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MeCP2 is required for global heterochromatic and nucleolar changes during activity-dependent neuronal maturation

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Abstract

Mutations in *MECP2*, encoding methyl CpG binding protein 2, cause the neurodevelopmental disorder Rett syndrome. MeCP2 is an abundant nuclear protein that binds to chromatin and modulates transcription in response to neuronal activity. Prior studies of MeCP2 function have focused on specific gene targets of MeCP2, but a more global role for MeCP2 in neuronal nuclear maturation has remained unexplored. MeCP2 levels increase during postnatal brain development, coinciding with dynamic changes in neuronal chromatin architecture, particularly detectable as changes in size, number, and location of nucleoli and perinucleolar heterochromatic chromocenters. To determine a potential role for MeCP2 in neuronal chromatin maturational changes, we measured nucleoli and chromocenters in developing wild-type and *Mecp2*-deficient mouse cortical sections, as well as mouse primary cortical neurons and a human neuronal cell line following induced maturation. *Mecp2*-deficient mouse neurons exhibited significant differences in nucleolar and chromocenter number and size, as more abundant smaller nucleoli in brain and primary neurons compared to wild-type, consistent with delayed neuronal nuclear maturation in the absence of MeCP2. Primary neurons increased chromocenter size following depolarization in wild-type, not *Mecp2*-deficient cultures. Wild-type *MECP2e1* over-expression in human SH-SY5Y cells was sufficient to induce significantly larger nucleoli, but not a T158M mutation of the methyl-binding domain. These results suggest that, in addition to the established role of MeCP2 in transcriptional regulation of specific target genes, the global chromatin-binding function of MeCP2 is essential for activity-dependent global chromatin dynamics during postnatal neuronal maturation.

Keywords

MeCP2; DNA methylation; maturation; chromatin; nucleoli; neuronal; epigenetic; neurodevelopmental; Rett syndrome

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INTRODUCTION

Mutations in the methyl CpG binding protein 2 gene (*MECP2*) cause Rett Syndrome (RTT), a progressive, neurodevelopmental disorder in females that occurs in 1 in 10,000–15,000 births (Amir et al., 1999). *MECP2* is located on the X-chromosome and a variety of different mutations and duplications are observed in a wider range of neurodevelopmental disorders in addition to RTT (Gonzales and LaSalle, 2010; Zoghbi, 2005). MeCP2 is an abundant nuclear protein expressed in numerous tissues and cell types throughout the body, however, the phenotypic consequences of *MECP2* mutation are most apparent in the central nervous system (CNS) during postnatal brain development when expression is elevated (Balmer et al., 2003; Shahbazian et al., 2002; Skene et al., 2010).

The neuronal nucleus undergoes dynamic, nonrandom changes in global chromatin during maturation that include changes in the location, size, and number of nuclear structures such as chromocenters and nucleoli (Manuelidis, 1984a; Manuelidis, 1985; Martou and De Boni, 2000; Solovei et al., 2004). Heterochromatic chromocenters consist of transcriptionally silent DNA frequently positioned around nucleoli. Nucleoli are euchromatic structures formed by transcriptionally active ribosomal DNA. Therefore, measurements of nucleolar size can be considered a direct measurement of active rDNA transcription in the neuronal nucleus. Nucleoli have other essential functions in the nucleus including the regulation of mitosis and cellular stress responses (Boisvert et al., 2007). Chromocenters and nucleoli are intimately linked as chromocenters consists of transcriptionally silent ribosomal DNA (rDNA) and nucleoli contain chromatin remodeling proteins involved in the formation of heterochromatin domains (Akhmanova et al., 2000; Caperta et al., 2007; Guetg et al., 2010; Santoro et al., 2002).

Neuronal nuclei undergo dynamic changes in compaction and histone deacetylation during postnatal neuronal maturation and MeCP2 has been shown to mediate changes to the local chromatin structure (Ishibashi et al., 2008; Nikitina et al., 2007a; Nikitina et al., 2007b; Thatcher and LaSalle, 2006). Brero and colleagues investigated MeCP2's role in global chromatin reorganization during terminal differentiation of myoblasts and found that MeCP2's methyl binding domain (MBD) was necessary and sufficient to induce clustering of chromocenters (Brero et al., 2005). However, the requirement for MeCP2 in global chromatin structural dynamics during postnatal neuronal maturation has not been previously investigated.

A complex interaction between genetics and the environment is necessary for normal brain development, and MeCP2 appears to be an important nuclear mediator of neuronal responsiveness. Previous studies in mouse brain sections and primary neuronal culture have shown changes in MeCP2's nuclear pattern across development and following potassium chloride (KCl) depolarization, suggesting that activity promotes neuronal maturation, leading to a redistribution of MeCP2 within the nucleus (Ballas et al., 2005; Martinowich et al., 2003; Shahbazian and Zoghbi, 2002). Previous studies have focused on changes to specific MeCP2 target genes following neuronal activity, but the role of MeCP2 in global chromatin changes following neuronal activity or with postnatal neuronal development has not yet been reported.

In this study, we show that *Mecp2*-deficiency impacted the size and number of nucleoli and chromocenters in brain and with neuronal activity. These results support the hypothesis that the increased levels of MeCP2 following neuronal activity act to globally reorganize chromatin for the morphologic differences observed with neuronal maturation.

RESULTS

***Mecp2* deficiency alters developmental maturational changes in nucleolar size and number in vivo and in vitro**

Neuronal maturation leads to observable and measurable changes in nuclear organization that may require MeCP2. To investigate differences in global chromatin and nucleolar changes in developing cortical neurons of mouse brain resulting from *Mecp2* deficiency, a developmental tissue microarray containing 600 μm diameter cores from *Mecp2*^{tm1.1Bird/y} and wild-type littermate control mice was immunostained for nucleolin to detect nucleoli and DAPI to detect nuclei and heterochromatic chromocenters. Fluorescent microscopy and image analysis was used to measure the diameter and number of nucleoli and chromocenters in cortical neurons for four developmental timepoints including embryonic day 15 (E15), and postnatal days 1, 21 and 28 (P1, P21, and P28). For each nucleus, all chromocenters $>0.63 \mu\text{m}$ and all detectable nucleoli were counted and the diameter measured for a total of 150 nuclei in 3 technical replicates per time-point.

Mecp2 deficiency resulted in significantly smaller nucleoli at E15 and P21 measured as both mean nucleolar diameter and the largest nucleolar diameter per nucleus (Fig. 1A, B). At P21, mean nuclear diameter was also significantly lower in *Mecp2* deficient nuclei at P1 (Fig. 1A). In contrast, nuclear diameters varied greatly, and *Mecp2*-deficient neurons were significantly larger or smaller than wild-type, depending on the developmental time-point (Fig. 1C). The variability in nuclear diameter could be indicative of the inherent heterogeneity within the developing cerebral cortex with different neuronal cell types and varying degrees of maturity, however the significantly reduced mean nucleolar diameter observed in *Mecp2*-deficient cortical neurons was independent of nuclear diameter. The number of nucleoli was also increased in the early developmental time-points (E15 and P1) in *Mecp2*-deficient compared to wild-type cortex (Fig. 1D). Significant differences between developmental time points were also observed for nucleolar size and number (Supplementary Figures 1 and 2).

To investigate whether the in vivo effects could be modeled in vitro, mouse cortical neurons from newborn *Mecp2*-deficient and wild-type littermate mice were isolated and cultured for 14 days in vitro (14 DIV). After fixation, replicate slides were immunostained for fibrillarin to detect nucleoli, a neuronal marker TU-20, and counterstained with DAPI. Image analysis was used to detect measurable changes in subnuclear structures between *Mecp2*-deficient and wild-type neurons. Similar to the results observed in brain sections, primary neurons cultured from *Mecp2*-deficient mice had significantly smaller nucleoli (Fig. 2A,B) and significantly more nucleoli (Fig. 2D) when compared to nuclei from wild-type littermates, although the mean nuclear diameter was not significantly different (Fig. 2C). These results support a role for MeCP2 in reorganizing neuronal nucleoli from small and numerous in the embryonic or immature neuronal nuclei to a single, large, centrally-located nucleolus in mature neuronal nuclei.

***Mecp2* deficiency alters developmental changes in chromocenter size and number in vivo and in vitro**

Because heterochromatic chromocenters are sites of MeCP2 localization and are found around nucleoli in neuronal nuclei, we investigated the effect of *Mecp2* deficiency on chromocenter size and number. *Mecp2*-deficient nuclei showed significant deficiency in the number of chromocenters at E15 and increased size of chromocenters at E15 and P1 compared to wild-type (Fig. 3A,B). In both the wild-type and *Mecp2*-deficient nuclei, there was an observed decrease in the number of chromocenters in postnatal stages (Fig. 3E,F), however, the decrease in the number of chromocenters was apparently delayed in *Mecp2*-

deficient nuclei, with the loss of a significant decrease in chromocenter number between E15 and P1 (Fig. 3F). Chromocenter diameter significantly increased with developmental stage in the cortical neurons of both wild-type and *Mecp2*-deficient mice (Fig. 3C,D), suggesting that the early stage significant differences in chromocenter formations in *Mecp2*-deficient neurons were transient rather than long-lived.

Cultured neurons from *Mecp2*-deficient and wild-type littermate mice also showed significant changes in chromocenter size, with *Mecp2*-deficient neurons having significantly smaller chromocenters compared to wild-type neurons (Fig. 4A). The number of chromocenters did not differ significantly (Fig. 4B). As was seen with the nucleolar measurements, the effects of *Mecp2* deficiency on chromocenters were observed both in vivo and in vitro.

Activity-induced changes in global chromatin and nucleoli are partially mediated by *Mecp2*

Previous studies have shown a redistribution of MeCP2 which correlates with derepression of MeCP2 target genes, such as *Bdnf*, following neuronal activity in vitro, suggesting that activity promotes neuronal maturation (Ballas et al., 2005; Martinowich et al., 2003). MeCP2 redistribution from a diffuse to punctate staining pattern was also reported to occur in the embryonic mouse brain and was hypothesized to serve as a mechanism for regulating gene silencing (Shahbazian et al., 2002). We therefore sought to investigate the effect of neuronal activity on global chromatin and nucleolar changes. Mouse cortical neurons were cultured for 21 days in vitro (21 DIV) and then treated with potassium chloride (KCl) for 10, 20, or 30 minutes or left untreated (control). The slides were then immunostained for nucleolin to detect nucleoli and the neuronal marker, TUJ1, and counterstained with DAPI. Changes in nuclear morphology between stimulated and unstimulated neurons were observed and the size and number of chromocenters and nucleoli measured.

We observed changes in nuclear morphology in the KCl-treated neurons, with a highly textured appearance of the nuclei, reflecting rapid global chromatin changes in response to KCl (Fig. 5C). The results from the measurements of chromocenter size show that KCl stimulation resulted in significantly larger chromocenters compared to control, untreated neurons (Fig. 5A). There were no significant differences in the number of chromocenters per nucleus in the control or treated samples (Fig. 5B).

To test if these measured global chromatin changes in response to activity required functional MeCP2, cortical neurons from *Mecp2*^{+/y} and *Mecp2*^{tm1.1Bird/y} mice were cultured for 14 DIV and treated with 50 mM KCl for 20 minutes or left untreated. As observed in Figure 5, KCl depolarization resulted in significantly larger chromocenters (Fig. 6A) without a change in the number of chromocenters (Fig. 6B) in wild-type neurons. In contrast, KCl had no effect on the size of chromocenters in *Mecp2*-deficient primary neurons (Fig. 6A). These results indicate that *Mecp2* participates in the neuronal response to activity by regulating the size and number of chromocenters, perhaps by aiding fusions between chromocenters during neuronal maturation, as has previously been observed in myoblasts (Brero et al., 2005). In addition to the measured changes in chromocenters, short-term KCl depolarization of 21 DIV primary cortical neurons resulted in neurons with significantly fewer but significantly smaller nucleoli and nuclei (Supplementary Fig. 3) in cultures of both genotypes (Supplementary Fig. 4).

Stable overexpression of *MECP2e1* in human SH-SY5Y cells results in significant changes in nucleolar size and number dependent on the MBD

We sought to investigate how MeCP2 gain of function and loss of MBD function affected nucleolar size and number by conducting experiments on human neuronal SH-SY5Y cells

induced to undergo differentiation. The parental SH-SY5Y cell line expressing endogenous MeCP2 was compared to two stably transfected cell lines. One cell line overexpressed the wild-type MeCP2e1 isoform, which is the most predominant form of MeCP2 in brain (Mnatzakanian et al., 2004). A second cell line overexpressed a T158M mutation in the MBD of the same *MECP2e1* construct, which is the most common point mutation in RTT (Bienvenu et al., 2002; Huppke et al., 2000). The SH-SY5Y cells were cultured for 72 hours with 16 nM of Phorbol-12-Myristate-13-acetate (PMA) to induce neuronal maturation. After fixation, the cells were stained for nucleolin and counterstained with DAPI and the diameter and number of nucleoli measured in each cell line. Unlike mouse nuclei, human nuclei do not have detectable chromocenters (Supplementary Figure 5).

Overexpression of MeCP2e1 resulted in significantly larger nucleoli compared to the parental cell line, while overexpression of the T158M mutant form of MeCP2 resulted in significantly smaller nucleoli (Fig. 7A,B). These results support the hypothesis that developmentally increased levels of MeCP2 aid in regulating the size of nucleoli in developing neurons and that mutation of the MBD impacts the MeCP2 induced nucleolar enlargement. The effect of MeCP2 T158M mutation appeared specific to nucleolar changes, as both MeCP2e1 and the T158M mutant overexpressing cells showed increased nuclear diameter and number of nucleoli (Fig. 7C,D, and Supplementary Fig. 5).

DISCUSSION

Global chromatin changes, observed as changes in chromocenter and nucleolar size and number, are essential for neuronal development as they underlie cellular changes in transcriptional activity and gene expression. Chromocenters and nucleoli are intimately linked as chromocenters contain the silent ribosomal DNA of the nucleolus and the nucleolus consist of a group of proteins known as the nucleolar remodeling complex or NoRC which has been shown to regulate chromocenter size in vitro (Akhmanova et al., 2000; Caperta et al., 2007; Guetg et al., 2010; Santoro et al., 2002). During neuronal maturation, the nuclear morphology of the neuron changes from a small, heterochromatic nucleus with many randomly-located chromocenters and nucleoli to a large, mostly euchromatic nucleus with fewer, larger chromocenters associated with a large, centrally-located nucleolus (Manuelidis, 1984a; Manuelidis, 1984b; Manuelidis, 1985; Martou and De Boni, 2000; Solovei et al., 2004). This non-random reorganization suggests that these changes occur via the clustering and relocation of these structures during terminal differentiation and these global chromatin changes have been observed in terminally-differentiating neurons in a variety of species, strongly indicating functional significance (Manuelidis, 1984b). As MeCP2 has previously been demonstrated to induce clustering of chromocenters in terminally-differentiating myoblasts (Brero et al., 2005), we sought to investigate the requirement of MeCP2 in inducing clustering of chromocenters and the maturation of nucleoli in developing neurons.

In this study, we have demonstrated in brain and cell culture that *Mecp2/MECP2* is required for maturational changes in both nucleolar and chromocenter size and number in neurons. This is the first quantitative study of developmental and activity-dependent changes in global subnuclear chromatin organization during postnatal neuronal maturation and as a result of *Mecp2*-deficiency. Our results are consistent with previous studies demonstrating dynamic changes in global chromatin and nucleoli during terminal differentiation of postnatal neurons (Manuelidis, 1984a; Manuelidis, 1985; Martou and De Boni, 2000; Solovei et al., 2004), but also designate a novel role for MeCP2 in these important activity-dependent developmental processes.

We report that *Mecp2*-deficiency results in significantly smaller nucleoli in primary neurons and mouse brain and that overexpression of *MECP2e1* is sufficient for increased nucleolar size in a human SH-SY5Y neuronal cell line. The nucleolus has been shown to be a multifunctional subnuclear compartment that is associated with ribosome and ribonucleoprotein particle biogenesis, chromocenter formation, mitosis, cell-cycle progression and proliferation, and stress response (Boisvert et al., 2007; Santoro et al., 2002). Given its multifunctional capacity, the absence of increased nucleolar size and decreased nucleolar number as a result of *Mecp2*-deficiency may contribute to Rett syndrome pathogenesis. Defects in nucleolar size and number are apparent as early as the embryonic stage of development, long before *Mecp2* null mice are symptomatic for the RTT-like phenotype (Chen et al., 2001; Guy et al., 2001). The developmental time-points used correspond to the first 3 to 4 weeks of life, a period of rapid brain growth and development known as the brain growth spurt (BGS) in mammals (Davison and Dobbing, 1968). Our results demonstrate that the seemingly normal development observed in RTT patients and mouse models of RTT at this time is not normal at the subnuclear level. We hypothesize that as neurons mature and become more euchromatic, a large nucleolus would be required to supply sufficient ribosomes to respond to the increase in gene transcription. Therapeutic interventions which target nucleolar proteins or ribosomal RNA transcription could be potentially beneficial in bypassing the nucleolar maturation defects caused by loss of function of *Mecp2*.

Measurements of nucleolar changes in differentiated SH-SY5Y cells showed that a mutation in MeCP2's methyl binding domain (MBD) is sufficient to negate the increased nucleolar size observed with *MECP2e1* overexpression. The MBD consists of 85 amino acids (spanning positions 78–162) and is located at the amino terminus of the MeCP2 protein (Nan et al., 1993). Previous studies have demonstrated that the MBD interacts with methylated cytosines in vitro and that the MBD alone is sufficient and necessary to induce clustering of chromocenters (Brero et al., 2005). The T158M mutation in MeCP2's MBD has previously been shown to be defective in chromatin binding in vivo (Kumar et al., 2008). Our results suggest that nucleolar maturation as a result of overexpression of the brain specific MeCP2e1 isoform requires an intact MBD.

Mature neuronal nucleoli are characterized by the appearance and enlargement of perinucleolar chromocenters that correlate with nucleolar enlargement and are significantly impacted by *Mecp2* deficiency. The abnormally increased chromocenter size in early developing neurons of *Mecp2*-deficient mouse cortex overtly appears to be discrepant with the measurements in primary neuronal cultures showing decreased chromocenter and nucleolar size in *Mecp2*-deficient neurons. Since inactive rDNA is contained within perinucleolar chromocenters (Akhmanova et al., 2000; Caperta et al., 2007; Guetg et al., 2010; Santoro et al., 2002), epigenetically regulated, and capable of reallocation to the nucleolus for transcription of rRNA genes (Caperta et al., 2007), we hypothesize that the significantly enlarged chromocenters of embryonic and early postnatal *Mecp2*-deficient cortical nuclei could be indicative of an abnormal increase in rDNA silencing and sequestration of rDNA within the perinucleolar chromocenters. This sequestration of rDNA in chromocenters and loss of reallocation to the nucleolus would result in decreased transcription of rRNA during critical periods of neuronal maturation, leading to significantly smaller nucleoli during later postnatal periods. In addition, other methyl CpG-binding domain (MBD) proteins may be overcompensating for the lack of MeCP2, inducing excessive clustering of chromocenters (Brero et al., 2005). MBD2 was previously demonstrated to occupy binding sites in the genome left vacant by the removal of MeCP2 (Klose et al., 2005). In addition, histone H1 competes for chromatin binding with MeCP2 and is increased in *Mecp2* deficient neuronal nuclei, suggesting a potential overcompensation (Ghosh et al., Skene et al., 2010). Experiments investigating MBD2 and

histone H1 proteins in the *Mecp2*-deficient brain at different developmental time-points could provide insights into possible functional redundancy and if so, potential therapeutic targets.

In our study, neuronal activity positively impacted the size of chromocenters and *Mecp2* deficiency abrogated these maturational changes. Previous studies focused on the neuronal activity-induced derepression of MeCP2 target genes such as *Bdnf* (Ballas et al., 2005; Chen et al., 2003; Martinowich et al., 2003; Zhou et al., 2006). Here, we observed and quantified global chromatin changes in response to neuronal activity. The effect of global changes to subnuclear chromatin with *Mecp2* deficiency may be a direct effect of the heterochromatin-binding of MeCP2, as MeCP2 induced changes require an intact MBD and numerous studies have demonstrated MeCP2's interactions with heterochromatic chromocenters around nucleoli (Klose et al., 2005; Kumar et al., 2008; Lewis et al., 1992; Nikitina et al., 2007a; Nikitina et al., 2007b; Skene et al., 2010). Alternatively, MeCP2 deficiency may be causing these changes indirectly through the aberrant expression of target genes that in turn regulate chromatin changes. However, Skene and colleagues recently demonstrated that MeCP2 deficiency resulted in global changes in neuronal chromatin structure which suggest that MeCP2, in accordance with its high abundance and global distribution in mature neurons, may not act as a gene-specific transcriptional repressor but as a global modulator of transcription throughout the entire genome (Skene et al., 2010). From this study, we conclude that the chromatin-binding function of MeCP2 is required for important developmental changes in global neuronal nuclear and nucleolar morphology. Moreover, these changes require an intact MBD, suggesting an epigenetic mechanism of regulation.

MATERIALS AND METHODS

Primary Neurons (Activity-dependent experiments, 21 DIV)

E15 C57BL/6J primary cortical mouse neurons (Lonza, <http://www.lonza.com>) were cultured according to supplier's protocol for 21 days on four chambered glass slides. Primary neurons were treated with 50 mM KCl for 10, 20, or 30 min or left untreated (control). Slides were fixed in Histochoice (Ameresco) for 15 minutes, washed in 1x PBS/0.5% Tween for 5 minutes and then stored in 70% EtOH.

Primary Neurons (*Mecp2*-deficient mice and wild-type littermates, 14 DIV)

Primary neuronal cultures were established from newborn *Mecp2*^{+/y} and *Mecp2*^{-/y} mice (P0, day of birth) obtained from breeding heterozygous mutant females and WT males (*Mecp2*^{tm1.1Bird} and C57BL/6J, Jackson Laboratories) (Guy et al., 2001). Newborn male mice were gendered by pigmentation of the scrotum. Tail samples were collected at the time of sacrifice for genomic DNA preparation and genotyping according to the vendor's PCR protocol (<http://jaxmice.jax.org>). Custom PCR primer sequences (Invitrogen) GTGAAGGAGTCTCCATACGGTC and TCTCCTTGCTTTTACGCCC are located in the *Mecp2* CDS, exon 4. Primers ATGGCCTGCCAGAGTCGT and CAATTGGAAGTGTCAACTACGGTT are located in the *Mecp2* 3'UTR.

Following sacrifice, brains were dissected to remove the cortical tissue. The cortical tissue was dissociated and then plated on poly-D-lysine coated 4-chambered glass slides in cell culture medium at densities up to 1×10^5 cells/ml. Neurons were maintained for 14 DIV in Neurobasal Medium containing B27 supplement, N2 supplement with transferrin, L-Glutamine, and gentamicin (Invitrogen). The cell culture medium was changed every 3–4 days. Prior to fixation, cells were treated for 20 minutes with 50 mM KCl or left untreated (control). All animal studies were performed in accordance with the University of California Davis Institutional Animal Care and Use and NIH guidelines.

Generation of SH-SY5Y MeCP2 stable cell lines and tissue culture

MeCP2e1-FLAG expression constructs were generated as previously reported (Adegbola et al., 2009). The constructs were transfected into SH-SY5Y cells and stably expressing pools were selected with 1mg/ml G418. SH-SY5Y cells were grown in complete minimal essential media with 15% fetal calf serum. Cells were plated onto four-chamber glass slides treated with poly-D-lysine at a density of 5×10^4 cells/ml. Cells were treated with 16nM Phorbol-12-Myristate-13-acetate (PMA) (EMD Biosciences) and fixed 72 hours later for 15 minutes in Histochoice (Ameresco) then washed in $1 \times$ PBS/0.5% Tween for 5 minutes and stored in 70% ethanol at -20°C . For immunofluorescent staining, SH-SY5Y cells were incubated with a primary antibody solution containing anti-FLAG M2 monoclonal antibody (Sigma-Aldrich) at a final concentration of 2 $\mu\text{g/ml}$ and anti-nucleolin (Abcam) detected with Oregon Green-conjugated anti-mouse and Texas Red-conjugated anti-rabbit antibodies and counterstained with DAPI.

Mouse Tissue Microarrays

Wildtype C57BL/6J and *Mecp2*^{tm1.1Bird/y} tissue for multiple age time points were obtained, fixed, and embedded in paraffin and sampled as described previously (Braunschweig et al., 2004). The mouse tissue microarray consisted of triplicate 600 μm cores of grey matter from the cerebral cortex of four age-matched male timepoints for both *Mecp2*^{+/y} and *Mecp2*^{tm1.1Bird/y}: embryonic day 15 (E15), postnatal day 1 (P1), postnatal day 21 (P21), and postnatal day 28 (P28).

Immunofluorescent Staining

A 1:100 dilution of primary antibodies including anti-nucleolin antibody or anti-fibrillarin and anti-TU-20 or TUJ-1 (Abcam), were diluted in IF staining buffer containing $1 \times$ phosphate buffered saline (PBS), 0.01% fetal calf serum (FCS), and 0.5% Tween. The slides were incubated in primary antibody solution overnight at 37°C , followed by three washes in $1 \times$ PBS/0.5% Tween three times for 5 min with agitation. A 1:100 dilution of secondary antibodies including Texas Red, Alexa 594 or Alexa 488-conjugated anti-rabbit antibody and Oregon Green or Alexa 488-conjugated anti-mouse antibody (Molecular Probes) were diluted in IF staining buffer and incubated on slides at 37°C for 1 hr followed by three additional washes in $1 \times$ PBS/0.5% Tween for 5 min with agitation. Slides were air dried and mounted in Progold antifade with DAPI (Invitrogen) or in Vectashield (Vector Laboratories) containing 5 $\mu\text{g/ml}$ DAPI and coverslipped. Paraffin embedded tissue slides were immunostained as described previously (Peddada et al., 2006), air dried, and mounted with Progold antifade with DAPI or 5 $\mu\text{g/mL}$ DAPI in Vectrashield and coverslipped.

Fluorescent Microscopy

Slides were visualized using $100\times$ oil immersion lenses on an Axioplan 2 fluorescence microscope (Carl Zeiss, Inc, NY) equipped with a Qimaging Retiga EXi high-speed uncooled digital camera, appropriate fluorescent filter sets, and automated xyz stage controls. The microscope and peripherals are controlled by a Macintosh running iVision (Scanalytics, Vienna, VA, USA) software with Multiprobe, Zeissmover and 3D extensions. Images were captured for blue, green, red, and long red filters. Each image was digitally deconvolved to remove out-of-focus light. Following haze removal, images of each fluorophore were merged to create an image representing all of the fluorescence within the section. All measurements were taken in real-time preview mode using a $100\times$ oil objective with a $1 \times$ zoom, using a drawing tool to measure each substructure so that each endpoint of the drawing tool was in the medial focal plane when defined. All measurements for a given experiment were taken with the same exposure times and microscope settings as was appropriate for the fluorescence intensity.

Data Analysis and Box Plots

For each cell nuclei, the nuclear diameter, size and number of each chromocenter, and size and number of each nucleoli were measured and saved as a text file. Thus, each cell nuclei generated 3 text files: nuclear diameter measurements, chromocenter measurements, and nucleolar measurements. Custom perl scripts (<http://www.perl.com/>) were used to automate the process of calculating means, determining the number of chromocenters and nucleoli per nucleus, extracting maximum values and organizing the data based on each condition, genotype, and timepoint. Custom perl scripts were also used to adjust for errors in XY value settings, overcome any file name extension discrepancies, and eliminate extraneous signals by excluding structures smaller than 0.63 microns to create a uniform data set. The 0.63 micron cut-off was determined by adjusting for different XY value settings on iVision (measurements taken under 63X magnification and 100X magnification settings). R software (<http://www.r-project.org/>) was used to generate box plots, which show the distribution of the data using a set of five numbers, the median, which is represented by the horizontal line in the middle of the box; the 25th percentile or lower quartile, represented as the lower side or bottom half of the box; the 75th percentile or upper quartile, represented as the upper side or top half of the box; and minimum and maximum data values represented by the bars at the ends of the vertical lines. Outliers, defined as points that are greater than 1.5 times the interquartile range beyond the first and third quartiles, are shown as open circles. Each box represents 3 technical replicates (150 total nuclei) for one experimental condition or genotype and timepoint.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

MeCP2	methyl-CpG binding protein 2
MeCP2e1	isoform of MeCP2
MBD	methyl-binding domain
RTT	Rett syndrome
DIV	days in vitro
rDNA	ribosomal DNA

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Figure 1. Loss of *Mecp2* results in significantly smaller and increased number of nucleoli in early postnatal cortical neurons *in vivo*

Cortical nuclei from different aged mice show developmental changes and effects of *Mecp2* deficiency in nucleolar size and number. Results in A–D represent the mean $\pm$ SEM for 150 nuclei per time point and genotype. Box –whisker plots of distributions are shown in Supplementary Figures 1 and 2. * = $p < 0.05$, ** = $p < 0.01$, and *** = $p < 0.001$ versus control (Wilcoxon rank sum). A) The mean nucleolar diameter per nucleus is significantly smaller in the *Mecp2*^{−/y} mice compared to *Mecp2*^{+/y} mice for all timepoints except P28. B) The largest nucleolus per nucleus is significantly smaller in the *Mecp2*^{−/y} mice compared to *Mecp2*^{+/y} mice for E15 and P21. C) The nuclear diameter is decreased significantly in the *Mecp2*^{−/y} neurons compared to *Mecp2*^{+/y} neurons of E15 and P21 mice, but is increased significantly in the *Mecp2*^{−/y} neurons compared to *Mecp2*^{+/y} neurons of P1 and P28 mice. D) The number of nucleoli per nucleus is significantly increased in the *Mecp2*^{−/y} mice compared to *Mecp2*^{+/y} mice at E15 and P1. E) Representative images from *Mecp2*^{+/y} and *Mecp2*^{−/y} mouse cortical neurons stained with anti-nucleolin (red fluorescence) and counterstained with DAPI (blue fluorescence). Individual nuclei are outlined in dashed white lines.

Figure 2. Loss of *Mecp2* results in significantly smaller nucleoli and significantly increased the number of nucleoli in primary cortical neurons

Mecp2^{-/y} and *Mecp2*^{+/y} primary mouse cortical neuronal cultures derived from an individual embryo of each genotype were maintained for 14 DIV. Three replicate slides were stained for fibrillarin and DAPI and nucleolar measurements taken from 50 nuclei per slide. Results in A–D represent the distribution as a box with the median (central line) for 150 nuclei per genotype. The whiskers run to the limit of the distribution, excluding outliers (circles), which is defined as points that are >1.5 times the interquartile range beyond the first and third quartiles. ***=p<0.001 versus control (Wilcoxon rank sum). A) The mean nucleolar diameter per nucleus is significantly smaller in the *Mecp2*^{-/y} neurons compared to *Mecp2*^{+/y} neurons. B) The largest nucleolus per nucleus is significantly smaller in the *Mecp2*^{-/y} neurons compared to *Mecp2*^{+/y} neurons. C) The nuclear diameter appears smaller in the *Mecp2*^{-/y} neurons compared to *Mecp2*^{+/y} neurons, but the difference is not significant. D) There are significantly more nucleoli in the *Mecp2*^{-/y} neurons compared to *Mecp2*^{+/y} neurons. E) Representative images from *Mecp2*^{-/y} and *Mecp2*^{+/y} primary mouse cortical neurons showing differences in nucleolar size and number. Sections were stained with anti-fibrillarin (red) to outline nucleoli. Nuclei were counterstained with DAPI (blue). Individual nuclei are outlined in dashed white lines.

Figure 3. Loss of *Mecp2* results in significant defects in chromocenter size and number in embryonic and neonatal brain

Nuclei from different aged mice show developmental changes and effects of *Mecp2* deficiency in chromocenter size and number, determined from DAPI staining. Results in A–F represent box-whisker plots for each genotype and timepoint. * = $p < 0.05$, ** = $p < 0.01$, and *** = $p < 0.001$ versus control (Wilcoxon rank sum). A) The mean chromocenter diameter per nucleus is significantly enlarged in the E15 and P1 *Mecp2*^{-/-y} mouse brain compared to *Mecp2*^{+/y}. B) There is a significant decrease in the number of chromocenters in the E15 *Mecp2*^{-/-y} mouse brain compared to *Mecp2*^{+/y}. C) In wild-type cortical neuronal nuclei, a significant increase was observed in chromocenter size at both early (E15 to P1) and later (P1 to P21 and P21 to P28) age transitions. D) In *Mecp2*^{-/-y} cortical neuronal nuclei, there is a similar significant increase in chromocenter size as *Mecp2*^{+/y} neuronal nuclei at all developmental transition except the E15 to P21 transition, which was not significant in *Mecp2*^{-/-y}. E) In wild-type cortical neuronal nuclei, there is a significant decrease in the number of chromocenters per nucleus at all postnatal timepoints compared to E15. F) The number of chromocenters per nucleus is not significantly decreased at P1 compared to E15 in the *Mecp2*^{-/-y} cortical neuronal nuclei, exhibiting a disruption of the developmental changes observed in the wild-type brain. G) Representative images from *Mecp2*^{+/y} and *Mecp2*^{-/-y} mouse cortical neurons stained with DAPI, showing differences in chromocenter size and number.

Figure 4. Loss of *Mecp2* results in significantly smaller chromocenters in primary cortical neurons

Mecp2^{-/-} and *Mecp2*^{+/+} primary mouse cortical neuronal cultures derived from an individual embryo of each genotype were maintained for 14 DIV. Three replicate slides were stained for DAPI and chromocenter measurements taken from 50 nuclei per slide. Results in A–B represent the box plot summary for 150 nuclei per genotype. ***= $p < 0.001$ versus control (Wilcoxon rank sum). A) The mean chromocenter diameter per nucleus is significantly smaller in *Mecp2*^{-/-} mouse primary neurons compared to *Mecp2*^{+/+} neurons. B) No significant difference in the number of chromocenters per nucleus was observed in *Mecp2*^{-/-} compared to *Mecp2*^{+/+} primary neuronal nuclei.

Figure 5. KCl depolarization of primary neuronal cultures results in significantly increased chromocenter size and changes to nuclear morphology

Wild-type primary mouse cortical neuronal cultures were derived from a single embryo and maintained for 21 DIV. Three replicate slides were depolarized with KCl for the indicated times and chromocenter measurements taken on 50 nuclei per slide. Results in A and B represent the box plot summary for 150 nuclei per condition. **= $p < 0.01$, ***= $p < 0.001$ compared to control (Ctrl), Wilcoxon rank sum. A) KCl stimulation resulted in significantly increased mean chromocenter diameter per nucleus in all timepoints compared to the untreated, control neurons. B) There were no significant differences in the number of chromocenters per nucleus between control and KCl-treated neurons. C) Representative images from control and KCl-treated neurons stained with DAPI. Individual nuclei are outlined in dashed white lines. In addition to the observed and measured changes in chromocenter diameter, DAPI fluorescence was more textured in appearance 10 min following KCl treatment, indicative of dynamic chromatin alterations following neuronal activity.

Figure 6. *Mecp2*-deficient neurons do not show an increase in chromocenter size following KCl treatment

Mecp2^{-/-} and *Mecp2*^{+/-} primary mouse cortical neuronal cultures were derived from a single embryo of each genotype and maintained for 14 DIV. Three replicate slides from each culture were depolarized with KCl for 20 minutes prior to fixation and DAPI staining. Results in A and B represent the box plot summary for 150 nuclei per genotype and condition. ***= $p < 0.001$, Wilcoxon rank sum. A) KCl stimulation in wild-type neurons resulted in significantly larger chromocenters when compared to control, untreated neurons. *Mecp2*-deficient neurons exhibited no significant change in chromocenter size following KCl treatment. B) KCl had no significant effect on the number of chromocenters per nucleus in wild-type neurons or *Mecp2*-deficient neurons. C) Representative images from control and KCl-treated neurons from wild-type and *Mecp2* deficient mice stained with anti-fibrillarin (red) and counterstained with DAPI (blue). Individual nuclei are outlined in dashed white lines.

Figure 7. Overexpression of *MECP2e1* results in significantly larger nucleoli in human SH-SY5Y cells, but mutation of the MBD of *MECP2* abrogates the effect

Human SH-SY5Y neuronal cells, either untransfected (Parental), or stably transfected with MECP2e1 (Flag MeCP2e1) or mutant MECP2e1 (Flag T158M) were differentiated with PMA and stained for anti-nucleolin and DAPI for nucleolar measurements. Results in A–D represent the box plot summary for 150 nuclei per cell line. * = $p < 0.05$, ** = $p < 0.01$, and *** = $p < 0.001$ compared to parental SH-SY5Y cells, Wilcoxon rank sum. A) SH-SY5Y cells stably overexpressing MeCP2e1 showed significantly larger mean nucleolar diameter per nucleus compared to the parental cell line. In contrast, overexpression of the T158M mutant form of MeCP2 resulted in significantly smaller mean nucleolar diameter per nucleus compared to the parental cell line. B) SH-SY5Y cells stably overexpressing MeCP2e1 showed significantly larger nucleoli than the parental cell line, reflected by the largest nucleolar diameter per nucleus. In contrast, overexpression of the T158M mutant form of MeCP2 resulted in significantly smaller largest nucleolar diameter per nucleus compared to the parental cell. C) SH-SY5Y cells stably overexpressing MeCP2e1 and MeCP2e1 T158M both have significantly larger nuclei compared to the parental cell line. While both MeCP2e1 transfected cell lines were significantly different from the untransfected control, the higher p value obtained for the MeCP2e1 compared to the MeCP2e1 T158M was because the means were different (12.44 vs. 12.21 microns compared to 11.53 microns mean of the SH-SY5Y parental cell line). D) SH-SY5Y cells stably overexpressing MeCP2e1 and MeCP2e1 T158M both have significantly more nucleoli compared to the parental cell line.