarod (Fig. 5A) and premature death starting around 8 weeks of age (Fig. 5B). However, in this colony, the body weight of *Mecp2* mutant was not different from controls (<2%, data not shown). Furthermore, the survival and motor coordination of *tauSetdb1* knock-in mice expressing wildtype *Mecp2* were not significantly different from controls (Fig. 5A). However, the *tauSetdb1* allele conferred a strong trend (Kaplan-Meier, $p < 0.07$) for further acceleration of premature death in *Mecp2* mutant mice (Fig. 5B). This Rett-like phenotype was observed in

both hetero- and homozygous *tauSetdb1* knock-in mice (Fig. 6B legend and data not shown). Thus, neither the *CK-Setdb1* transgene nor the *tauSetdb1* knock-in allele ameliorated the symptoms induced by *Mecp2* deficiency.

Levels of trimethyl H3-lysine 9 in pericentromeric heterochromatin are maintained in neurons from *Mecp2* deficient brain

As mentioned, transgene-derived expression of *myc-Setdb1* cDNA resulted in H3K9 hypertri-methylation at pericentric repeats. Of note, MeCP2 is highly concentrated in pericentromeric heterochromatin (Lewis et al., 1992). In the mouse, pericentric heterochromatin is mainly defined by tandem repeats of A/T-rich major satellite repeats comprised of approximately 10^5 copies of a 234 base-pair unit (Waterston et al., 2002). Among the various repressive histone methylation marks, H3K9me3 is highly and consistently enriched at sites of major satellite repeats in different cell types (Martens et al., 2005). Importantly, immunoprecipitation studies revealed association of MeCP2 with Suppressor of variegation 3–9 homolog 1 (Suv39H1) (Lunyak et al., 2002), a histone methyl-transferase essential for H3K9 trimethylation at pericentric repeats (Peters et al., 2001). Together, these findings imply that MeCP2, bound to the methylated CpG's of the major satellite, regulates H3K9me3 at these repeats via an interaction with Suv39H1. Therefore, loss of MeCP2 could affect orderly methylation of H3K9 in chromatin surrounding major satellite repeats. However, until now this hypothesis has not been tested in *Mecp2* deficient brain. Importantly, MeCP2 is localized almost exclusively in mature neurons (Akbarian et al., 2001; Chen et al., 2001; Jung et al., 2003; LaSalle et al., 2001; Shahbazian et al., 2002b), which reside in brain together with glia and other cells mostly in a 2:1 – 1:2 ratio (Herculano-Houzel et al., 2006; Sherwood et al., 2006); therefore, a conclusive assessment of MeCP2 association with neuronal heterochromatin would require separation from non-neuronal elements. Here, we extracted neuronal nuclei from whole forebrain of 8 week old *Mecp2*^{-f/y} and male controls; at this stage, the *Mecp2* mutant mice already are symptomatic and show deficits in locomotor activity (Fig. 3B). The neuronal nuclei were sorted via FACS after immunotagging with anti-NeuN antibody (Fig. 6A, B), employing a previously described protocol (Jiang et al., 2008). Subsequently, neuronal nuclei were subjected to digestion with micrococcal nuclease and, after ChIP with anti-H3K9me3 antibody, the mono-nucleosomal DNA from immunoprecipitates, input, and no antibody control samples were blotted without prior amplification. Overall DNA yields of input samples were similar between mutant and control samples, indicating that the nucleosomal organization of the genome is grossly maintained in the absence of MeCP2 (data not shown). Furthermore, levels of H3K9me3 at major satellite repeats were completely preserved in neurons from *Mecp2* mutant brain, compared to wildtype controls (Fig. 6C). We conclude that MeCP2 is not essential for the establishment or maintenance of the H3K9me3 mark at major satellite DNA in neuronal nuclei.

DISCUSSION

Using genetically engineered mice, we demonstrate that persistent up-regulation of neuronal *Setdb1* expression in adult brain results in hypertrimethylation of H3K9 at pericentromeric repeat DNA, and worsens the phenotype of *Mecp2* deficient mice. Furthermore, we show that H3K9 trimethylation levels remain unaltered in pericentromeric chromatin of neuronal nuclei from symptomatic *Mecp2* deficient mice. Therefore, loss of *Mecp2* is not associated with a generalized deficit in H3K9 tri-methylation at DNA repeats in diseased neurons. These negative findings of the present study, however, do not rule out that other types of histone modifications show global alterations in the *Mecp2* deficient neurons. Indeed, a recent study reported that chromatin of mature *Mecp2*^{-/-} neurons is affected by a generalized increase in histone acetylation (Skene et al., 2010).

The finding reported here—normal levels of trimethylated H3K9 in pericentric heterochromatin of *Mecp2* deficient neurons—is in good agreement with recent findings that methylation of H3K9 and other histone residues are maintained at normal levels (or show only minimal changes) in bulk chromatin extracted from whole tissue of selected brain regions of *Mecp2* null mutant mice (Urdinguio et al., 2007) or peripheral blood cells of RTT patients (Kaufmann et al., 2005). On the other hand, a study utilizing quantitative immunofluorescence for H3K9me3 found a generalized decrease in *Mecp2* deficient neuronal nuclei, although the punctate staining of heterochromatic foci was preserved in the mutants (Thatcher and LaSalle, 2006). Likewise, no consistent changes in global histone acetylation were reported in *Mecp2/MECP2* deficient brain (Shahbazian et al., 2002a; Thatcher and LaSalle, 2006; Urdinguio et al., 2007), blood cells, or cell lines (Balmer et al., 2002; Kaufmann et al., 2005; Wan et al., 2001), and it remains possible that this variation may be in part due to differences in the genetic background and type of *Mecp2/MECP2* mutations, or even the specific histone molecules or residues examined.

In the present study, genetically engineered mice with a sustained up-regulation of *Setdb1* expression in adult brain displayed hyper-trimethylation of H3K9 in neuronal chromatin, yet did not develop an overt neurological phenotype. However, in the *Mecp2* mutant mice, the *CK-Setdb1* transgene resulted in a significant worsening of motor deficits (Fig. 3B), and the *tauSetdb1* allele conferred a strong trend ($P < 0.07$) towards further shortening of the survival time of these animals (Fig. 5B). These findings effectively rule out the hypothetical possibility that the artificial elevation of H3K9 methylation in our *Setdb1* overexpressing mice was not sufficiently high in order to compensate for the loss of MeCP2. Of note, *Setdb1* transgenes in mice expressing wildtype *Mecp2* appear target a select set of genes and appear to exert a therapeutic, antidepressant-like effect on mood-related behaviors without adverse consequences on the animals health (Jiang et al., 2010). These findings, taken together, imply that brains of mice with normal levels of MeCP2 are able to tolerate the artificial hypermethylation of H3K9, but the same manipulation applied to the *Mecp2* deficient brain then leads to additional dysfunction and further decompensation of the animals' RTT phenotypes. Presently, it remains unknown whether or not the *Setdb1* and *Mecp2* proteins functionally interact or co-localize in neuronal chromatin remodeling complexes. Our finding that neuronal overexpression of *Setdb1* is tolerated in an otherwise normal brain, but not in RTT, is of interest from the viewpoint of a related finding in Huntington's disease, a neurodegenerative disorder caused by polyglutamine repeats which is associated with transcriptional dysregulation (Butler and Bates, 2006). Interestingly, both *Setdb1* expression and levels of H3K9me3 are increased in Huntington's striatum (Lee et al., 2008; Ryu et al., 2006; Stack et al., 2007). These findings are largely of a correlative nature and while further investigations are required, the evidence thus far would suggest dysregulation of *Setdb1*-mediated histone methylation worsens neuronal disease in the Huntington's brain as it does in the *Mecp2* mutant mice of the present study, while a similar level of *Setdb1* activity in normal brain does not result in an overt phenotype.

To date, little is known about the molecular signaling pathways regulating *Setdb1* expression and function in brain. A number of chromatin-associated proteins that interact with *Setdb1*, including mSin3B (Yang et al., 2003), histone deacetylases (HDAC) 1 and 2 (Ng and Bird, 2000), and tripartite motif protein 28 (*Trim28*), also known as KAP-1 co-repressor (Schultz et al., 2002), are expressed at moderate or high levels in adult brain (Lein et al., 2007). Furthermore, gene deletion studies suggest that the histone acetyl-transferase, CREB-binding protein (CBP), inhibits *Setdb1* expression in striatal neurons by limiting Ets-2 transcription factor activity (Lee et al., 2008). However, the precise role of *Setdb1* in developing and mature brain, including its potential contribution to neurological disease, requires further investigation. Notably, in the *Setdb1* overexpressing mice of the present study, histone trimethylation at pericentric repeats was increased, which is in good

agreement with a recent report linking *CBP* deficiency in brain to increased expression of *Setdb1*, which was accompanied by increased H3K9me3 and expansion of pericentric heterochromatin (Lee et al., 2008). Therefore, at least in brain, *Setdb1* could be involved in the epigenetic regulation of pericentromeric satellites and other DNA repeats, in addition to its well described interaction with repressor and silencing complexes in euchromatin (Ayyanathan et al., 2003; Li et al., 2006; Lyst et al., 2006; Takada et al., 2007; Wang et al., 2007; Wang et al., 2003) which in mature neurons are likely to affect less than 1% of annotated genes (Jiang et al., 2010). Indeed, a role for *Setdb1* in the regulation of DNA repeat chromatin is supported by the observation that this methyl-transferase is recruited by heterochromatin protein HP-1 to mediate histone methylation in repetitive DNA in various mammalian cell lines (Verschure et al., 2005), and at pericentromeric repeats in murine embryonic stem cells lacking *Suv(3)9h1/2* histone methyl-transferase (HMTase) activity (Kourmouli et al., 2005). Given that *Suv(3)9h1* interacts with MeCP2 (Lunyak et al., 2002), one could speculate that neuronal overexpression of this particular methyl-transferase—in contrast to the *Setdb1* experiments presented here—could rescue the RTT phenotype of *Mecp2* mutant mice. However, because H3K9 methylation levels in the pericentromeric satellite repeats of mature neurons lacking *Mecp2* did not differ from wildtype controls, a more likely scenario is that neither *Suv(3)9h1/2* nor *Setdb1* or other H3K9-specific methyl-transferase transgenes prevent or ameliorate neuronal disease resulting from the loss of *Mecp2*.

However, it is likely that the *Setdb1* transgenes of the present study affected histone H3K9 methylation, in addition to pericentric heterochromatin and other repeats, also at several single copy genes. The small subset of annotated genes targeted by *Setdb1* in mature neurons includes the N-methyl-D-aspartate (NMDA) receptor subunit *Grin2b* and additional glutamate receptor genes (Jiang et al., 2010). Therefore, it remains possible that *Setdb1*-mediated histone H3K9 hypermethylation and repressive chromatin remodeling could result in decreased excitatory neurotransmission in the *Mecp2* deficient mice of the present study, thereby further potentiating the neuronal signaling defects in the animals (Dani et al., 2005; Nelson et al., 2008).

Of note, there is evidence that in some brain regions, including the hypothalamus, *Mecp2* both positively and negatively regulates a substantial number of mRNAs, suggesting that this protein may play a more complex role in transcriptional regulation than repression alone (Chahrour et al., 2008). Furthermore, a recent case diagnosed with the congenital variant of RTT was linked to a truncating mutation in *FOXG1* (Ariani et al., 2008), which encodes a brain-specific transcriptional factor that interacts with JARID1B (Roesch et al., 2008), a histone demethylase with specificity for the H3K4 residue (Xiang et al., 2007; Yamane et al., 2007). H3K4 methylation is commonly associated with open chromatin and active gene expression (Shilatifard, 2008). Thus, it remains possible that artificial manipulation of histone methylation marks other than H3K9 could profoundly modify the RTT phenotype of *Mecp2* mutant mice.

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Figure 1. *Setdb1* expression is down-regulated during the course of neuronal maturation

A, Representative immunoblots showing *Setdb1* (upper band), and for loading control, histone H4 (modification-independent) (lower band) immunoreactivity from E15.5 cortical plate (lanes 1, 2) and adult (postnatal week 8–12) cerebral cortex (lane 3). Band in lane 4 is same as in lane 3, shown again after a longer film exposure. 5 μ g (lane 1) or 20 μ g (lane 2, 3) of protein was loaded. **B**, Bar graphs showing relative *Setdb1* mRNA levels as determined by qRT-PCR in mouse cerebral cortex at different stages as indicated: embryonic day (E) 12.5, 15.5, 18.5, postnatal (P) 7, and adult (8 weeks and older). Data are expressed as mean $\pm$ SEM after normalization to 18S rRNA. N = 5/age group. **C**, Representative *Setdb1* immunoblots from cerebral cortex at different stages as indicated. Data are expressed as mean $\pm$ SEM after normalization to GADPH. N = 4/age group. * p <0.05, # p <0.01 (B, C) as compared to E 12.5, one-way ANOVA. Notice that both *Setdb1* mRNA and protein decline during the course of cortical maturation. **D–F**, Primary cultures of cortical neurons from E. 15.5 mouse cortical plate. **D**, Immunofluorescence of TuJ1 neuronal marker (red), GFAP (green) and DAPI (blue) at DIV 4. **E–F**, Representative blots (top) and bar graphs (bottom) showing *Setdb1* (E) and MeCP2 (F) protein levels at DIV 2, 7, and 14. Data are expressed as mean $\pm$ SEM after normalization to H4. Notice opposing patterns of expression over time, as recognized by decline in *Setdb1* and increase in MeCP2 expression. N = 4/age group. * p <0.05, # p <0.01 as compared to DIV 2, one-way ANOVA.

Figure 2. Widespread *Setdb1* expression in the developing mouse brain

Representative images showing *Setdb1* mRNA expression in coronal sections from frozen (A, B) or paraffin-embedded (C) tissue blocks at E12.5 (A) or E15.5 (B, C). Sections were hybridized with digoxigenin-labeled anti-sense (a) and sense (b) *Setdb1* RNA probes. Notice robust expression in all compartments of the developing brain. Abbreviations: CI: cerebral cortex, layer 1, CP: caudoputamen, CxP: cortical plate, cpn: caudate putamen neuroepithelium, GP: globus pallidus, H: hippocampus, iz: intermediate zone, LV: lateral ventricle, nc: neocortical neuroepithelium, SP: subplate, svz: subventricular zone (striatum), svzc: subventricular zone (neocortex), V3: third ventricle, nc: neocortical plate, iz: intermediate zone, vz: ventricular zone. Scale bars: 200 μ m (A, B), 50 μ m (C).

Figure 3. *CK-Setdb1* worsens the RTT phenotype of *Mecp2* mutant mice

A, Body weight (at 4 weeks of age); **B**, locomotor activity (8 weeks of age); and **C**, rotarod performance (11 weeks of age) of wildtype, *CK-Setdb1*, *Mecp2*^{-/-} and *CK-Setdb1/Mecp2*^{-/-}. N=23–31/genotype (locomotor activity), N=13–16/genotype (rotarod). *p<0.05 (*Mecp2*^{-/-}), #p<0.01 (*CK-Setdb1/Mecp2*^{-/-}) as compared to wildtype; *p<0.05 (*CK-Setdb1/Mecp2*^{-/-}) as compared to *Mecp2*^{-/-}, one-way ANOVA. **D**, Kaplan-Meier survival curves. N=19–24/genotype. Notice worsening of neurological deficits (**A–C**) and no prolonged survival (**D**) of *CK-Setdb1/Mecp2*^{-/-} mice, as compared to *Mecp2*^{-/-}.

Figure 4. *tauSetdb1* mice

A, Targeting vector, including myc-tagged *Setdb1* cDNA (*myc-Setdb1*), neomycin resistance cassette (*NEO^R*), and homologous flanking sequences of *Mapt* (*tau*) (gray box). Insertion of myc-*Setdb1*-*NEO^R* cassette at *tau* exon 2 ATG start (marked by vertical line). Locations of 5'-external (1), internal (2) and 3'-external (3) probes used for Southern blot analysis as indicated. **B**: BamHI; E: EcoRI. **B**, Representative images showing Southern blot analysis of WT and targeted embryonic stem cell clones (F1, D3, G11, E12) after digestion with BamHI or EcoRI. **a**) Hybridization with the internal probe (2) results in a 9887-bp band for the targeted allele (ki). **b**) Hybridization with the 5'-external probe (1) results in a 9175-bp WT band and a 4432-bp band for the targeted allele. **c**) Hybridization with the 3'-external probe (3) results in a 9175-bp WT band and a 6768-bp band for the targeted allele. **C**, Immunohistochemical stains with anti-myc antibody, notice labeling of neuronal nuclei in

cerebral cortex (a), striatum (c), hippocampus (e) and cerebellum (g) of *tauSetdb1* mice; (b, d, f, h) show corresponding sections from wildtype controls. Scale bar, 10 μ m. **D**, Bar graphs showing *Setdb1* mRNA expression in cerebral cortex (a) and striatum (b) from wildtype and *tauSetdb1* mice. N=3–4/genotype. #p<0.01, Mann Whitney test. **E**, Anti-myc immunoblot showing expected 180kDa band in cerebral cortex (CC), striatum (Str), hippocampus (Hipp), cerebellum (Cbl), thalamus (Thal), hypothalamus (Hypo), periaqueductal gray (PAG), and substantia nigra (SN) in adult *tauSetdb1* (+), but not in WT mice (–).

Figure 5. *tauSetdb1* accelerates premature death of *Mecp2* deficient mice
A, Rotarod performance from wildtype, *tauSetdb1*, *Mecp2*^{-/-}, and *tauSetdb1/Mecp2*^{-/-} at 12 weeks of age. N=9–17/genotype. *p<0.05 as compared to Control, one-way ANOVA. **B**, Kaplan-Meier survival curves. N=11–25/genotype. The group of *tauSetdb1/Mecp2*^{-/-} mice was comprised of 8 hetero- and 17 homozygous *tauSetdb1* knock-in mice. Notice left shift in survival curve of *tauSetdb1/Mecp2*^{-/-} mutant mice (Kaplan-Meier, P<0.07). Notice further no significant effect of *tauSetdb1* on rotarod performance.

Figure 6. H3K9 tri-methylation is normal in neuronal heterochromatin of the *Mecp2* deficient brain

A, Representative image showing immunofluorescence of MeCP2/DAPI counterstain (a/b, c/d, green/blue), and NeuN/DAPI (e/f, g/h, green/blue) in nuclei after hypotonic extraction from adult *Mecp2*^{2lox/y}/*Nestin-cre* conditional knockout (*Mecp2*^{-/-}) (a/b, e/f) and wildtype (c/d, g/h) forebrains. Notice lack of MeCP2 immunoreactivity in the mutants. Scale bar, 10 μ m. **B**, Representative FACS scatter plots from (a) negative control (NeuN-) processed without NeuN antibody, and (b) sample processed with NeuN antibody (NeuN+). Only the population of nuclei in sector P3, showing robust immunofluorescence, were collected during FACS. **C**, Chromatin of FACS-sorted NeuN+ nuclei from wildtype and *Mecp2*^{-/-} were subjected to anti-H3K9me3 ChIP, and then processed together with input by slot-blot hybridized to major satellite DNA probe. Bar graph showings no changes in H3K9me3 levels in pericentromeric heterochromatin of neuronal nuclei extracted from 8 weeks old wildtype and *Mecp2*^{-/-} mice. N=8/genotype. Data shown as mean $\pm$ SEM.