Inhibitors of differentiation (ID1, ID2, ID3 and ID4) genes are neuronal targets of MeCP2 that are elevated in Rett syndrome

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Rett syndrome (RTT) is an X-linked dominant neurodevelopmental disorder caused by mutations in MECP2, encoding methyl-CpG-binding protein 2. MeCP2 is a transcriptional repressor elevated in mature neurons and is predicted to be required for neuronal maturation by regulating multiple target genes. Identifying primary gene targets in either Mecp2-deficient mice or human RTT brain has proven to be difficult, perhaps because of the transient requirement for MeCP2 during neuronal maturation. In order to experimentally control the timing of MeCP2 expression and deficiency during neuronal maturation, human SH-SY5Y cells undergoing mature neuronal differentiation were transfected with methylated MeCP2 oligonucleotide decoy to disrupt the binding of MeCP2 to endogenous targets. Genome-wide expression microarray analysis identified all four known members of the inhibitors of differentiation or inhibitors of DNA-binding (ID1, ID2, ID3 and ID4) subfamily of helix–loop–helix genes as novel neuronal targets of MeCP2. Chromatin immunoprecipitation analysis confirmed binding of MeCP2 near or within the promoters of ID1, ID2 and ID3, and quantitative RT-PCR confirmed increased expression of all four Id genes in Mecp2-deficient mouse brain. All four ID proteins were significantly increased in Mecp2-deficient mouse and human RTT brain using immunofluorescence and laser scanning cytometric analyses. Because of their involvement in cell differentiation and neural development, ID genes are ideal primary targets for MeCP2 regulation of neuronal maturation that may explain the molecular pathogenesis of RTT.

INTRODUCTION

Rett syndrome (RTT) is an X-linked dominant disorder caused by mutation in methyl-CpG-binding protein 2 (MECP2) located on Xq28 (1,2). RTT primarily affects females, who are mosaic for expression of the MECP2 mutation because of random X-inactivation (3). The onset of symptoms is delayed until 6–18 months of age and includes severe mental retardation with absence of speech, stereotypic hand movements, epileptic seizures and respiratory dysfunction (4–6).

MeCP2 belongs to a family of methyl-CpG-binding proteins consisting of MBD1, MBD2, MBD3 and MBD4, all of which contain a conserved methyl-CpG-binding domain (MBD) (7,8). Mice lacking MBD1 show deficits in adult neurogenesis and hippocampal function (9). Mice lacking MBD2 show mild maternal behavior deficits, whereas MBD3−/− mice die at an early embryonic stage (10). Mice deficient in MBD4, a mismatch repair enzyme, show deficits in DNA repair and increased tumor formation (11). Mecp2-deficient and mutant mouse models recapitulate the neurodevelopmental symptoms of RTT (12–14).

MeCP2, the most extensively studied member of MBD family, is a nuclear protein dynamically expressed during postnatal mammalian brain development and is a marker for neuronal maturity (15–18). Elevated MeCP2 expression is hypothesized to be required for neuronal differentiation by the regulation of multiple target genes (19). MeCP2 binds to DNA through its MBD and complexes with the transcriptional repressor Sin3A and histone deacetylase through the transcriptional repressor domain (20–22). The involvement of MeCP2 in methylation-specific transcriptional repression (20,23) suggested that MeCP2 deficiency in RTT would result in wide spread gene dysregulation. This hypothesis was previously tested using gene expression microarray analysis with...
Mecp2-deficient mouse brain (24), RTT patient cell lines (25) and post-mortem RTT brain tissue (26). Subtle and non-overlapping transcriptional changes were observed in each of these studies, indicating that MeCP2 deficiency does not result in obvious high levels of genome-wide transcriptional dysregulation. The changes in gene expression observed due to MeCP2 deficiency in human and mouse brains could be representative of events downstream of direct MeCP2 target genes and therefore may be inherently noisy.

Alternative approaches have also been used to identify primary targets of MeCP2. In mammals, BDNF has been identified by a candidate gene approach (27,28) and DLX5 was identified by cloning fragments from MeCP2 chromatin immunoprecipitation (ChIP) (29). In Xenopus laevis, the neuronal repressor xHairy2a was identified as a target of MeCP2 in differentiating neuroectoderm (30). A cDNA microarray analysis was performed on lymphoblastoid cell lines derived from RTT patients with and without MeCP2 mutations, followed by ChIP to distinguish the direct targets of MeCP2 from indirect targets (31). Significantly reduced expression of UBE3A and GABRB3 genes within the human 15q11–13 region was shown using multiple quantitative methods in MeCP2-deficient mice and RTT patients (32). Finally, a recent study demonstrated increased expression of serum glucocorticoid-inducible kinase 1 (Sgk) and FK506-binding protein 5 (Fkbp5) in Mecp2-deficient mouse brain, suggesting MeCP2 as a modulator of glucocorticoid-inducible gene expression (33). Each of these studies identified various genes dysregulated due to MeCP2 deficiency but none of the target genes could completely explain the primary neurodevelopmental defect observed in RTT patients.

Because the phenotypic effects of MeCP2 deficiency appears to be limited to a precise stage of postnatal neuronal maturation, we used an alternative approach to experimentally control the timing of MeCP2 expression and inhibition in a neuronal cell culture system. Microarray analysis of transcriptional changes, during maturational differentiation, that were specifically altered by MeCP2 inhibition identified ID1, ID2, ID3 and ID4 genes as primary targets of MeCP2. All four ID genes belong to the same class of helix–loop–helix (HLH) transcriptional regulators, encoding known inhibitors of differentiation or inhibitors of DNA binding that block the function of tissue-specific basic helix–loop–helix (bHLH) transcription factors involved in regulation of important neuronal differentiation genes such as NEUROD1. We report significantly increased protein expression of all four ID genes in both Mecp2-deficient mice and RTT human brain tissue compared with wild-type mice or age-matched controls. Further understanding the role of ID genes as neuronal targets of MeCP2 may explain the arrest in postnatal neuronal maturation seen in RTT.

RESULTS
Microarray analysis performed to identify the primary targets of MeCP2 during SH-SY5Y differentiation

In order to identify genes specifically regulated by MeCP2 at a precise stage of neuronal maturational differentiation, expression microarray experiments were conducted on human SH-SY5Y neuronal cells transfected with a methylated oligonucleotide decoy to block MeCP2 binding to endogenous target genes during phorbol 12-myristate 13-acetate (PMA)-induced maturational differentiation. SH-SY5Y human neuroblastoma cells were chosen because they can be induced by PMA to produce a functional sympathetic neuronal phenotype (34), resulting in the 2-fold increase in MeCP2 expression by 48 h (35). The MeCP2 decoy (MD) is a 22 mer double-stranded DNA molecule containing two methylated CpG sites adjacent to AT-runs (36). A control sequence with CpG sites mutated to AT was used as a specificity and transfection control decoy (CD). The decoy approach of blocking MeCP2 binding in SH-SY5Y cells has been previously validated in our laboratory and the specificity to MeCP2 was demonstrated by ChIP at the SNURF/SNRPN locus (37).

Four different SH-SY5Y treatments were compared by gene expression-profiling experiments: (i) undifferentiated (UD), (ii) 48 h differentiated and untransfected (D-UT), (iii) 48 h differentiated and MeCP2 decoy transfected (D-MD) and (iv) 48 h differentiated and control decoy transfected (D-CD). For each cell treatment, total RNA was isolated from triplicate biological experiments and labeled cRNA was hybridized to Affymetrix HG U133 plus 2.0 arrays (12 arrays in total). Data analysis was performed using dChip analysis software and significant differences between different cell treatments were identified.

As MeCP2 was hypothesized to regulate genes involved in neuronal maturation, we first chose to examine genes significantly changed following SH-SY5Y differentiation that could be potential targets of MeCP2. A Boolean logic approach was used to identify transcript levels significantly affected during differentiation by the D-MD but not the D-CD transfection. Table 1 demonstrates the pairwise analyses that were useful in determining the genes altered specifically by the MeCP2 decoy. First, transcripts showing ≥2.0 or ≤2.0-fold significant changes (P ≤ 0.05) between UD and D-UT are selected. PMA-induced differentiation of human SH-SY5Y neuronal cells resulted in upregulation of 183 genes and downregulation of 45 genes compared with undifferentiated cells. Of this selected list of 228 genes, 24 genes (20 increased and four decreased upon differentiation) were found to have significant (P ≤ 0.05) differences between undifferentiated and MeCP2 decoy but not undifferentiated and control decoy (UD versus D-MD NOT D-CD). Interestingly, from the MeCP2 target candidate gene list, four genes were from the same family of transcriptional regulators called ‘inhibitors of differentiation’ or ‘inhibitors of DNA binding’ (ID) involved in cellular proliferation and differentiation (38,39). Expression levels of three out of four genes decreased with differentiation (ID1, ID2, ID3) and one out of 12 genes increased with differentiation (ID4) were significantly increased with MeCP2 decoy treatment. The fold changes and P-values of all four ID genes from the microarray analysis are shown in Table 2 and raw data from microarray are shown in Supplementary Material, Figure S1. The complete lists of genes from the above-mentioned analysis are shown in Supplementary Material, Tables S1–S5. The simplistic pairwise analysis of D-MD versus D-CD revealed some differentially expressed transcripts (Supplementary Material, Table S6), but because the control decoy (D-CD) had an unexpected non-specific effect on MBD1 and MBD2...
Table 1. Selection for primary target genes of MeCP2 during SH-SY5Y cell differentiation

<table>
<thead>
<tr>
<th>Criteria for selection</th>
<th>Number of probe sets</th>
<th>Number of genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Significant ($P \leq 0.05$) AND increased by $\geq 2.0$-fold change in expression upon differentiation (UD versus D-UT)</td>
<td>261</td>
<td>183</td>
</tr>
<tr>
<td>Out of 183 genes, genes significant ($P \leq 0.05$) AND increased by $\geq 1.2$-fold change in expression with MeCP2 decoy NOT control decoy</td>
<td>14</td>
<td>12</td>
</tr>
<tr>
<td>Significant ($P \leq 0.05$) AND decreased by $\geq 2.0$-fold change in expression upon differentiation (UD versus D-UT)</td>
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<td>8</td>
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<td>Out of 45 genes, genes significant ($P \leq 0.05$) AND increased by $\geq 1.2$-fold change in expression with MeCP2 decoy NOT control decoy</td>
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<td>4</td>
</tr>
<tr>
<td>Significant ($P \leq 0.05$) AND decreased by $\geq 2.0$-fold change in expression upon differentiation (UD versus D-UT)</td>
<td>60</td>
<td>45</td>
</tr>
<tr>
<td>Out of 45 genes, genes significant ($P \leq 0.05$) AND increased by $\geq 1.2$-fold change in expression with MeCP2 decoy NOT control decoy</td>
<td>0</td>
<td>0</td>
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Table 2. All members of the ID gene family are significantly increased by MeCP2 decoy

<table>
<thead>
<tr>
<th>Gene description</th>
<th>Gene symbol</th>
<th>Chromosomal location</th>
<th>Undifferentiated versus differentiated and untransfected (UD versus D-UT)</th>
<th>Differentiated and untransfected versus MeCP2 decoy transfected (D-UT versus D-MD)</th>
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</thead>
<tbody>
<tr>
<td>Inhibitor of DNA binding 1</td>
<td>ID1</td>
<td>20q11</td>
<td>$-2.91$</td>
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<tr>
<td>Inhibitor of DNA binding 2</td>
<td>ID2</td>
<td>2p25</td>
<td>$-3.22$</td>
<td>0.00166</td>
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<td>ID3</td>
<td>1p36.13–p36.12</td>
<td>$-2$</td>
<td>0.00099</td>
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<tr>
<td>Inhibitor of DNA binding 4</td>
<td>ID4</td>
<td>6p22–p21</td>
<td>$2.53$</td>
<td>0.00152</td>
</tr>
</tbody>
</table>

Validation of ID gene expression microarray results by quantitative RT-PCR in human SH-SY5Y neuronal cells

The microarray results were confirmed by performing quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR) on all four ID genes in the SH-SY5Y MeCP2 decoy experimental system. Results shown in Figure 1A are relative fold changes compared with undifferentiated SH-SY5Y cells (UD) set at 1.0, represented by the hatched bar. Consistent with the microarray results, qRT-PCR data for ID1, ID2 and ID3 genes showed a decrease in transcript level in differentiated SH-SY5Y cells (D-UT), but increased expression in differentiated cells transfected with MeCP2 decoy (D-MD) relative to D-UT. The ID4 transcript levels increased following SH-SY5Y differentiation and further increased in the MeCP2 decoy (D-MD) transfected cells also compared with D-UT.

qRT-PCR of ID genes in MeCP2-deficient (MeCP2<sup>tm<sup>1.1Bird</sup></sup>y) mouse brain

To test the relevance of ID genes as neuronal targets of MeCP2 in a more relevant biological system, qRT-PCR was conducted using cDNA from postnatal 28, 49 and 70 day MeCP2<sup>−/−</sup> and littermate MeCP2<sup>+/+</sup> brain. Figure 1B shows the relative fold change of MeCP2<sup>−/−</sup> compared with MeCP2<sup>+/+</sup> brain cDNA (MeCP2<sup>+/+</sup> set to 1.0, hatched bar) for all four Id genes. At the youngest time point (P28), increases in Id1 (2.5-fold), Id2 (1.3-fold), Id3 (1.9-fold) and Id4 (3.5-fold) transcripts were observed in MeCP2<sup>−/−</sup> compared with MeCP2<sup>+/+</sup> brain samples. Interestingly, in the later P70 time point, increased transcript levels were observed only for Id1 in MeCP2<sup>−/−</sup> brain compared with MeCP2<sup>+/+</sup>. Decreased expression levels were observed for Id2, Id3 and Id4 by P70. These results not only show the reproducibility of the microarray results using qRT-PCR in a different experimental system for inhibiting MeCP2, but also suggest that the transcriptional changes in the ID genes caused by MeCP2 deficiency may be developmental stage-specific and transient.

qRT-PCR on a downstream target of ID genes in MeCP2-deficient mouse brain

To test the hypothesis that aberrantly increased expression of ID genes has long-lived consequences on the expression of the downstream genes important for neuronal maturation, qRT-PCR was also performed on an autoregulated bHLH transcription factor, neurogenic differentiation factor 1 (Neurod1) (42). Figure 1C demonstrates progressive 20–75% decreased expression of Neurod1 in MeCP2<sup>−/−</sup> brain from P28 to P70 relative to MeCP2<sup>+/+</sup> brain. It has been shown previously that ID2 in cultured HeLa and HIT cells (43) and ID2 and ID3 in Xenopus embryos (44) act as inhibitors of NEUROD1 activity. These results suggest that the transient increase in ID transcript levels associated with MeCP2 deficiency could trigger a cascade of downstream events that affect postnatal neuronal maturation.

binding (37), this comparison was less informative. The microarray data discussed in this article have been deposited in NCBI’s Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) and are accessible through GEO Series accession number GSE4600.

The effect of MeCP2 deficiency and the binding sites for MeCP2 were further characterized for the ID gene family because of the common relationship between all four ID genes (40) and their known involvement in cellular differentiation (39,41).

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from two pairs of mice per time point. Graphs (B) and (C) represent mean ± SEM of three replicate experiments. (B) The qRT-PCR data for ID1, ID2 and ID3 genes show a decreased expression with differentiation, in untransfected SH-SY5Y cells (D-UT), and increased expression with MeCP2 decoy (D-MD). qRT-PCR data of ID4 show an increased expression level following SH-SY5Y differentiation. ID4 was further increased in the D-MD transfected cells compared with 48 h D-UT. Results shown represent mean ± SEM of three replicate experiments. (B) The qRT-PCR was conducted in cDNA samples from MeCP2−/− and MeCP2+/+ mice brains. The relative fold change differences for all four ID genes were higher in the MeCP2−/− compared with MeCP2+/+ at P28 time point and reached significance by *t*-test for ID3 and ID4 (*P < 0.05*). No increase in cDNA was observed at later time points (P49 and P70). (C) The qRT-PCR results on a downstream target of ID genes, NeuroD1, showed lower expression in all three postnatal time points (P28, P49, and P70) in MeCP2−/− brain compared with the MeCP2+/+ control brain, with significantly reduced expression at P49 and P70 by *t*-test (*P ≤ 0.05*). Each time point in bar graphs (B) and (C) represents mean ± SEM of three replicate experiments from two pairs of mice per time point.

**Figure 1.** qRT-PCR results for ID1–4 and NeuroD1, a downstream target of ID genes in two different systems. (A, B and C) Graphical representation of all four ID genes’s qPCR data reflects fold changes relative to the control (set to 1.0, indicated by hatched bar), following normalization of the data to GAPDH housekeeping control using the comparative CT method. (A) The qRT-PCR data for ID1, ID2 and ID3 genes show a decreased expression with differentiation, in untransfected SH-SY5Y cells (D-UT), and increased expression with MeCP2 decoy (D-MD). qRT-PCR data of ID4 show an increased expression level following SH-SY5Y differentiation. ID4 was further increased in the D-MD transfected cells compared with 48 h D-UT. Results shown represent mean ± SEM of three replicate experiments. (B) The qRT-PCR was conducted in cDNA samples from MeCP2−/− and MeCP2+/+ mice brains. The relative fold change differences for all four ID genes were higher in the MeCP2−/− compared with MeCP2+/+ at P28 time point and reached significance by *t*-test for ID3 and ID4 (*P < 0.05*). No increase in cDNA was observed at later time points (P49 and P70). (C) The qRT-PCR results on a downstream target of ID genes, NeuroD1, showed lower expression in all three postnatal time points (P28, P49, and P70) in MeCP2−/− brain compared with the MeCP2+/+ control brain, with significantly reduced expression at P49 and P70 by *t*-test (*P ≤ 0.05*). Each time point in bar graphs (B) and (C) represents mean ± SEM of three replicate experiments from two pairs of mice per time point.

**ID protein expression changes in MeCP2-deficient mouse model using immunofluorescence and quantitative laser scanning cytometry**

Interestingly, the results from qRT-PCR data for MeCP2-deficient mice showed increased transcript for all four ID genes at P28. To examine and quantify ID protein expression, immunofluorescence followed by quantitation by laser scanning cytometry (LSC) was performed for all four ID genes on P28 day sagittal brain sections of three MeCP2−/−1.1Birdy mice and corresponding MeCP2+/+ littermate controls. The approach of immunofluorescence on tissue microarrays and brain sections followed by analysis using LSC has been previously validated in our laboratory in several studies (15,32).

Briefly, immunofluorescence was performed on the 5 μm sagittal mouse tissue sections by staining the slides with each of the four ID antibodies, followed by secondary fluorescent antibody staining and detection using LSC. The fluorescently stained cells were analyzed by LSC that creates contours around the nuclei to identify individual cells. Quantitative data regarding mean fluorescence intensities (representative of total protein expression) were recorded for each cell in different fluorescent channels. Each brain region was individually gated to obtain the mean fluorescence of each ID protein. Figure 2 shows representative LSC images for all four ID protein expressions, showing differences in regional distribution in MeCP2−/− compared with MeCP2+/+. Each pixel on the LSC image represents an individual cell, colored on the basis of each of the ID protein’s max pixel’ histograms of whole brain. Max pixel is the maximum fluorescence intensity of each contoured nucleus, as described previously (15,45). Blue-colored nuclei (negative) were gated on the basis of overlap with IgG negative control histogram. Green (low) and red (high) regions were colored on the basis of the right half max of the major peak of the ID protein max pixel histogram. Significantly increased expression of all four ID proteins was observed in multiple brain regions (cerebrum, cerebellum, thalamus and hypothalamus, medulla oblongata and pons and hippocampus) of P28 MeCP2−/− mouse brain compared with MeCP2+/+ littermate control. Numerical data from separately gated brain regions for all four ID proteins at time point P28 from three pairs of mice are shown in Table 3.

To determine whether ID protein expression changes observed in MeCP2−/− were transient or long-lived, LSC analysis was performed on a tissue microarray consisting of cerebral cortex samples from MeCP2−/− mice and corresponding MeCP2+/+ littermate controls at various developmental time points ranging from embryonic day 15 (E15) to postnatal day 70 (P70). For each time point and ID protein, fluorescence max pixel values of cells from MeCP2−/− mouse cerebral cortex tissue cores were compared with those from MeCP2+/+ cores (Table 4). The protein expression of ID1 in MeCP2−/− mouse brain showed significant increases compared with controls in E15 and in four postnatal time points (P7, P28, P49, and P70), whereas ID2 showed significant increases at E15 and five postnatal time points (P7, P28, P49, P56 and P70). The protein expression of ID3 increased significantly in P7, P56 and P70, whereas ID4 protein expression increased significantly in four postnatal time points (P28, P49, P56 and P70) of MeCP2−/− mice compared with MeCP2+/+ littermate controls.

**ID protein expression changes in human RTT and control cerebral samples using immunofluorescence and quantitative LSC**

In order to determine whether ID protein expression is also significantly higher in MeCP2-deficient RTT human brain samples, a similar approach of immunofluorescence followed by quantitation on LSC was performed. A tissue microarray containing human post-mortem cerebral cortex samples in triplicates from different RTT individuals, all with known MECP2 deficiencies (46) of different ages and corresponding age-matched control individuals, was used. Figure 3 demonstrates that one male and two females with known MECP2...
deficiencies (gray, filled histograms) show significantly higher ID protein expression compared with age-matched controls (black, open histogram). Increased ID protein expression was observed in all the RTT individuals compared with controls, except for ID1 in RTT 4312 brain tissue. The observed dysregulation of ID protein expression in RTT brains further supports the ID gene family as relevant neuronal targets of MeCP2 and suggests a role in the pathogenesis of RTT.

These combined results demonstrate that increased expression of the ID family of transcriptional regulators is observed in Mecp2-deficient mouse brain and human RTT cortical tissue. These results validate the microarray results and suggest that the novel finding of ID genes as targets of MeCP2 is not simply an artifact of the SH-SY5Y cell culture system.

**DISCUSSION**

MeCP2 protein levels are significantly higher in central nervous system (CNS) tissues compared with non-CNS tissues, addressing a major paradox in the pathogenesis of RTT regarding how mutations in ubiquitously transcribed MECP2 result in a phenotype specific to the CNS (19,45). Several studies have shown that elevated MeCP2 expression is acquired during postnatal brain development, suggesting that MeCP2 might play an important role during neuronal maturation and synaptogenesis (16,17,19). In order to identify primary MeCP2 targets in the CNS and understand the cascade of events that follows their dysregulation, studying the right tissue at the right stage of differentiation is critical.

In this study we performed expression microarray analysis to identify novel gene targets of MeCP2 during neuronal maturational differentiation in a human SH-SY5Y neuronal cell culture system. MeCP2 binding to endogenous targets at a precise stage of neuronal maturational differentiation was specifically inhibited by transfection with an MeCP2 decoy. Both transcriptionally up- and downregulated MeCP2 target genes have been observed by microarray analysis, consistent with previous studies (24–26), and arguing against a single role for MeCP2 as a transcriptional repressor.
Table 3. Significant expression differences of ID1, ID2, ID3 and ID4 proteins in different brain regions of postnatal day 28 (P28) Mecp2<sup>−/−</sup> mice compared with the Mecp2<sup>+/+</sup> mice

<table>
<thead>
<tr>
<th>Brain region</th>
<th>Pair 1 Mecp2&lt;sup&gt;−/−&lt;/sup&gt;</th>
<th></th>
<th>Pair 2 Mecp2&lt;sup&gt;−/−&lt;/sup&gt;</th>
<th></th>
<th>Pair 3 Mecp2&lt;sup&gt;−/−&lt;/sup&gt;</th>
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<tr>
<td></td>
<td>Mean ± SEM</td>
<td>Cell number</td>
<td>Mean ± SEM</td>
<td>Cell number</td>
<td>Mean ± SEM</td>
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<tr>
<td>Inhibitor of differentiation 1—ID1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Whole brain 6308 ± 8</td>
<td>52657</td>
<td>Whole brain 7435 ± 9***</td>
<td>43790</td>
<td>Whole brain 5497 ± 8</td>
<td>51079</td>
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<td>Thalamus and hypothalamus</td>
<td>7480 ± 26</td>
<td>Thalamus and hypothalamus</td>
<td>4322</td>
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<tr>
<td></td>
<td>Hippocampus 6793 ± 24</td>
<td>4210</td>
<td>Hippocampus 9519 ± 42***</td>
<td>2119</td>
<td>Hippocampus 5921 ± 29</td>
<td>3144</td>
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<tr>
<td>Inhibitor of differentiation 3—ID3&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Whole brain 5727 ± 8</td>
<td>37924</td>
<td>Whole brain 6625 ± 8***</td>
<td>43220</td>
<td>Whole brain 5265 ± 7</td>
<td>48152</td>
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<td>4146</td>
<td>Cerebellum 7374 ± 23***</td>
<td>4281</td>
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<td>Medulla oblongata and pons</td>
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<td>6451 ± 25***</td>
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<td>2725</td>
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<td>Inhibitor of differentiation 4—ID4&lt;sup&gt;d&lt;/sup&gt;</td>
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<td>40365</td>
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<td>9192</td>
<td>Medulla oblongata and pons</td>
<td>6951 ± 15***</td>
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<sup>a</sup>Significance determined by $\chi^2$ and $P$-values denoted by asterisk. *$P \leq 0.05$, **$P \leq 0.005$, ***$P \leq 0.0005$.

<sup>b</sup>ID1 rabbit monoclonal antibody provided by Dr Robert Benezra.

<sup>c</sup>ID2, ID3, ID4 rabbit polyclonal antibodies obtained from commercially available source (Santa Cruz Biotechnologies, Inc.).
Using microarray analysis, we have identified all four known members of the ID subfamily of HLH proteins as novel neuronal targets of MeCP2. The microarray results reported in this study were consistent with published data showing that ID1, ID2 and ID3 transcript levels decreased upon SH-SY5Y neuronal differentiation (47) and ID4 expression level increased following neuronal differentiation (48). Our findings are novel in demonstrating that MeCP2 regulates ID gene transcriptional changes during SH-SY5Y neuronal differentiation. However, we also showed significantly increased ID transcript levels compared with the controls in all postnatal time points tested. The transient upregulation of ID proteins results in long-lived effects on ID protein expression in the brain. We also observed decreased mRNA expression of Neurod1, a neurodevelopmentally essential gene target of ID proteins in Mecp2+/− mice compared with Mecp2+/+ controls in all postnatal time points tested. The transient upregulation of ID proteins results in disrupting the autoregulatory loop and leading to more lasting declines in proteins such as Neurog1. These results suggest that the MeCP2-deficient neurons in the mouse model and in RTT patients might become arrested in an immature stage of maturational differentiation and inappropriately high ID protein levels could either cause or reflect improper neuronal maturation.

Several tissue-specific bHLH transcription factors such as Ascl1, Neurod1 and Neurog1 control the determination and differentiation of neurons in the CNS and peripheral nervous system (49,50). bHLH proteins dimerize, bind to E-box sequences containing CANNTG and activate transcription.
of genes (50). The function of bHLH proteins is blocked by ID proteins that are structurally similar to bHLH, except that they lack the basic DNA binding-domain (38,39,51,52). Four members, ID1 through ID4, have been identified in mammals (38,48,53) as inhibitors of differentiation or inhibitors of DNA binding.

In the absence of ID proteins, bHLH factors function prematurely and terminal differentiation is improperly induced (54). Targeted deletions of all four ID genes have been performed in mouse. Deletion of either *Id1* or *Id3* individually does not lead to an observable phenotype, but when both are inactivated, telencephalic neurogenesis and angiogenesis are greatly perturbed (55). Mice lacking *Id2* are viable, although they have an abnormal immune system and a lactation defect (56,57). Mice lacking *Id4* have a small brain and compromised proliferation of stem cells in the ventricular zone (58).

The role of ID proteins during neuronal differentiation and the overall effect of MeCP2 deficiency on ID gene expression are illustrated in the model as shown in Figure 5. In this model, the 2-fold increase in MeCP2 expression during neuronal maturation would serve to downregulate expression of all four ID genes, resulting in the release of more bHLH transcription factors for binding to the E-box of genes involved in neuronal maturational differentiation. Mutation of *MECP2*...
would therefore increase the levels of ID proteins, thus tipping the balance away from cellular differentiation.

In a recent study, the levels of BDNF protein were reduced in Mecp2-deficient mouse brain and deletion of Bdnf in Mecp2-deficient mice caused an earlier onset of RTT-like symptoms. In contrast, over-expression of conditional Bdnf transgene in the Mecp2-deficient mice extended lifespan, rescued a locomotor defect and reversed an electrophysiological deficit (59). Interestingly, within rat BDNF promoter III, a Ca\(^{2+}\)-responsive E-box element (CaRE2) that binds to two bHLH transcription factors, upstream stimulatory factors 1 and 2 (USF1/2), was identified (60). In addition, a previous study showed that neurotrophins such as BDNF stimulate neuronal differentiation by altering the balance of expression of various bHLH transcription factors (61). Reduced levels of BDNF and increased levels of ID proteins in Mecp2-deficient mice might result in lower expression of bHLH proteins, resulting in improper neuronal differentiation.

ID proteins are central to pathways regulating proliferation, differentiation, angiogenesis, migration, invasion and cell–cell interaction. Therefore, by understanding the role of ID proteins during neuronal maturation, they might be therapeutically targeted to reverse the arrest in neuronal maturation seen in RTT and other neurodevelopmental disorders.

**MATERIALS AND METHODS**

**Cell culture and MeCP2 decoy transfections**

SH-SY5Y human neuroblastoma cells (ATCC) were grown in complete minimal essential media with 15% fetal calf serum in T 175 cm\(^2\) large tissue culture flasks until 70–80% confluency. The cells were transfected with decoy mixture prepared in serum-free MEM containing 3 µl FuGENE 6 (Roche) transfection reagent and 1 µM of either methylated MD or CD, incubated at room temperature for 30 min before addition to the flasks. MeCP2 decoy (MD) and control decoy (CD) were obtained commercially (GeneDetect.com). Both the control (5’ AATCTAGTCTAGACTAGATTA 3’) and MeCP2 decoy (5’ AATCCGGTCTAGA CCGGATTA 3’) double-stranded phosphorothioate oligodeoxynucleotides were treated with HpaII methylase (New England Biolabs) (1 U per 1 µg decoy DNA) overnight to methylate the CpG sites. The methylase-treated decoys, MD and CD decoy were digested with HpaII (New England Biolabs) and analyzed by PAGE to confirm methylation. Twelve hours after transfection, cells were treated with 16 nM phorbol 12-myristate 13-acetate (PMA) (Sigma). Total RNA was isolated 48 h after PMA was added using TRIzol reagent (Invitrogen Life Technologies) from four different experimental conditions: UD, D-UT, D-MD and D-CD.

**Microarray**

Total RNA yield was quantified by spectrophotometric analysis and quality was verified on a gel. A cleanup of RNA was performed using RNaseasy Mini kit (QIAGEN) before proceeding to cDNA synthesis. One microgram of RNA under each condition was first reverse-transcribed using T7-oligo (dT) promoter primer followed by RNase-H-mediated second-stranded cDNA synthesis using One-Cycle cDNA Synthesis kit (Affymetrix). The double-stranded cDNA was purified using sample cleanup modules and served as a template in the subsequent in vitro transcription reaction for complementary RNA (cRNA) amplification and biotin labeling. The biotinylated cRNA targets were cleaned, fragmented and hybridized to Affymetrix HG-U133 plus 2.0 arrays. Detailed procedure of all the steps mentioned earlier are described in the GeneChip expression analysis technical manual available on the Affymetrix website (http://www.affymetrix.com). A total of 12 array hybridizations were performed consisting of three biological replicates of four experimental conditions each. The hybridizations were performed in a GeneChip hybridization oven 640 and Gene chip fluidics station 450 at...
PCR reactions consisted of 1× DNA master SYBR Green I reaction buffer (Roche), 1.5–2 mM MgCl₂, 0.5 μM of primers and 10–20 ng of cDNA. First, stranded cDNA was synthesized using reverse transcriptase (Invitrogen) according to the manufacturer’s instructions. Primer sequences specific to all four human ID genes and the control housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), are listed in Supplementary Material, Table S7. The primer sequences for mouse ID genes were obtained from PCR primer bank (62). PCR reactions consisted of 1× DNA master SYBR Green I reaction buffer (Roche), 1.5–2 mM MgCl₂, 0.5 μM of primers and 10–20 ng of cDNA. First, stranded cDNA was synthesized using reverse transcriptase (Invitrogen) according to the manufacturer’s protocol from total RNA of SH-SY5Y cells or brain tissue from MeCP2<sup>+/y</sup> and MeCP2<sup>−/y</sup> mice. Detection of the PCR product on LightCycler is carried out at the end of the 72°C extension period. To confirm amplification specificity, the PCR products of each primer pair were subjected to a melting curve analysis. The qRT-PCR data were analyzed using the LightCycler analysis software version 2.0. Final quantification was performed using the comparative CT method (Applied Biosystems) and primer pair were subjected to a melting curve analysis. The qRT-PCR was performed using a LightCycler rapid thermal cycler system (Roche, Indianapolis, USA) according to the manufacturer’s instructions. Primer sequences specific to all four human ID genes and the control housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), are listed in Supplementary Material, Table S7. The primer sequences for mouse ID genes were obtained from PCR primer bank (62).

**Quantitative Real-Time RT–PCR**

qRT-PCR was performed using a LightCycler rapid thermal cycler system (Roche, Indianapolis, USA) according to the manufacturer’s instructions. Primer sequences specific to all four human ID genes and the control housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), are listed in Supplementary Material, Table S7. The primer sequences for mouse ID genes were obtained from PCR primer bank (62). PCR reactions consisted of 1× DNA master SYBR Green I reaction buffer (Roche), 1.5–2 mM MgCl₂, 0.5 μM of primers and 10–20 ng of cDNA. First, stranded cDNA was synthesized using reverse transcriptase (Invitrogen) according to the manufacturer’s protocol from total RNA of SH-SY5Y cells or brain tissue from MeCP2<sup>+/y</sup> and MeCP2<sup>−/y</sup> mice. Detection of the PCR product on LightCycler is carried out at the end of the 72°C extension period. To confirm amplification specificity, the PCR products of each primer pair were subjected to a melting curve analysis. The qRT-PCR data were analyzed using the LightCycler analysis software version 2.0. Final quantification was performed using the comparative CT method (Applied Biosystems) and primer pair were subjected to a melting curve analysis. The qRT-PCR was performed using a LightCycler rapid thermal cycler system (Roche, Indianapolis, USA) according to the manufacturer’s instructions. Primer sequences specific to all four human ID genes and the control housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), are listed in Supplementary Material, Table S7. The primer sequences for mouse ID genes were obtained from PCR primer bank (62). PCR reactions consisted of 1× DNA master SYBR Green I reaction buffer (Roche), 1.5–2 mM MgCl₂, 0.5 μM of primers and 10–20 ng of cDNA. First, stranded cDNA was synthesized using reverse transcriptase (Invitrogen) according to the manufacturer’s protocol from total RNA of SH-SY5Y cells or brain tissue from MeCP2<sup>+/y</sup> and MeCP2<sup>−/y</sup> mice. Detection of the PCR product on LightCycler is carried out at the end of the 72°C extension period. To confirm amplification specificity, the PCR products of each primer pair were subjected to a melting curve analysis. The qRT-PCR data were analyzed using the LightCycler analysis software version 2.0. Final quantification was performed using the comparative CT method (Applied Biosystems) and primer pair were subjected to a melting curve analysis. The qRT-PCR was performed using a LightCycler rapid thermal cycler system (Roche, Indianapolis, USA) according to the manufacturer’s instructions. Primer sequences specific to all four human ID genes and the control housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), are listed in Supplementary Material, Table S7. The primer sequences for mouse ID genes were obtained from PCR primer bank (62).

**Chromatin immunoprecipitation**

Chromatin from human SH-SY5Y neuroblastoma cells from all four conditions (UD, D-UT, D-MD and D-CD) used for microarray was experiment was isolated using a procedure previously described (37). ChIP DNA was amplified using primers designed to span 5′ CpG islands near or overlapping the promoter of each ID gene and promoter of SNURF/SNRPN as a positive control for ChIP assay conditions. Primer sequences are listed in Supplementary Material, Table S7.

**MeCP2-deficient mice**

MeCP2<sup>−/y/Bird/+</sup> (Jackson Laboratories) heterozygous female mice were mated with wild-type C57BL/6J mice (Jackson Laboratories) to produce the four possible genotypes. Genotyping was performed using protocols provided by the commercial vendors. Mice were euthanized at different developmental time points (P1 corresponds to day of birth). Whole brain specimens were cut sagitally at the midline using a sterile scalpel, and one half was placed in 10% neutral-buffered formalin for 2–3 days and then embedded in paraffin blocks; the other half was placed in TRIzol and stored at −80°C for protein and nucleic acid extractions. Sections of 1 mm were removed from each block followed by sectioning at 5 μm for experimental slides. Hematoxylin and eosin (H & E) slides were prepared for each brain specimen to aid in identification of brain regions. Epitope exposure was carried out as described previously (45).

**Mouse tissue microarray**

Wild-type C57BL/6J and MeCP2<sup>−/y/Bird/+</sup> tissue for multiple age time points was obtained, fixed and embedded in paraffin and sampled as described previously (16). Mouse tissue array included triplicate 600 μm cores of gray matter from cerebral cortex of six age-matched male mice time points for MeCP2<sup>+/y</sup> and MeCP2<sup>−/y/Bird/+</sup> (E 15, P1, P28, P49, P56 and P70).

**Human tissue microarray**

Tissues were received frozen and were fixed, embedded and arrayed as described earlier (46). The human tissue array used contained triplicate 600 μm cores of gray matter from frontal cortex, Broadman area 9, ≤30 h PMI. Tissues included on the human array were received from Maryland brain bank and the Harvard brain bank. The array consisted of three cores each of RTT brain samples, RTT 1238 (2 y), RTT 1420 (21 y), RTT 4312 (24 y), and three cores each of three age-matched controls.

**Immunofluorescence**

Tissue microarrays or whole brain sections were cut into 5 μm sections, placed on glass slides and baked overnight at 56°C. Slides underwent 4× 5 min xylene washes, 2× 5 min 100% ethanol washes, dried on a slide warmer (Hybaid) at 50°C, then placed in a 1:10 dilution of DAKO antigen retrieval solution at 95°C for 1 h, cooled to room temperature and washed for 5 min in 0.2× SSC. Primary antibodies were diluted in immunofluorescence buffer (IF buffer) (1× PBS, 0.5% Tween, 0.01% fetal calf serum) added to slides, coverslipped and incubated at 37°C for 2 h, washed three times for 5 min in 1× PBS/0.5% Tween. Secondary antibodies diluted in the same IF buffer along with 250 μg/ml RNAsed and 3 μg/ml propidium iodide (PI) were then added to slides, coverslipped and incubated at 37°C for 1 h, followed by 3× 5 min 1× PBS/0.5% Tween washes. Slides were mounted with nuclear counter stain PI (7 μg/ml PI in 50% glycerol/50% 1× PBS) for analyzing the slides on LSC. Primary antibodies used: anti-ID1 (Santa Cruz, rabbit polyclonal) and anti-ID1 (rabbit monoclonal, kind gift from Dr Robert Benezra) 1:100, anti-ID2 (Santa Cruz, rabbit polyclonal) 1:100, anti-ID3 (Santa Cruz, rabbit polyclonal) 1:100, anti-ID4 (Santa Cruz, rabbit polyclonal) 1:100, anti-MeCP2 (Aves, C-terminal, chicken polyclonal) 1:10 000 and anti-Histone H1 (Upstate, mouse monoclonal) 1:100, anti-MeCP2 (Aves, C-terminal, chicken polyclonal) 1:100, anti-MeCP2 (Aves, C-terminal, chicken polyclonal) 1:10 000 and anti-Histone H1 (Upstate, mouse monoclonal) 1:100. Secondary antibodies used: goat anti-rabbit IgG-Oregon Green (Molecular Probes) 1:100, donkey anti-rabbit IgG-CY5 (Molecular Probes) 1:100, donkey anti-chicken IgY-CY5 (Jackson) 1:100 and goat anti-mouse IgG-Cascade Blue (Molecular Probes) 1:100. Rabbit IgG (Upstate), Chicken IgY (Aves) and Mouse IgG (Upstate) were used at equivalent or higher concentrations than the primary antibodies with the same secondary to test for background levels of staining.
Laser scanning cytometry

Slides stained with the earlier-mentioned antibodies were scanned using a Compucyte Laser Scanning Cytometer, as described previously (15,45,46). Settings for voltage, PMT and threshold were identical between negative control and experimental samples. Nuclei, stained red with PI, were contoured within each core and fluorescence was measured for each nucleus for all channels (red, green, blue and long red). Data were analyzed using Wincyte software from Compucyte.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online.

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Conflict of Interest statement. None declared.

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