

## DLX5 and DLX6 Expression Is Biallelic and Not Modulated by MeCP2 Deficiency

Birgitt Schüle, Hong Hua Li, Claudia Fisch-Kohl, Carolin Purmann, and Uta Francke

Mutations in *MECP2* and *Mecp2* (encoding methyl-CpG binding protein 2 [MeCP2]) cause distinct neurological phenotypes in humans and mice, respectively, but the molecular pathology is unclear. Recent literature claimed that the developmental homeobox gene *DLX5* is imprinted and that its imprinting status is modulated by MeCP2, leading to biallelic expression in Rett syndrome and twofold overexpression of *Dlx5* and *Dlx6* in *Mecp2*-null mice. The conclusion that *DLX5* is a direct target of MeCP2 has implications for research on the molecular bases of Rett syndrome, autism, and genomic imprinting. Attempting to replicate the reported data, we evaluated allele-specific expression of *DLX5* and *DLX6* in mouse × human somatic cell hybrids, lymphoblastoid cell lines, and frontal cortex from controls and individuals with *MECP2* mutations. We identified novel single-nucleotide polymorphisms in *DLX5* and *DLX6*, enabling the first imprinting studies of *DLX6*. We found that *DLX5* and *DLX6* are biallelically expressed in somatic cell hybrids and in human cell lines and brain, with no differences between affected and control samples. We also determined expression levels of *Dlx5* and *Dlx6* in forebrain from seven male *Mecp2*-mutant mice and eight wild-type littermates by real-time quantitative reverse-transcriptase polymerase chain reaction assays. Expression of *Dlx5* and *Dlx6*, as well as of the imprinted gene *Peg3*, in mouse forebrain was highly variable, with no consistent differences between *Mecp2*-null mutants and controls. We conclude that *DLX5* and *DLX6* are not imprinted in humans and are not likely to be direct targets of MeCP2 modulation. In contrast, the imprinting status of *PEG3* and *PEG10* is maintained in MeCP2-deficient tissues. Our results confirm that MeCP2 plays no role in the maintenance of genomic imprinting and add *PEG3* and *PEG10* to the list of studied imprinted genes.

Rett syndrome (RTT [MIM #312750]) is a neurodevelopmental disorder that affects females almost exclusively. An apparently normal early-postnatal period is followed by developmental stagnation and then regression, with loss of motor skills and speech, autonomic dysfunction, and seizures.<sup>1</sup> De novo recurrent loss-of-function mutations of the X-linked gene *MECP2* (MIM +300005) are found in almost all girls who receive a clinical diagnosis of RTT<sup>2</sup> (for review, see the work of Francke<sup>3</sup>). The incidence is estimated to be 1 in 10,000 female births, and the availability of molecular diagnostic testing leads to earlier diagnosis, before the clinical picture is fully developed. *MECP2* encodes methyl-CpG binding protein 2 (MeCP2), a multifunctional protein that is expressed ubiquitously but at the highest levels in neurons. Since *MECP2* is subject to X inactivation, affected females are mosaic for cells that either have normal MeCP2 levels or lack MeCP2 function completely. Classic RTT is associated with random X-inactivation patterns, but skewed X-chromosome inactivation leads to phenotypic variants.<sup>4,5</sup> Most males with inactivating *MECP2* mutations have congenital encephalopathy, with lack of postnatal development, and respiratory insufficiency that usually leads to early death.<sup>6</sup> Recent elegant experiments with genetically manipulated mouse models revealed that the symptoms can be pre-

vented or delayed<sup>7</sup> and even reversed<sup>8</sup> when normal MeCP2 function is restored postnatally.

One of the functions of MeCP2 is to repress transcription of methylated genes by recruiting a chromatin remodeling complex to promoter regions.<sup>9</sup> Therefore, lack of MeCP2 is expected to cause abnormal expression of genes that affect postnatal neuronal function. Although large-scale misregulation of gene expression has not been observed, a few target genes have been identified by global gene-expression studies.<sup>10-17</sup> Imprinted genes that are transcribed exclusively from the maternal or the paternal allele are attractive candidates for potential MeCP2 modulation, because their uniparental expression pattern is controlled by parent-of-origin-specific methylation of specific sites (differentially methylated regions [DMRs]).<sup>18</sup> Of the ~80 known imprinted genes in mammals,<sup>19</sup> 5 (*SNRPN*, *IPW*, *NDN*, *H19*, and *IGF2*) were previously studied in RTT tissues. These genes showed monoallelic expression in MeCP2-deficient clonal cell lines and brain.<sup>20</sup> Although Samaco et al.<sup>21</sup> and Makedonski et al.<sup>22</sup> reported decreased expression levels of the brain-imprinted genes *UBE3A* and *Ube3a* in MeCP2-deficient human and mouse brain tissues, respectively, these data could not be replicated in a more extensive study of *Ube3a* RNA and protein expression in *Mecp2*-mutant mouse brains.<sup>23</sup>

Hailed as a breakthrough discovery with a major impact

From the Departments of Genetics (B.S.; H.H.L.; C.F.-K.; C.P.; U.F.) and Pediatrics (U.F.), Stanford University School of Medicine, Stanford  
Received April 6, 2007; accepted for publication May 21, 2007; electronically published August 2, 2007.

Address for correspondence and reprints: Dr. Uta Francke, Departments of Genetics and Pediatrics, Stanford University School of Medicine, 279 Campus Drive, Stanford, CA 94305-5323. E-mail: ufrancke@stanford.edu

*Am. J. Hum. Genet.* 2007;81:492–506. © 2007 by The American Society of Human Genetics. All rights reserved. 0002-9297/2007/8103-0007\$15.00  
DOI: 10.1086/520063

on RTT research was the work by Horike et al.<sup>24</sup> that reported that MeCP2 regulates the expression of the distal-less homeobox 5 gene (*DLX5*). Under the assumption that *DLX5* is imprinted, the authors claimed loss of imprinting in MeCP2-deficient cell lines and brain. In an *Mecp2*-mutant mouse model,<sup>25</sup> expression levels of *Dlx5* and *Dlx6* were reported to be increased twofold in the frontal cortex. This increase was said to be the result of “relaxed imprinting,” although *Dlx5* is known to be biallelically expressed in mice.<sup>26</sup> Horike et al.<sup>24</sup> concluded that *DLX5* is a direct target of MeCP2 modulation, and this conclusion is now widely quoted in the literature.

Using a wide range of methodologies, Horike et al.<sup>24</sup> reported a series of observations that are not coherent. First, searching for in vivo binding sites of MeCP2, they precipitated urea-gradient-purified, formaldehyde-cross-linked chromatin, derived from whole brains of 1-d-old normal mice, with anti-MeCP2 antibody and cloned the precipitated DNA fragments. Of 100 randomly sequenced clones, only 3 contained CpG dinucleotides and were consistent with promoters. The others were located in introns or up to 100 kb away from the ends of the nearest transcription unit. Scanning the vicinity of apparent MeCP2-binding sequences for biologically interesting candidates, Horike et al.<sup>24</sup> focused on *DLX5* for two reasons. First, *DLX5* directly regulates expression of glutamic acid dehydroxylase and promotes differentiation of GABAergic neurons,<sup>27</sup> and, second, *DLX5* had previously been reported to be imprinted in human lymphoblasts and brain tissues,<sup>28</sup> although not in mouse brain.<sup>26</sup>

*DLX* genes encode a family of transcription factors that contain a homeobox DNA-binding domain related to that of the *Drosophila* gene *distal-less*.<sup>29</sup> In mammalian genomes, the six known *DLX* genes occur in pairs that are closely linked. *DLX5* and *DLX6* are located within a 20-kb region in a tail-to-tail configuration on human chromosome 7q21.3 and mouse chromosome 6A1. During development, *DLX5* and *DLX6* are expressed in defined regions of the brain and in skeletal structures. *DLX5* induces bone formation and is expressed in later stages of osteoblast differentiation. *Dlx5*-knockout mice have multiple defects in their ears, noses, mandibles, and skull bones and die shortly after birth.<sup>30</sup> A proportion of them have exencephaly.<sup>31</sup> Early developmental defects in neurogenesis have also been recognized.<sup>32</sup> Notably, developmental defects were seen only in homozygous mutants, not in heterozygotes with a parent-of-origin-dependent effect, as would be expected if *Dlx5* were an imprinted gene.

Evidence that *DLX5* is imprinted in humans was first reported by Okita et al.,<sup>28</sup> who studied somatic cell hybrid (SCH) lines containing a single human chromosome 7 of defined parental origin. *DLX5* was not expressed in SCHs containing a paternal chromosome 7 but was expressed in cells containing a maternally derived chromosome 7. The hybrid cell studies of 76 ESTs from the 7q21-q31 region revealed monoallelic expression for six transcripts,

but *DLX5* was the only one for which imprinting status could be confirmed by studies of human lymphoblastoid cell lines (LCLs) and brain. Specifically, Okita et al.<sup>28</sup> studied LCLs from 15 individuals who were heterozygous for an intragenic polymorphism in *DLX5* (c.\*163dupC [rs5886002]; the alleles are referred to as “-/G” in dbSNP). RT-PCR products were sequenced and were said to reveal monoallelic expression in 13 of the 15 samples. In all eight cases in which the parental origin of the alleles could be determined, *DLX5* was reported to be expressed only from the maternal allele. The authors also examined the allelic expression of *DLX5* in brain tissue samples from three c.\*163dupC heterozygotes. Sequence analysis of RT-PCR products revealed biallelic but unequal expression, preferentially from a single allele of unknown parental origin. Nevertheless, Okita et al. concluded that “*DLX5* is imprinted and maternally expressed in normal human lymphoblasts and brain tissues.”<sup>28(p557)</sup>

Horike et al.<sup>24</sup> used the same c.\*163dupC polymorphism to assess the imprinting status of *DLX5* in LCLs from individuals with RTT and controls. By using RT-PCR with 40 cycles, they reported monoallelic expression in two normal control LCLs and in only one of four RTT LCLs. They interpreted these results to indicate “loss of imprinting” in RTT.

In the mouse, Kimura et al.<sup>26</sup> reported that *Dlx5* is not imprinted. They studied offspring of interspecies crosses, distinguished by a SNP in the 3' UTR of *Dlx5*, as well as heterozygous *Dlx5*-knockout mice that had inherited the knockout allele from either parent. Both approaches revealed that *Dlx5* is expressed in a biallelic fashion in cerebral cortex, diencephalon, olfactory bulb, hippocampus, and testis, with no allele-specific preference. Furthermore, the CpG islands of the *Dlx5* and *Dlx6* promoters were unmethylated on both alleles in wild-type and *Mecp2* Y/mice.<sup>24</sup> Moreover, no differential methylation was detected at other CpG sites in the *Dlx5/Dlx6* region between the two alleles, either in wild-type or in *Mecp2*-mutant mice. The absence of DMRs is consistent with the observed lack of imprinting of *Dlx5* and *Dlx6* in mouse brain.

Horike et al.<sup>24</sup> assessed expression of *Dlx5* and *Dlx6* in mouse brain, first by quantitative RT-PCR (qRT-PCR) experiments on brain samples from an unspecified number of 8-wk-old wild-type and *Mecp2*-mutant mice. When relative expression levels for *Dlx5* and *Dlx6*, as well as for other imprinted and nonimprinted genes, were compared, only *Dlx5* and *Dlx6* showed an approximately twofold increase in the mutant samples compared with in the wild-type samples. To determine whether the increased expression was allele specific, Horike et al. examined the SNP in the 3' UTR of *Dlx5* in interspecies crosses, as was done previously by Kimura et al.<sup>26</sup> On the basis of the intensity of restriction-enzyme fragments of RT-PCR products, Horike et al. confirmed that *Dlx5* was biallelically transcribed in frontal cortex, but transcript levels were said to be higher for the maternal allele. However, in an *Mecp2*-mutant male mouse, which was heterozygous for the *Dlx5*

SNP, both parental-specific restriction fragments were present at equal levels, which led the authors to conclude that MeCP2 deficiency abolished the albeit incomplete imprinting pattern. Notably, the transcript levels and parental-specific expressions of four other, truly imprinted genes (*Calcr*, *Sgce*, *Peg10*, and *Asb4*), located in the same gene cluster on chromosome 6, were not affected by MeCP2 deficiency.

To examine the validity of the fundamental claims that led to the identification of *DLX5* as a primary target of MeCP2, we attempted to reproduce the reported data in a systematic fashion. First, in two sets of SCHs with single paternal or maternal copies of chromosome 7, we found expression of both alleles of human *DLX5* and *DLX6*. Second, both genes were consistently expressed in a biallelic fashion in LCLs and brain samples from normal individuals and in clonal RTT LCLs. To evaluate whether MeCP2 deficiency relaxes the imprinting of truly imprinted genes in humans, we studied allele-specific expression of paternally expressed gene 3 (*PEG3*) on chromosome 19 and paternally expressed gene 10 (*PEG10*) near the *DLX5* and *DLX6* genes on 7q. We demonstrated strictly monoallelic expression of *PEG3* and *PEG10* in tissues from males and females with *MECP2* mutations. Third, to evaluate the alternative hypothesis—also put forward by Horike et al.,<sup>24</sup> that differential chromatin loop formation affecting the expression of both alleles, rather than allele-specific imprinting, could control *Dlx5* transcript levels in different brain regions—we compared expression levels of *Dlx5* and *Dlx6* in the forebrain of *Mecp2*-mutant male mice and normal male littermates. We saw no differences when we combined data from seven different litters of mice aged 7–9 wk. Our results provide strong evidence against the claim that *DLX5* and *DLX6* are targets of MeCP2 and play a role in the pathogenesis of RTT.

## Material and Methods

### SCHs

Human blood leukocytes from unaffected donors were fused with the *Hprt*-deleted mouse cell line A9. SCHs were selected in hypoxanthine-aminopterin-thymidine medium, which forces retention of the human X chromosome. Hybrid cell clones were genotyped for eight microsatellite markers on human chromosome 7, to identify those that had retained a single copy of chromosome 7<sup>33</sup> (performed by GMP Genetics). Genotyping of the donors' parents for the loci with distinguishing alleles allowed us to assign maternal or paternal origin of the chromosome 7 that was present. Chromosome 7 retention was not selected for in the hybrid clones, and we did not determine the copy numbers. Hybrid cell lines were grown in Dulbecco's modified Eagle medium with 10% fetal calf serum, 2 mM glutamine, and 1 × hypoxanthine and thymidine and were subcultured at 1:3 with TrypLE (GIBCO), by use of standard tissue-culture techniques.

### LCLs and Brain Samples

For detection and genotyping of *DLX5* and *DLX6* polymorphisms, we sequenced 14 LCLs previously established in our lab-

oratory from RTT-affected individuals with known *MECP2* mutations, 22 unaffected control LCLs, and six fetal and six adult control brain samples. For *DLX5*, 15 additional control brain samples were genotyped. For two *PEG3* SNPs, we genotyped the RTT LCLs and seven brain samples from individuals with RTT, including two hemizygous males with known *MECP2* mutations (University of Maryland Brain and Tissue Bank for Developmental Disorders [numbers 1238, 1420, 1748, 1815, 4516, and 4852]). One sample from the Harvard tissue bank is brain sample number b4160. Only the brain samples were genotyped for *PEG10* SNPs. Frozen tissue used for the expression studies was from frontal cortex. All human materials were obtained and studied under a protocol approved by the Stanford Human Research Protection Program.

### DNA Extraction and Genotyping

We extracted DNA from cultured cells, by using phenol-chloroform, and from brain tissue, by using DNA Stat 60 (Tel-Test). For PCR, we used Promega Go *Taq* in accordance with the manufacturer's instructions, with the primers listed in table 1, in a 25- $\mu$ l reaction. The PCR cycling program included an initial denaturation at 95°C for 4 min, followed by five cycles at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s. The annealing temperature was reduced by 0.5°C in the second to fifth cycle. For 30 additional cycles, the annealing temperature was 54°C for the *DLX5* genomic DNA (gDNA) primers and was 57°C for the *DLX5* cDNA primers, as well as for the *DLX6*, *PEG3*, and *PEG10* primers. The gel-purified PCR amplicons were sequenced using BigDye Terminator chemistry on an ABI 3100 sequencer (Applied Biosystems). For confirmatory studies of the *DLX5* c.\*163dupC polymorphism, we used a proofreading DNA polymerase (Platinum Pfx [Invitrogen]) to minimize slippage errors during the amplification of the mononucleotide strings.

### RNA Extraction, cDNA Synthesis, and RT-PCR Assay

We extracted total RNA from mouse × human SCH lines and frozen human cortex, using RNA Stat 60 (Tel-Test). From the LCLs, we extracted mRNA by use of Oligotex Direct mRNA Mini Kit (Qiagen). We treated the RNA with 20 U RNase-free DNaseI (Roche) for 20 min, followed by 10 min of inactivation of the enzyme at 75°C. For reverse transcription, 5  $\mu$ g total RNA or 1  $\mu$ g mRNA was incubated with Superscript III (Invitrogen), as recommended by the manufacturer, in a 40- $\mu$ l reaction. For each reaction, we used a "minus RT" control, to which no Superscript III was added. For *DLX5*, *DLX6*, *PEG3*, and *PEG10* RT-PCR assays, we used gene-specific primers (table 1) and 4  $\mu$ l of the cDNA reaction with Promega Go *Taq* Flexi, in accordance with the manufacturer's instructions. PCR cycling and sequencing of amplicons were done as described above.

### Mecp2-Mutant Mice

Female mice heterozygous for the *Mecp2*<sup>tm1.1Bird</sup> mutation<sup>25</sup> were purchased from the Jackson Laboratory and were bred with C57BL/6J male mice. Tail DNA samples were genotyped for *Mecp2*, as described elsewhere.<sup>23</sup> Mutant male mice and wild-type male littermates at age 7–9 wk were euthanized with CO<sub>2</sub>. Forebrains (the frontal one-third of the cortex) were dissected and were snap frozen on dry ice. The Animal Care Committee of Stanford University approved all experimental procedures.

**Table 1. Primers for Genotyping and Expression Analysis**

| Organism and Gene/SNP                       | Alleles | gDNA Primer (5'→3')      |                         |         | Product Size (bp)       | cDNA Primer (5'→3')     |     |  | Product Size (bp) |
|---|---------|--------------------------|-------------------------|---------|-------------------------|-------------------------|-----|--|-------------------|
|   |         | Forward                  | Reverse                 | Forward |                         | Reverse                 |     |  |                   |
| Human:                                      |         |                          |                         |         |                         |                         |     |  |                   |
| <i>DLX5</i> c.*163dupC ( <i>rs5886002</i> ) | -/G     | TTTTTTGGGACTACTGTGTTTTGC | AGATTTCAAGGCACCATTGAAAG | 203     | GCTGGGATTGACACAAACAC    | AGGCACCATTGAAAGTGTC     | 568 |  |                   |
| <i>DLX5</i> c.*142T→C                       | T/C     | Same as above            | Same as above           |         | Same as above           | Same as above           |     |  |                   |
| <i>DLX5</i> 5' UTR                          | ...     | ...                      | ...                     | ...     | GCCACAACAGCAAGGACAG     | TTTGCCATTACCATTCTCA     | 440 |  |                   |
| <i>DLX6</i> c.*9A→G ( <i>rs3213654</i> )    | A/G     | CTCCAGTCTGGGACGTTTCT     | GCTCTCCTAAGCCTGCTCCT    | 232     | TCGCTTTCAGCAGACACAGT    | CGGCTTCTTGCCACTTAT      | 457 |  |                   |
| <i>DLX6</i> c.*775dupC                      | -/C     | AAGGGAATGCTGCATGTTTT     | TAGCTTTGTGAATGCCACCA    | 202     | Same as for gDNA        | Same as for gDNA        | ... |  |                   |
| <i>DLX6</i> c.*771C→T ( <i>rs2272280</i> )  | C/T     | Same as above            | Same as above           |         | Same as above           | Same as above           |     |  |                   |
| <i>PEG3</i> c.*703A→G ( <i>rs1055359</i> )  | A/G     | CTTGTGAAGCTGTAGGCATGA    | CTGGGTCACAAAAGCCAAT     | 163     | Same as for gDNA        | Same as for gDNA        | ... |  |                   |
| <i>PEG3</i> c.42C→T ( <i>rs1860565</i> )    | C/T     | GGGATGGGTACTIONCACCCTG   | CAGGTCATTCCAACCATGTG    | 218     | AAGCCGGAGAACTGTGAGAA    | CTTCTGGGTTCTGGTGTG      | 239 |  |                   |
| <i>PEG10</i> c.*2923T→C ( <i>rs13073</i> )  | C/T     | GTGTCATTTCTGCTGCTGGT     | AGGAGCCTCTCATTACAGC     | 410     | Same as for gDNA        | Same as for gDNA        |     |  |                   |
| Mouse:                                      |         |                          |                         |         |                         |                         |     |  |                   |
| <i>Dlx5</i>                                 | ...     | ...                      | ...                     | ...     | TCTTAGGACTGACGCAAACA    | GTTACACGCCATAGGGTCGC    | 132 |  |                   |
| <i>Dlx6</i>                                 | ...     | ...                      | ...                     | ...     | TTCCGAGAGAGCCGAAC       | GTGGTTACTACCCTGTTCA     | 117 |  |                   |
| <i>Peg3A</i>                                | ...     | ...                      | ...                     | ...     | CACGAAGACGACACCAACAG    | GTCTCGAGGCTCCACATCTC    | 150 |  |                   |
| <i>Peg3B</i>                                | ...     | ...                      | ...                     | ...     | ACAGTGACGACGACATGAGC    | GTCTCGAGGCTCCACATCTC    | 122 |  |                   |
| <i>Rps28</i>                                | ...     | ...                      | ...                     | ...     | TAGGGTAACCAAAGTCTGGGCAG | GACATTTCCGGATGATAGAGCGG | 103 |  |                   |
| <i>β-actin</i>                              | ...     | ...                      | ...                     | ...     | TGACCTGAAGTACCCCATTTGA  | CCATGTCGTCCCAGTTGGTAAC  | 54  |  |                   |
| <i>Snca</i>                                 | ...     | ...                      | ...                     | ...     | GCAGCCAGAAGTCGGAAA      | TGACACATCCATGGCTAAAGA   | 58  |  |                   |



### Real-Time qRT-PCR of *Dlx5*, *Dlx6*, and *Peg3* in Mouse Brain

We extracted total RNA from frozen brain tissues by using TRIzol reagent (Invitrogen). We treated 2  $\mu$ g of total RNA with 2 U DNase I (Ambion) and reverse transcribed it with random hexamers and Superscript II (Invitrogen). After real-time qRT-PCR with Sybr Green on an ABI 5700 instrument (Applied Bioscience), we analyzed melting curves for each reaction, to ensure a single peak. *Actb* and *Rps28* served as RNA controls for relative quantitation (User Bulletin 2 [Applied Biosystems]). Details of the methods were as described in previous articles from our laboratory.<sup>12,34</sup> Primer sequences are listed in table 1. *Dlx5* primers (nt 617–636 and nt 748–729 of GenBank accession number NM\_198854.1) span the last two exons—that is, exons 2 and 3 or exons 3 and 4, depending on the transcript variant. *Dlx6* primers (nt 339–319 and nt 223–241 of GenBank accession number BC114342.1) span exons 2 and 3. For *Peg3*, two different primer sets were used: Peg3A, spanning exons 2, 3, and 4, and Peg3B, spanning exons 3 and 4.

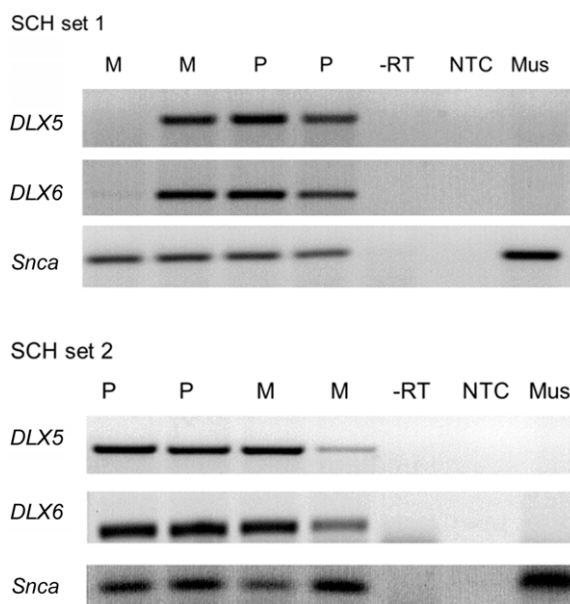
## Results

### Expression of Human *DLX5* and *DLX6* from Maternal and Paternal Chromosome 7 in Mouse $\times$ Human SCH Lines

We examined eight SCH lines, four each derived from two unrelated individuals. In both sets, two lines had retained the maternal copy of chromosome 7 and two had retained the paternal copy, as determined by STR genotyping. *DLX5* and *DLX6* were consistently expressed from both alleles in seven of the eight SCH clones (fig. 1). Quantitative expression differences between clones are likely caused by different retention frequencies of chromosome 7 in the SCH lines, and the one exceptional clone with no transcript had apparently lost chromosome 7 during propagation of the culture. For a loading control, we studied mouse *Snca* expression. The *DLX5* primers specifically amplify the human transcript, including the 5' UTR, exon 1, and part of exon 2. The forward primer is complementary only to human *DLX5*, and the reverse primer has seven mismatches with the mouse *Dlx5* sequence. The two *DLX6* primers from the 3' UTR had four and two mismatches with the *Dlx6* sequence. Both sets of primers did not amplify mouse cDNA, since no transcripts were obtained from the SCH without chromosome 7 or from mouse control brain. When we sequenced *DLX5* and *DLX6* RT-PCR products from SCH lines, both were 100% identical to the human cDNA sequences. Thus, our results provide clear evidence of biallelic expression of *DLX5* and *DLX6* from both parent-specific copies of human chromosome 7 retained in mouse  $\times$  human SCHs. Our data contradict a previous report of maternal-specific imprinted expression of *DLX5*.<sup>28</sup> The imprinting status of *DLX6* had not been determined previously.

### Biallelic Expression of *DLX5* in RTT LCLs, Normal LCLs, and Normal Human Brain

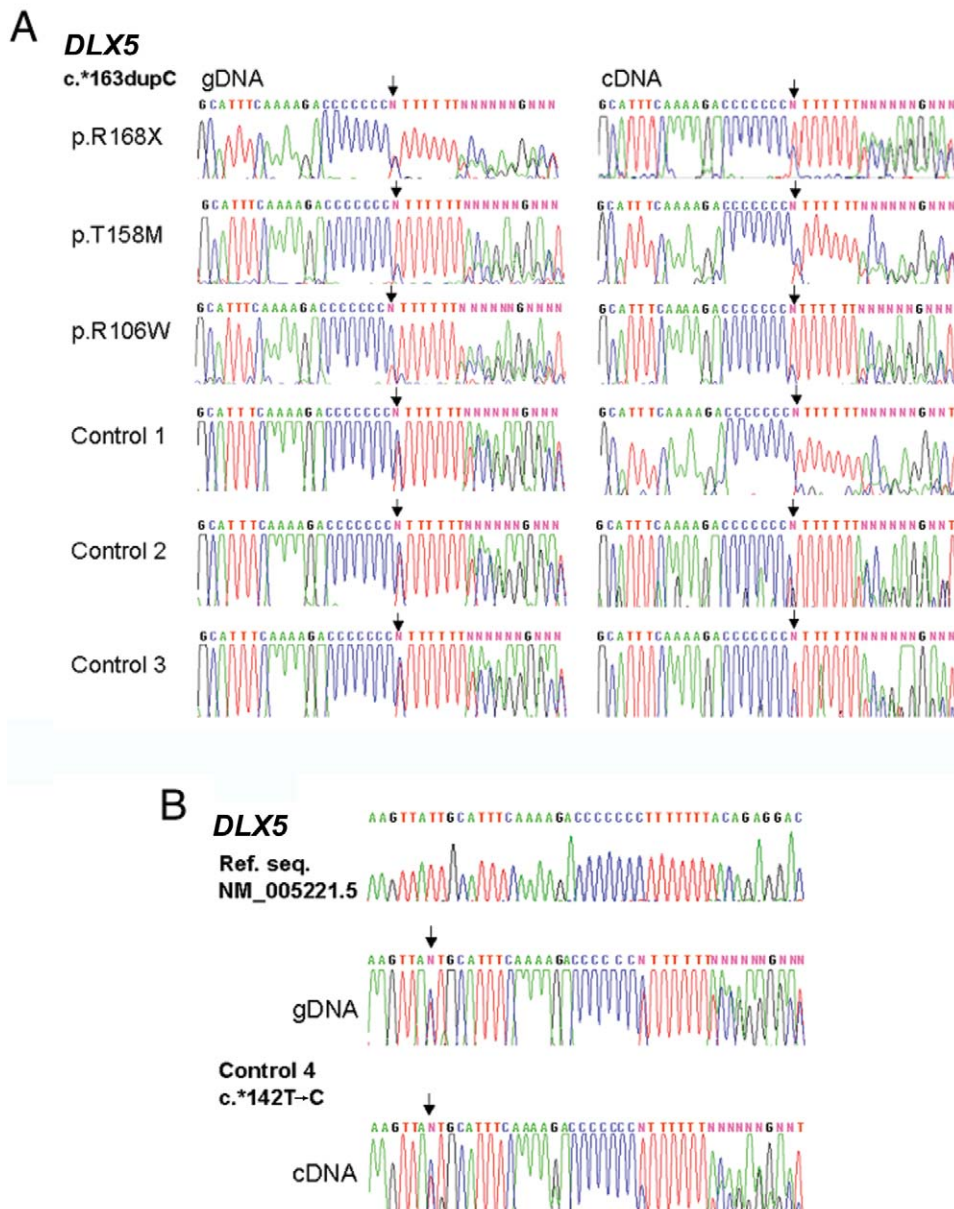
Next, we tested expression and imprinting status of *DLX5* in LCLs and brain samples by studying the same c.\*163dupC polymorphism that previously was used to



**Figure 1.** Absence of imprinting of *DLX5* and *DLX6* in SCH lines. RT-PCR analysis of human *DLX5* and *DLX6* expression in mouse  $\times$  human SCHs containing a single copy of human chromosome 7 shows that both genes are expressed from the maternally (M) as well as the paternally (P) derived chromosome 7. One SCH line in set 1 had apparently lost the maternal chromosome 7, and one line in set 2 had retained the maternal chromosome 7 at a low copy number. Primers used do not amplify the mouse (Mus) orthologues in C57BL/6 cortex. Mouse *Snca* expression is shown as a loading control. NTC = no template control; -RT = first-strand synthesis without reverse transcriptase.

distinguish the two alleles<sup>24,28</sup> (fig. 2A). To identify heterozygotes, we sequenced gDNA from 36 human LCLs and 27 brain samples. We confirmed the results with a second round of sequencing, using a proofreading polymerase. Three LCLs initially designated as heterozygous were found to be C7 homozygotes after resequencing. Frequencies of c.\*163dupC heterozygotes were 5/14 for RTT LCLs, 6/22 for normal LCLs, and 6/27 for normal brains. A novel SNP, c.\*142T→C, was discovered in one sample (fig. 2B). Allele frequencies are provided in table 2.

By RT-PCR analysis, *DLX5* was biallelically expressed in all 13 heterozygous samples that showed expression levels sufficiently high for analysis (figs. 2 and 3). The results are unlikely to be caused by gDNA contamination, because the primers used for amplification spanned an intron, and no PCR product was obtained in the absence of reverse transcriptase in the first-strand cDNA synthesis reaction. The sequence tracings suggest apparently equal amplification of transcripts from both alleles in most LCLs from individuals with RTT and unaffected controls, except for the sample with p.R106W and control 1, for which the cDNA results suggest preferential amplification of the C7 allele. Similar skewing is apparent, however, for the gDNA samples with p.T158M and p.R106W and likely is caused



**Figure 2.** Absence of imprinting of *DLX5* in LCLs. *A*, Biallelic expression revealed by *DLX5* genotyping (of gDNA) and expression analysis (of cDNA) of LCLs from three females with RTT and common *MECP2* mutations, as indicated, and three normal controls. All six LCLs are heterozygous for the *DLX5* c.\*163dupC polymorphism (*rs5886002*) (left panel), and all show that both alleles are expressed (right panel). The sequence shown is the sense strand. Arrows indicate the positions of nucleotide changes. *B*, Novel *DLX5* SNP c.\*142T→C detected in a normal LCL, denoted by an arrow in the gDNA. Comparison with the reference sequence (Ref. seq. [GenBank accession number NM\_005221.5]) shows that the SNP is located upstream of *rs5886002*. LCLs from control 4 are heterozygous for both SNPs, and both alleles are expressed in cDNA.

by PCR artifacts during amplification of the C7/C8 alleles (fig. 2). There was no obvious difference between the heterozygous clonal RTT LCLs that expressed either mutant *MECP2* in 100% of cells (p.R168X and p.T158M) or wild-type *MECP2* in 100% of cells (p.R106W).<sup>12</sup>

The low expression levels of *DLX5* and *DLX6* in LCLs presented a considerable challenge. Initially, when we raised the PCR cycle number to  $\geq 40$ , we occasionally saw

expression of only one or the other allele, but not in a parent-of-origin-specific manner. These results were not reproducible and were probably caused by the very low copy number of transcripts. We obtained consistent and reproducible biallelic expression only when we performed RNA extraction, first-strand cDNA synthesis, and PCR amplification in one session, avoiding freeze-thaw cycles of the samples. In contrast, RT-PCR of all six frontal cortex

**Table 2. DLX5 and DLX6 Sequence Variants**

| SNP                    | dbSNP            | No. of Unrelated Individuals Studied | Allele Frequencies |
|------------------------|------------------|--------------------------------------|--------------------|
| <i>DLX5</i> c.*163dupC | <i>rs5886002</i> | 64                                   | - = .87 C = .13    |
| <i>DLX5</i> c.*142T→C  | No record        | 50                                   | T = .99 C = .01    |
| <i>DLX6</i> c.*9A→G    | <i>rs3213654</i> | 46                                   | A = .98 G = .02    |
| <i>DLX6</i> c.*775dupC | No record        | 50                                   | - = .99 C = .01    |
| <i>DLX6</i> c.*771C→T  | <i>rs2272280</i> | 50                                   | C = .98 T = .02    |

NOTE.—Reference sequences were *DLX5* (GenBank accession number NM\_005221.5) and *DLX6* (GenBank accession number NM\_005222.2). All variants are described in accordance with guidelines from den Dunnen and Antonarakis<sup>35</sup>; updates can be found at HGVS Nomenclature for the Description of Sequence Variants.

samples from non-RTT donors generated strong and unambiguous biallelic expression of *DLX5* (fig. 3). We conclude that *DLX5* is not imprinted in human LCLs and cortex.

#### Biallelic Expression of *DLX6* in Normal Human Brain

The imprinting status of *DLX6* had not been determined previously because of a lack of suitable polymorphisms.<sup>24</sup> We screened our LCLs and brain samples for heterozygosity at two rare SNPs (c.\*9A→G [*rs3213654*] and c.\*771C→T [*rs2272280*]) that are located ~800 nt apart in the 3' UTR and are in linkage disequilibrium. One of 22 normal LCLs and 1 of 12 non-RTT brain samples were heterozygous for both SNPs. In addition, one fetal brain gDNA showed a novel c.\*775dupC variant. By RT-PCR, *DLX6* expression in the LCLs was too low for reproducible results; however, we obtained reliable data for the two brain samples, and both showed biallelic expression of *DLX6* (fig. 4).

#### Monoallelic Expression of Imprinted Genes *PEG3* and *PEG10* in *MeCP2*-Deficient Tissues

Because we were unable to confirm that *DLX5* and *DLX6* are imprinted in humans, our results could not address the question of whether *MeCP2* deficiency leads to “relaxation of imprinting” in our samples. Therefore, we initially examined the bona fide imprinted gene *PEG3* as a positive control. Of 13 RTT LCLs, 7 were heterozygous for the common c.42C→T polymorphism (*rs1860565*). We tested the allele-specific *PEG3* expression of three LCLs carrying different *MECP2* mutations (p.R106W, p.R168X, and c.806delG). By using the androgen-receptor assay and *MECP2* expression analyses, we had previously discovered completely skewed X-inactivation patterns in these three LCLs.<sup>12</sup> Although the p.R168X LCLs expressed the mutant *MECP2* allele in 100% of cells, the p.R106W and c.806delG LCLs were 100% wild type for *MECP2* expression. All three LCLs showed strictly monoallelic expression of *PEG3* (fig. 5A), indicating that imprinting of this gene is maintained in the absence of *MeCP2* protein.

To assess the *PEG3* imprinting status in brain, we tested five brain samples from females with RTT for two *PEG3*

SNPs, c.\*703A→G (*rs1055359*) and c.42C→T (*rs1860565*), and identified three heterozygotes. One had no known *MECP2* mutation, the second had missense mutation c.451G→T (p.D151Y), and the third was reported to have c.378-2A→G, affecting the 5' splice site of exon 4. By RT-PCR analysis of this brain sample, we were able to document that this mutation leads to abnormal splicing, causing a frameshift and premature stop codon. The mutant and wild-type *MECP2* transcripts were amplified to similar degrees (data not shown). We also included two brain samples from males with congenital encephalopathy who were hemizygous for c.806delG and c.1154\_1185del32 *MECP2* mutations and heterozygous for one of the *PEG3* SNPs. All five brain samples showed strictly monoallelic expression of *PEG3* (fig. 5A).

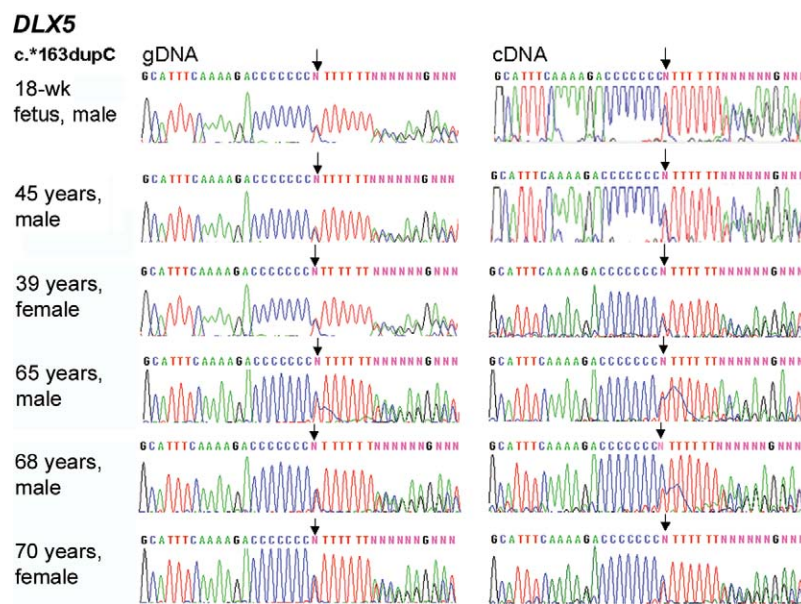
To extend the control studies to another imprinted gene, we selected *PEG10* because it is located on chromosome 7 near the *DLX5* and *DLX6* loci. Three of our six *MECP2*-mutant brain samples genotyped for SNPs, one male and two female, were heterozygous for the common *PEG10* SNP c.\*2923T→C (*rs13073*). Only one *PEG10* allele was expressed in each of the samples (fig. 5B). We conclude that the *MeCP2* protein is not necessary for the maintenance of *PEG3* and *PEG10* imprinting in human brain.

#### Highly Variable Expression Levels of *Dlx5*, *Dlx6*, and *Peg3* in Mouse Brain

Although *Dlx5* is not imprinted in mouse, Horike et al.<sup>24</sup> reported a twofold increase in expression of *Dlx5* and *Dlx6* in *Mecp2*-mutant brain compared with that in control brain. To explain that increase, they postulated that *MeCP2* induces a specific “silent chromatin loop” formation that affects expression levels, presumably on both alleles. As the first step to evaluate the validity of this model, we attempted to reproduce the reported expression changes.

We established robust qRT-PCR assays on mouse forebrain in which *Dlx5* and *Dlx6* are highly expressed. Both the *Dlx5* and *Dlx6* primers span an intron, and the *Dlx5* primers amplify both splice variants. We used mice with the same mutant allele (*Mecp2*<sup>tm1.1Bird</sup>) on the same strain background (C57BL/6J) and bred them with mice of the same age (7–9 wk) as in the previously published report. We normalized qRT-PCR data for *Dlx5* and *Dlx6* to two housekeeping genes whose expression is not affected by *MeCP2* deficiency at that age: *Actb* (encoding  $\beta$ -actin) and *Rps28*.<sup>17</sup> We then compared the relative transcript levels of *Dlx5* and *Dlx6* in 15 male mice (7 *Mecp2*-mutant and 8 wild-type mice) from seven different litters. When all data were combined, *P* values were not significant (fig. 6A). We show the actual data, to allow comparisons among littermates. The range of variation (“biological noise”) is quite striking. Although selective presentation of a subset of these data could yield statistically significant results, by studying a larger number of animals, we were





**Figure 3.** Absence of imprinting of *DLX5* in frontal cortex. *DLX5* genotyping (of gDNA) and expression analysis (of cDNA) of frontal cortex brain samples from unaffected controls revealed biallelic expression. All six donors (age and sex indicated) were heterozygous for the c.\*163dupC (*rs5886002*) polymorphism (left panel). In all brain samples, both alleles are expressed at a similar level (right panel). Arrows indicate the positions of nucleotide changes.

unable to confirm that expression of *Dlx5* and *Dlx6* is increased in *Mecp2*-mutant brain.

To assess whether a similar range of variation would be observed for expression of an imprinted gene, we performed qRT-PCR analyses on the same mouse forebrain samples, with two different intron-spanning primer sets for *Peg3* (fig. 6B). An increase in *Peg3* expression in mutants was seen in some litters but not in others. The similarity of patterns obtained with the *Peg3A* and *Peg3B* primer sets suggests that technical variability plays a minor role. The variation has a biological basis and is unrelated to the *Mecp2* genotype. Therefore, we saw no justification for engaging in chromatin modification and chromatin loop assays to evaluate the remaining aspects of the model described in the previous report. The data we obtained argue against the foundation of the proposed model. *DLX5* is not imprinted, and there is no relaxation of imprinting in RTT cell lines or brains and no consistent overexpression of *Dlx5* and *Dlx6* in *Mecp2*-mutant mouse brain. It is highly unlikely, therefore, that *DLX5* is a direct target of MeCP2.

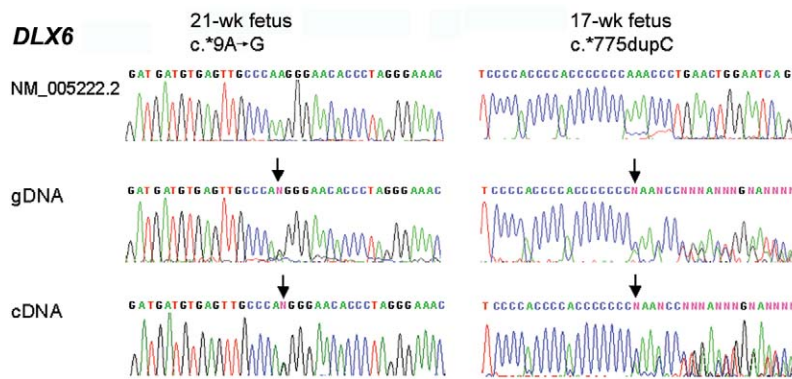
## Discussion

A previous report identified *DLX5* as a target of MeCP2 and drew wide-ranging conclusions linking RTT with genomic imprinting. The purported “loss of imprinting” of *DLX5* was speculated to have implications for GABA neurotransmission and for autism.<sup>24</sup> Numerous articles have cited the conclusions of Horike et al.<sup>24</sup> as facts, with consequences for research on autism, RTT, Angelman syn-

drome, and genomic imprinting.<sup>36–43</sup> It is the purpose of this article to set the record straight. We provide unequivocal evidence against the claim that *DLX5* is imprinted in humans and is a direct target of MeCP2 modulation. As summarized in table 3, we report consistent biallelic expression in interspecies SCHs, LCLs, and cortex from normal donors, with no difference between them and RTT samples. We report the first evidence that *DLX6* is not imprinted either. This result is expected, since *Dlx5* and *Dlx6* are coordinately regulated under the control of *Dlx1* and *Dlx2* via conserved intergenic enhancers.<sup>44</sup> We also point out the inconsistencies of the previous work, calling into doubt the claimed subtle preference of maternal allele expression in normal mouse brain and the reported twofold increase of expression of *Dlx5* and *Dlx6* in *Mecp2*-null brain. By comparing forebrain samples from 15 mice from seven litters, we found that expression of *Dlx5* and *Dlx6* was highly variable, with no significant differences between male *Mecp2*-mutants and normal male littermates. Thus, our serious attempts to replicate the reported data have yielded absolutely no evidence of imprinting of *DLX5* or evidence of any influence of MeCP2 deficiency on the expression of *DLX5/Dlx5* or *DLX6/Dlx6*.

Therefore, any postulated models to explain the non-reproducible data, such as “silent chromatin loop formation,”<sup>24(p38)</sup> are unfounded. Attempting to explain their finding of increased *Dlx5* and *Dlx6* expression in *Mecp2*-deficient mouse brain, Horike et al.<sup>24</sup> identified a small region in an intron that contains a single methylated CpG





**Figure 4.** Absence of imprinting of *DLX6* in fetal brain. *DLX6* genotyping (of gDNA) and expression analysis (of cDNA) in two fetal brain samples revealed biallelic expression. The 21-wk fetus (*left panel*) was heterozygous for the known c.\*9A→G SNP (*rs3213654*). The 17-wk fetus (*right panel*) was heterozygous for a novel SNP, c.\*775dupC. The upper trace displays the reference sequence, GenBank accession number NM\_005222.2. Arrows indicate the positions of nucleotide changes.

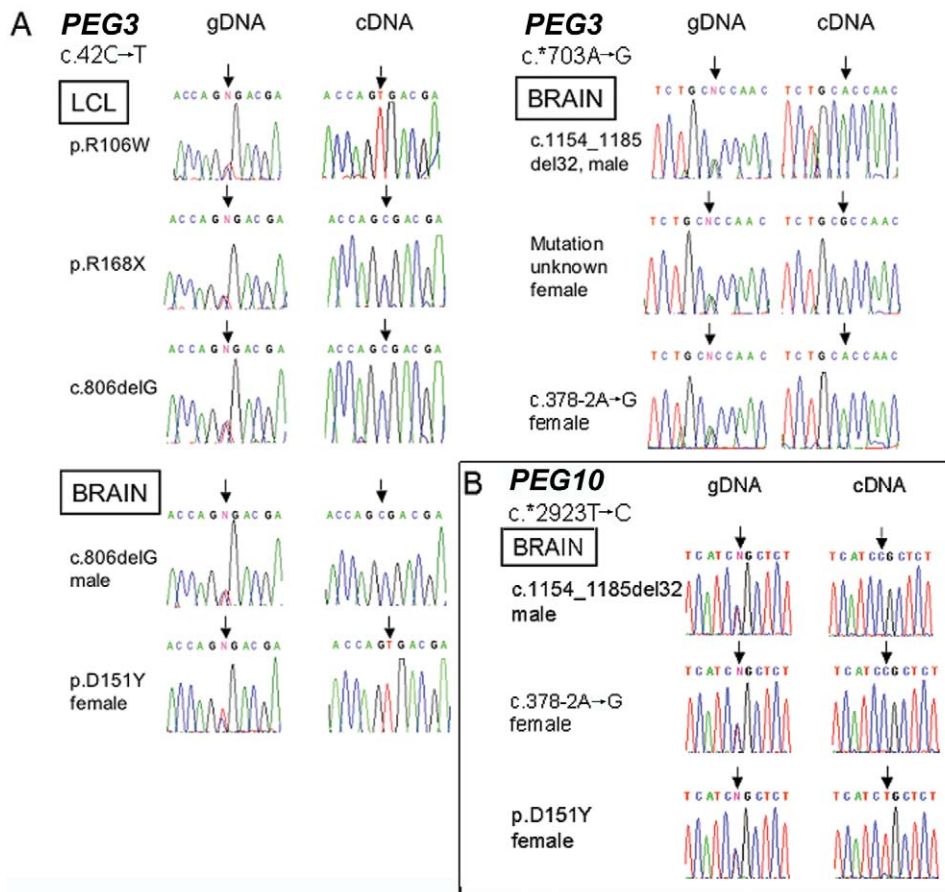
site to which MeCP2 binds. Histone-acetylation and histone-methylation patterns associated with this region were reported to be different between mutant and wild-type mouse brains. It was, therefore, claimed that the chromatin modification differences at this site result in increased *Dlx5* expression. On the basis of their prior work with the nuclear matrix binding protein SATB1 (i.e., special AT-rich sequence binding 1) that regulates genes by folding chromatin into loop domains in thymocytes,<sup>45</sup> Horike et al.<sup>24</sup> then used a chromosome conformation capture (3C) method to generate evidence of differential chromatin loop formation in wild-type versus *Mecp2*-mutant brains. The proposed model of an 11-kb loop of “silent chromatin” in normal cells and its absence in *Mecp2*-deficient cells does not, however, correlate with the difference in allele-specific *Dlx5* expression reported in the same article. If it were true that MeCP2 facilitates a chromatin loop formation that induces transcriptionally silent chromatin, this should lead to reduced expression of *Dlx5* and *Dlx6* in normal brain compared with in *Mecp2*-null brain, *without any allelic difference*, because the CpG in the intron to which MeCP2 presumably binds is equally methylated on both alleles.<sup>24</sup> The model proposed is inconsistent with the fact that *Dlx5* is expressed at high levels in normal forebrain. If the proposed silencing mechanism were active in a brain region, such as the cerebellum, where *Dlx5* is not expressed, then MeCP2 deficiency should lead to *Dlx5* expression in the mutant cerebellum. But Horike et al.<sup>24</sup> reported only qRT-PCR data for frontal cortex from 8-wk-old *Mecp2*-mutant mouse. In our cerebellum microarray studies, *Dlx5* expression was not increased in 2-wk-old, 4-wk-old, and 8-wk-old *Mecp2* mutants,<sup>17</sup> and the level of *Dlx5* expression in cerebellum was too low to be reliably measured by use of qRT-PCR. Therefore, the proposed mode of MeCP2 modulation of *Dlx5* expression does not fit the cerebellum expression data either.

That MeCP2 may function in chromatin compaction

was first proposed by Georgel et al.,<sup>46</sup> an article not cited by Horike et al.<sup>24</sup> By elegant in vitro studies, Georgel et al. showed that MeCP2 assembles novel secondary chromatin structures from nucleosome arrays but does so independent of DNA methylation. They identified the C-terminal domain of MeCP2 as the region necessary for the nucleosome compaction and suggested that the ability of MeCP2 to silence chromatin may be related to its effect on large chromatin organization. Recently, complex interactions of MeCP2 with DNA and chromatin were revealed by the study of mutant constructs, and they could be divided into methylation-dependent and methylation-independent interactions, with multiple chromatin binding sites identified.<sup>47</sup> The functional implications of these interactions are yet unknown.

#### *Expression of Human Genes in Rodent × Human SCHs That Retain a Reduced Number of Human Chromosomes*

When the inability to reproduce published data is reported, the discussion needs to focus on experimental design and methodology. Imprinting of human *DLX5* was first reported by Okita et al.,<sup>28</sup> who studied expression in SCHs containing virally tagged single human chromosomes. In contrast, we found biallelic expression of *DLX5* in two recently generated sets of SCHs that segregate human chromosomes randomly. Historically, SCHs were a very useful tool in genetic research. The first decade of human gene mapping relied on the expression of human genes located on the human chromosomes retained in interspecies hybrid cell lines. Distinction between the human and rodent gene products was achieved by electrophoretic separation of proteins that either were stained for activity by substrate binding, in cases of enzymes, or were immunologically detected with human-specific antibodies.<sup>48</sup> Faithful expression of human genes in the rodent cell environment, regardless of the number or iden-



**Figure 5.** Imprinting of *PEG3* and *PEG10* in *MECP2* mutants. *A*, Maintenance of imprinting of *PEG3* in cell lines and brains from males and females with *MECP2* mutations. Samples on the left are heterozygous for the *PEG3* c.42C→T SNP (*rs1860565*) (gDNA); samples on the right are heterozygous for the c.\*703A→G SNP (*rs1055359*) (gDNA). Arrows indicate the locations of variant nucleotides. As shown in the right-hand traces (cDNA), all samples express *PEG3* from only one allele. *B*, Maintenance of imprinting of *PEG10* in frontal cortex from one male and two females with *MECP2* mutations who are heterozygous for the c.\*2923C→T SNP (*rs13073*). RT-PCR analyses reveal strictly monoallelic expression (cDNA). Arrows indicate the positions of variant nucleotides.

tity of other human chromosomes present, led to the assignment of numerous gene loci to chromosomes or chromosome regions.<sup>49</sup> Extensive work by many researchers solidified the notion that the rodent genome contains all the transcriptional machinery required for expression of human genes from individual human chromosomes retained.

The use of rodent × human SCHs to detect allele-specific expression of imprinted genes was pioneered by Meguro et al.<sup>50</sup> By use of microcell-mediated chromosome-transfer methods, the authors generated monochromosomal hybrids with mouse A9 cells that retained only a single virally tagged human chromosome 15 of either parental origin. Working with these cell lines, Meguro et al. reported that the three GABA<sub>A</sub> receptor subunit genes in the Prader-Willi syndrome/Angelman syndrome deletion region (*GABRB3*, *GABRA5*, and *GABRG3*) are imprinted and paternally expressed. This finding, although still widely cited, was not confirmed in a later study that used

a large set of preexisting mouse A9 × human SCHs.<sup>51</sup> Gabriel et al.<sup>51</sup> validated the general approach, however, by demonstrating that imprinted expression patterns were faithfully retained in SCHs containing either maternal or paternal copies of chromosomes 11 and 15, although the allele-specific DNA methylation patterns were rather variable.

In 1999 and 2001, a Japanese group reported that they made large sets of monochromosomal hybrids that would allow the systematic detection of imprinted genes and other epigenetic phenomena.<sup>52,53</sup> A subset of these hybrids was used to study the imprinted gene cluster on chromosome 7 and led to the report that *DLX5* is imprinted,<sup>28</sup> a result we could not confirm. The successful use of SCH lines for the identification of imprinted genes relies on several prerequisites. First, the parental origin of the single human chromosome retained needs to be reliably identified. Second, the transcription unit to be studied has to be retained intact, because hybrid cells, cultured over ex-

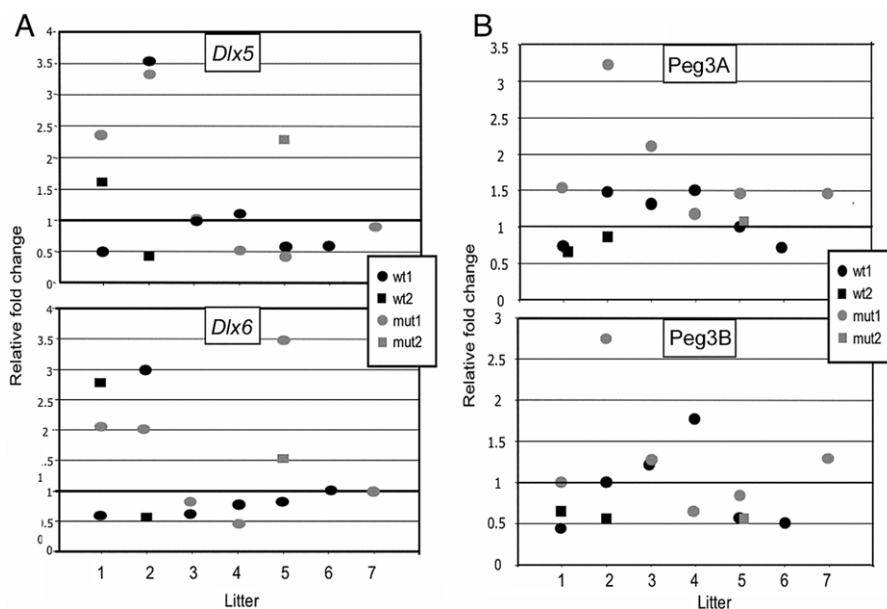
tended periods, may undergo chromosome losses, rearrangements, and mutations. Third, the method for detection of expression must reliably distinguish the human and the rodent gene products. In the studies reported here, we used well-characterized hybrid cells that were derived from two unrelated human donors for whom the parental origin of chromosome 7 alleles had been unequivocally established by genotyping all four parents. The SCH lines had been in culture for only a limited time. Originally, we chose two SCHs from each donor that had retained the paternal chromosome 7 and two that had retained the maternal copy, but one of the four lines with the maternal chromosome 7 subsequently lost this chromosome, as determined by PCR amplification of gDNA. Our *DLX5* primers were specific to cDNA, not amplification of gDNA. Our primers also were designed with sufficient mismatches against the mouse sequence, to ensure that no mouse transcript would be amplified, and we used mouse brain RNA as a negative control. We detected unambiguous amplification of only human-specific transcripts of *DLX5* and *DLX6* in SCH lines containing either the maternal (three lines) or the paternal (four lines) chromosome 7, contradicting the findings of Okita et al.<sup>28</sup> We note that other

**Table 3. Human *DLX5*, *DLX6*, *PEG3*, and *PEG10* Expression**

| Gene and Sample      | Expression  | No. of Samples  |
|----------------------|-------------|-----------------|
| <i>DLX5</i> :        |             |                 |
| SCHs                 | Biallelic   | 3 mat and 4 pat |
| RTT LCLs             | Biallelic   | 3               |
| Normal LCLs          | Biallelic   | 4               |
| Normal fetal brain   | Biallelic   | 1               |
| Normal adult cortex  | Biallelic   | 5               |
| <i>DLX6</i> :        |             |                 |
| SCHs                 | Biallelic   | 3 mat and 4 pat |
| Fetal brain          | Biallelic   | 2               |
| <i>PEG3</i> :        |             |                 |
| RTT LCLs             | Monoallelic | 3               |
| RTT brain            | Monoallelic | 3               |
| Male MeCP2 -/Y brain | Monoallelic | 2               |
| <i>PEG10</i> :       |             |                 |
| RTT brain            | Monoallelic | 2               |
| Male MeCP2 -/Y brain | Monoallelic | 1               |

NOTE.—mat = maternal; pat = paternal.

data in their article are doubtful as well. Although the authors show imprinted paternal-specific expression for *PEG10*, confirming previous studies in humans and mice, they report biallelic expression for *SGCE*. This gene is well



**Figure 6.** No increase in *Dlx5* and *Dlx6* expression in *Mecp2*-mutant mouse brain. *A*, Expression of *Dlx5* and *Dlx6* genes in frontal cortex of *Mecp2* mutants (mut) compared with that of wild-type (wt) males from seven different litters, at age 7 wk (litters 1–5) or 9 wk (litters 6 and 7). For litters with more than one wild type or more than one mutant, individual animals are identified by different symbols (designated wt1 and wt2 or mut1 and mut2, respectively). Real-time qRT-PCR data from each mouse were converted to relative fold changes by comparison with a normalized sample, a wild-type mouse whose level of gene expression was closest to the mean value for all wild-type data—that is, wt1 in litter 3 for *Dlx5* and wt1 in litter 6 for *Dlx6*. *P* values calculated by two-tailed, unequal variance are  $P = .4984$  for *Dlx5* and  $P = .5134$  for *Dlx6*. *P* values calculated by one-tailed, unequal variance are  $P = .2492$  for *Dlx5* and  $P = .2567$  for *Dlx6*. The plots illustrate the wide variability within and between litters. *B*, Expression of the imprinted gene *Peg3*, studied in the same forebrain samples used in fig. 6*A*, by qRT-PCR with two different primer sets (*Peg3A* and *Peg3B*). Expression levels were normalized to the same housekeeping gene, *Rps28*. The wide range of expression levels in individual mice and the similarity of patterns obtained with *Peg3A* and *Peg3B* primer sets indicate biological noise rather than technical variation.

known to be imprinted and paternally expressed in human and mouse.<sup>54-57</sup> Okita et al. further claimed paternal-only expression for paraoxonase 1 (*PON1*), a gene that was not confirmed to be imprinted in a subsequent study.<sup>58</sup>

#### Use of LCLs for Imprinting Studies

LCLs are derived from peripheral blood B cells by transformation with Epstein-Barr virus.<sup>59</sup> They are immortal, although variable in growth rates. Given their transformed nature, LCLs may express tissue-specific genes that are not normally expressed in untransformed B cells. Therefore, the study of neurodevelopmental genes, like *DLX5* and *DLX6*, that are expressed ectopically and at a very low level in LCLs, could lead to failure to amplify RT-PCR products from both alleles (as reported by Okita et al.<sup>28</sup> and Horike et al.<sup>24</sup>). Horike et al. reported monoallelic expression of *DLX5* in two normal LCLs and biallelic expression in three of four RTT LCLs. In our experience, only after multiple optimization steps were we able to document consistent biallelic expression of this ectopically expressed locus in all normal control and RTT LCLs. This point is very well illustrated in a recent article by Itaba-Matsumoto et al.,<sup>39</sup> which reported studies of 12 LCLs from individuals with RTT and identified *MECP2* mutations who were heterozygous for *DLX5* c.\*163dupC/rs5886002. The authors estimated the degree of X-inactivation mosaicism in these LCLs by deducing relative mutant/wild-type *MECP2* expression levels from cDNA sequence tracings. Six LCLs were completely skewed and expressed only wild-type *MECP2*. Three of those showed biallelic *DLX5* expression. Moreover, of five LCLs with 25%–75% mutant *MECP2* expression, two showed biallelic *DLX5* expression, as did some LCLs from unaffected individuals. Itaba-Matsumoto et al. concluded that they could not reproduce the data reported by Horike et al., but only with regard to *MECP2* mutation-type-specific loss of imprinting of *DLX5*. Itaba-Matsumoto et al.<sup>39</sup> did not question the imprinting status of this gene in normal cells and wrote in their abstract, “a sample with high expression of mutated *MECP2* in TRD mutation showed biallelic [sic] expression of *DLX5* suggesting loss of imprinting.”<sup>39</sup> Since these authors were able to amplify only one *DLX5* allele in 50% of their cell lines, with no relationship to mutant *MECP2* expression levels, their data suggest technical difficulties with RT-PCR caused by low *DLX5* expression levels in LCLs.

Although lymphoblast DNA may acquire differential methylation patterns after establishment in culture,<sup>60</sup> LCLs have been used successfully for global gene-expression analyses.<sup>61</sup> Previous studies reported that monoallelic expression patterns of imprinted genes on chromosomes 11 and 15 are maintained in LCLs.<sup>51</sup> Moreover, monoallelic expression was reported for imprinted genes in LCLs from individuals with RTT.<sup>20</sup> To further validate the use of LCLs for imprinting studies, we analyzed the expression of *PEG3*, an imprinted locus on chromosome 19. We

found *PEG3* to be strictly monoallelically expressed in RTT LCLs that were completely skewed for X inactivation, expressing either 100% mutant or 100% wild-type MeCP2.

#### Partial Imprinting or Allelic Imbalance

In studies of the forebrain, in which *DLX5* is highly expressed, low cDNA copy number should not be an issue for transcript amplification. Indeed, biallelic expression was found in two of three human brain samples studied by Okita et al.<sup>28</sup> However, on the basis of inspection of band intensities on sequencing gels, the authors concluded that expression is unequal, with preferential expression of a single allele of unknown parental origin. Our studies revealed unambiguous expression of both *DLX5* alleles in six cortex samples from heterozygous individuals, ranging in age from 18 fetal wk to 70 years. Likewise, *DLX6* was biallelically expressed in two fetal brains that were heterozygous for a known and a novel SNP, respectively. Therefore, *DLX5* and *DLX6* are not imprinted in human brain, and we have not observed apparent unequal expression.

Consistent with our results in human, *Dlx5* was reported to be biallelically expressed in multiple brain regions, with no allele-specific preference, in F1 mice of interspecies crosses.<sup>26</sup> By using the same approach, Horike et al., however, reported “preferential expression from the maternal allele”<sup>24(p33)</sup> in control mouse brains. This pattern was said to be changed to “equal expression” of *Dlx5* alleles in a single *Mecp2*-mutant brain. This result led the authors to conclude that “MeCP2 deficiency leads to complete loss of relaxed imprinting in brain.”<sup>24(p33)</sup> The evidence consists of restriction-enzyme digestion of RT-PCR products from heterozygous templates. In this system, quantitation of alleles is difficult because, at the end of the PCR cycles, when the sample is cooling down, denaturation and re-naturation may occur, leading to the formation of heteroduplex molecules that are not susceptible to restriction-enzyme cleavage. This phenomenon results in the larger uncleaved band appearing relatively stronger. In addition, the cleaved allele is represented by two smaller bands whose combined fluorescent intensity should approximate that of the uncleaved allele. The pattern expected from a template with both alleles equally represented is illustrated by the gDNA results for the four imprinted genes in figure 2*b* in the work of Horike et al.,<sup>24</sup> in which the largest fragment is always the most intense one. In figure 2*a*, a single *Mecp2*-mutant RNA sample is shown with exactly the same intensity of two bands, representing the 636-bp uncut and 533-bp cut *Dlx5* allelic fragments. The smaller 103-bp restriction fragment, representing part of the cleavable allele, is cut off. This result would not be expected if both alleles were expressed equally. Therefore, the data do not support the claim of parent-of-origin-specific allelic imbalance of *Dlx5* expression in normal brain and a role for MeCP2 in maintaining a maternal preference indicating relaxed imprinting.



Allelic variation in gene expression for nonimprinted genes is increasingly being recognized. The magnitude varies from a 1.3-fold to 9-fold difference in 20%–50% of the genes tested.<sup>62–66</sup> Interestingly, allelic transcript variants may follow Mendelian inheritance because of variation in *cis*-acting elements or “regulatory SNPs.”<sup>67</sup> Therefore, unequal allelic expression levels of any gene should not be casually interpreted as “partial imprinting.” Confusion could arise for genes that are imprinted in a cell type–specific fashion, if the sample tested were a mixture of cells affected by the imprinting and cells that are not. There is no evidence that the genes discussed here fall into this category.

Horike et al.<sup>24</sup> provided circumstantial evidence in support of the fact that *Dlx5* is not imprinted in mouse brain, by showing that there are no DMRs in the vicinity of *Dlx5/Dlx6* and that the promoters of both genes are unmethylated on both alleles. Evolutionary considerations lend additional support. As epigenetic phenomena evolve in mammalian species, there are well-established epigenetic differences between humans and mice. In humans, ~15% of X-linked genes are biallelically expressed—that is, escape from X inactivation—which is not the case in mice.<sup>68</sup> Likewise, the list of known imprinted genes contains many that are imprinted in mice but biallelically expressed in humans.<sup>19</sup>

#### No Role for MeCP2 in Imprinting

To determine whether truly imprinted genes maintain their imprinting status in MeCP2-deficient brain, we studied *PEG3* and *PEG10* expression in cortex samples from mosaic females with RTT, as well as from nonmosaic males with congenital encephalopathy who are hemizygous for *MECP2* mutations. The strictly monoallelic expression of *PEG3* and *PEG10* confirms that the lack of normal MeCP2 protein does not abolish or relax the imprinting status of imprinted loci (table 3). Ten imprinted transcripts have previously been shown to maintain their imprinting status in MeCP2-deficient tissues from humans and/or mice: *H19*, *IGF2*, *SNRPN*, *IPW*, and *NDN*,<sup>20</sup> as well as *KCNQ1OT1* (or *LIT1*), *Sgce*, *Peg10*, *Asb4*, and *Calcr*.<sup>24</sup> Although the role of DMRs for the maintenance of imprinting is undisputed, MeCP2 is unlikely to be the CpG binding molecule that translates this methylation signal.

#### Acknowledgments

We are grateful to Katherine Carlsmith Harrison, Johannes Richter, ChaRandle Jordan, and Anita Beck, for contributing data, and to Alessandra Splendore and Feng Ding, for constructive comments on the manuscript. This work was supported in part by research grants from the National Institutes of Health and the Rett Syndrome Research Foundation (to U.F.) and fellowships from the Deutsche Forschungsgemeinschaft and the Stanford Medical School Chandler Fund (to B.S.).

#### Web Resources

Accession numbers and URLs for data presented herein are as follows:

dbSNP, <http://www.ncbi.nlm.nih.gov/projects/SNP/>  
GenBank, <http://www.ncbi.nlm.nih.gov/Genbank/> (for *DLX5* [accession number NM\_005221.5], *DLX6* [accession number NM\_005222.2], and *Dlx6* [accession number BC114342.1] reference sequences)

HGVs Nomenclature for the Description of Sequence Variants, <http://www.hgvs.org/mutnomen/>

Online Mendelian Inheritance in Man (OMIM), <http://www.ncbi.nlm.nih.gov/Omim/> (for RTT and *MECP2*)

#### References

1. Rett A (1966) Über ein eigenartiges hirnatrophisches Syndrom bei Hyperammonämie im Kindesalter. *Wien Med Wochenschr* 116:723–738
2. Amir RE, Van den Veyver IB, Wan M, Tran CQ, Francke U, Zoghbi HY (1999) Rett syndrome is caused by mutations in X-linked *MECP2*, encoding methyl-CpG-binding protein 2. *Nat Genet* 23:185–188
3. Francke U (2006) Neurogenetics of MeCP2 deficiency. *Nat Clin Pract Neurol* 2:212–221
4. Wan M, Lee SS, Zhang X, Houwink-Manville I, Song HR, Amir RE, Budden S, Naidu S, Pereira JL, Lo IF, et al (1999) Rett syndrome and beyond: recurrent spontaneous and familial *MECP2* mutations at CpG hotspots. *Am J Hum Genet* 65:1520–1529
5. Carney RM, Wolpert CM, Ravan SA, Shahbazian M, Ashley-Koch A, Cuccaro ML, Vance JM, Pericak-Vance MA (2003) Identification of MeCP2 mutations in a series of females with autistic disorder. *Pediatr Neurol* 28:205–211
6. Kankirawatana P, Leonard H, Ellaway C, Scurlock J, Mansour A, Makris CM, Dure LS, Friez M, Lane J, Kiraly-Borri C, et al (2006) Early progressive encephalopathy in boys and *MECP2* mutations. *Neurology* 67:164–166
7. Giacometti E, Luikenhuis S, Beard C, Jaenisch R (2007) Partial rescue of MeCP2 deficiency by postnatal activation of MeCP2. *Proc Natl Acad Sci USA* 104:1931–1936
8. Guy J, Gan J, Selfridge J, Cobb S, Bird A (2007) Reversal of neurological defects in a mouse model of Rett syndrome. *Science* 315:1143–1147
9. Nan X, Campoy FJ, Bird A (1997) MeCP2 is a transcriptional repressor with abundant binding sites in genomic chromatin. *Cell* 88:471–481
10. Colantuoni C, Jeon OH, Hyder K, Chenchik A, Khimani AH, Narayanan V, Hoffman EP, Kaufmann WE, Naidu S, Pevsner J (2001) Gene expression profiling in postmortem Rett syndrome brain: differential gene expression and patient classification. *Neurobiol Dis* 8:847–865
11. Tudor M, Akbarian S, Chen RZ, Jaenisch R (2002) Transcriptional profiling of a mouse model for Rett syndrome reveals subtle transcriptional changes in the brain. *Proc Natl Acad Sci USA* 99:15536–15541
12. Traynor J, Agarwal P, Lazzaroni L, Francke U (2002) Gene expression patterns vary in clonal cell cultures from Rett syndrome females with eight different *MECP2* mutations. *BMC Med Genet* 3:12
13. Ballestar E, Roperio S, Alaminos M, Armstrong J, Setien F, Agrelo R, Fraga MF, Herranz M, Avila S, Pineda M, et al (2005)

- The impact of *MECP2* mutations in the expression patterns of Rett syndrome patients. *Hum Genet* 116:91–104
14. Nuber UA, Kriaucionis S, Roloff TC, Guy J, Selfridge J, Steinhoff C, Schulz R, Lipkowitz B, Ropers HH, Holmes MC, et al (2005) Up-regulation of glucocorticoid-regulated genes in a mouse model of Rett syndrome. *Hum Mol Genet* 14:2247–2256
  15. Delgado IJ, Kim DS, Thatcher KN, LaSalle JM, Van den Veyver IB (2006) Expression profiling of clonal lymphocyte cell cultures from Rett syndrome patients. *BMC Med Genet* 7:61
  16. Deng V, Matagne V, Banine F, Frerking M, Ohliger P, Budden S, Pevsner J, Disen GA, Sherman LS, Ojeda SR (2007) *FXYD1* is a MeCP2 target gene overexpressed in the brains of Rett syndrome patients and *Mecp2*-null mice. *Hum Mol Genet* 16:640–650
  17. Jordan C, Li H-H, Kwan H, Francke U (2007) Cerebellar gene expression profiles of mouse models for Rett syndrome reveal novel MeCP2 targets. *BMC Med Genet* 8:36
  18. Reik W, Walter J (1998) Imprinting mechanisms in mammals. *Curr Opin Genet Dev* 8:154–164
  19. Morison IM, Ramsay JP, Spencer HG (2005) A census of mammalian imprinting. *Trends Genet* 21:457–465
  20. Balmer D, Arredondo J, Samaco RC, LaSalle JM (2002) MECP2 mutations in Rett syndrome adversely affect lymphocyte growth, but do not affect imprinted gene expression in blood or brain. *Hum Genet* 110:545–552
  21. Samaco RC, Hogart A, LaSalle JM (2005) Epigenetic overlap in autism-spectrum neurodevelopmental disorders: *MECP2* deficiency causes reduced expression of *UBE3A* and *GABRB3*. *Hum Mol Genet* 14:483–492
  22. Makedonski K, Abuhatzira L, Kaufman Y, Razin A, Shemer R (2005) MeCP2 deficiency in Rett syndrome causes epigenetic aberrations at the PWS/AS imprinting center that affects *UBE3A* expression. *Hum Mol Genet* 14:1049–1058
  23. Jordan C, Francke U (2006) *Ube3a* expression is not altered in *Mecp2* mutant mice. *Hum Mol Genet* 15:2210–2215
  24. Horike S, Cai S, Miyano M, Cheng JF, Kohwi-Shigematsu T (2005) Loss of silent-chromatin looping and impaired imprinting of *DLX5* in Rett syndrome. *Nat Genet* 37:31–40
  25. Guy J, Hendrich B, Holmes M, Martin JE, Bird A (2001) A mouse *Mecp2*-null mutation causes neurological symptoms that mimic Rett syndrome. *Nat Genet* 27:322–326
  26. Kimura MI, Kazuki Y, Kashiwagi A, Kai Y, Abe S, Barbieri O, Levi G, Oshimura M (2004) *Dlx5*, the mouse homologue of the human-imprinted *DLX5* gene, is biallelically expressed in the mouse brain. *J Hum Genet* 49:273–277
  27. Stuhmer T, Anderson SA, Ekker M, Rubenstein JL (2002) Ectopic expression of the *Dlx* genes induces glutamic acid decarboxylase and *Dlx* expression. *Development* 129:245–252
  28. Okita C, Meguro M, Hoshiya H, Haruta M, Sakamoto YK, Oshimura M (2003) A new imprinted cluster on the human chromosome 7q21-q31, identified by human-mouse monochromosomal hybrids. *Genomics* 81:556–559
  29. Panganiban G, Rubenstein JL (2002) Developmental functions of the *Distal-less/Dlx* homeobox genes. *Development* 129:4371–4386
  30. Acampora D, Merlo GR, Paleari L, Zerega B, Postiglione MP, Mantero S, Bober E, Barbieri O, Simeone A, Levi G (1999) Craniofacial, vestibular and bone defects in mice lacking the *Distal-less*-related gene *Dlx5*. *Development* 126:3795–3809
  31. Depew MJ, Liu JK, Long JE, Presley R, Meneses JJ, Pedersen RA, Rubenstein JL (1999) *Dlx5* regulates regional development of the branchial arches and sensory capsules. *Development* 126:3831–3846
  32. Perera M, Merlo GR, Verardo S, Paleari L, Corte G, Levi G (2004) Defective neuronogenesis in the absence of *Dlx5*. *Mol Cell Neurosci* 25:153–161
  33. Yan H, Papadopoulos N, Marra G, Perrera C, Jiricny J, Boland CR, Lynch HT, Chadwick RB, de la Chapelle A, Berg K, et al (2000) Conversion of diploidy to haploidy. *Nature* 403:723–724
  34. Gallagher RC, Pils B, Albalwi M, Francke U (2002) Evidence for the role of *PWCR1/HBII-85* C/D box small nucleolar RNAs in Prader-Willi syndrome. *Am J Hum Genet* 71:669–678
  35. den Dunnen JT, Antonarakis SE (2000) Mutation nomenclature extensions and suggestions to describe complex mutations: a discussion. *Hum Mutat* 15:7–12
  36. Hamilton SP, Woo JM, Carlson EJ, Ghanem N, Ekker M, Rubenstein JL (2005) Analysis of four *DLX* homeobox genes in autistic probands. *BMC Genet* 6:52
  37. Schanen NC (2006) Epigenetics of autism spectrum disorders. *Hum Mol Genet Spec* 2 15:R138–R150
  38. Badcock C, Crespi B (2006) Imbalanced genomic imprinting in brain development: an evolutionary basis for the aetiology of autism. *J Evol Biol* 19:1007–1032
  39. Itaba-Matsumoto N, Maegawa S, Yamagata H, Kondo I, Oshimura M, Nanba E (2007) Imprinting status of paternally imprinted *DLX5* gene in Japanese patients with Rett syndrome. *Brain Dev* (electronically published March 17, 2007) (accessed May 19, 2007)
  40. Chadwick LH, Wade PA (2007) MeCP2 in Rett syndrome: transcriptional repressor or chromatin architectural protein? *Curr Opin Genet Dev* 17:121–125
  41. Itoh M, Ide S, Takashima S, Kudo S, Nomura Y, Segawa M, Kubota T, Mori H, Tanaka S, Horie H, et al (2007) Methyl CpG-binding protein 2 (a mutation of which causes Rett syndrome) directly regulates insulin-like growth factor binding protein 3 in mouse and human brains. *J Neuropathol Exp Neurol* 66:117–123
  42. Kuwajima T, Nishimura I, Yoshikawa K (2006) Necdin promotes GABAergic neuron differentiation in cooperation with *Dlx* homeodomain proteins. *J Neurosci* 26:5383–5392
  43. Bienvenu T, Chelly J (2006) Molecular genetics of Rett syndrome: when DNA methylation goes unrecognized. *Nat Rev Genet* 7:415–426
  44. Zhou QP, Le TN, Qiu X, Spencer V, de Melo J, Du G, Plews M, Fonseca M, Sun JM, Davie JR, et al (2004) Identification of a direct *Dlx* homeodomain target in the developing mouse forebrain and retina by optimization of chromatin immunoprecipitation. *Nucleic Acids Res* 32:884–892
  45. Yasui D, Miyano M, Cai S, Varga-Weisz P, Kohwi-Shigematsu T (2002) SATB1 targets chromatin remodelling to regulate genes over long distances. *Nature* 419:641–645
  46. Georgel PT, Horowitz-Scherer RA, Adkins N, Woodcock CL, Wade PA, Hansen JC (2003) Chromatin compaction by human MeCP2: assembly of novel secondary chromatin structures in the absence of DNA methylation. *J Biol Chem* 278:32181–32188
  47. Nikitina T, Shi X, Ghosh RP, Horowitz-Scherer RA, Hansen JC, Woodcock CL (2007) Multiple modes of interaction between the methylated DNA binding protein MeCP2 and chromatin. *Mol Cell Biol* 27:864–877
  48. Francke U, Pellegrino MA (1977) Assignment of the major

- histocompatibility complex to a region of the short arm of human chromosome 6. *Proc Natl Acad Sci USA* 74:1147–1151
49. Francke U, Busby N, Shaw D, Hansen S, Brown MG (1976) Intrachromosomal gene mapping in man: assignment of nucleoside phosphorylase to region 14cen leads to 14q21 by interspecific hybridization of cells with a t(X;14)(p22;q21) translocation. *Somatic Cell Genet* 2:27–40
  50. Meguro M, Mitsuya K, Sui H, Shigenami K, Kugoh H, Nakao M, Oshimura M (1997) Evidence for uniparental, paternal expression of the human GABAA receptor subunit genes, using microcell-mediated chromosome transfer. *Hum Mol Genet* 6:2127–2133
  51. Gabriel JM, Higgins MJ, Gebuhr TC, Shows TB, Saitoh S, Nicholls RD (1998) A model system to study genomic imprinting of human genes. *Proc Natl Acad Sci USA* 95:14857–14862
  52. Kugoh H, Mitsuya K, Meguro M, Shigenami K, Schulz TC, Oshimura M (1999) Mouse A9 cells containing single human chromosomes for analysis of genomic imprinting. *DNA Res* 6:165–172
  53. Inoue J, Mitsuya K, Maegawa S, Kugoh H, Kadota M, Okamura D, Shinohara T, Nishihara S, Takehara S, Yamauchi K, et al (2001) Construction of 700 human/mouse A9 monochromosomal hybrids and analysis of imprinted genes on human chromosome 6. *J Hum Genet* 46:137–145
  54. Piras G, El Kharroubi A, Kozlov S, Escalante-Alcalde D, Hernandez L, Copeland NG, Gilbert DJ, Jenkins NA, Stewart CL (2000) *Zac1* (*Lot1*), a potential tumor suppressor gene, and the gene for  $\epsilon$ -sarcoglycan are maternally imprinted genes: identification by a subtractive screen of novel uniparental fibroblast lines. *Mol Cell Biol* 20:3308–3315
  55. Ono R, Kobayashi S, Wagatsuma H, Aisaka K, Kohda T, Kaneko-Ishino T, Ishino F (2001) A retrotransposon-derived gene, *PEG10*, is a novel imprinted gene located on human chromosome 7q21. *Genomics* 73:232–237
  56. Müller B, Hedrich K, Kock N, Dragasevic N, Svetel M, Garrels J, Landt O, Nitschke M, Pramstaller PP, Reik W, et al (2002) Evidence that paternal expression of the  $\epsilon$ -sarcoglycan gene accounts for reduced penetrance in myoclonus-dystonia. *Am J Hum Genet* 71:1303–1311
  57. Grabowski M, Zimprich A, Lorenz-Depiereux B, Kalscheuer V, Asmus F, Gasser T, Meitinger T, Strom TM (2003) The  $\epsilon$ -sarcoglycan gene (SGCE), mutated in myoclonus-dystonia syndrome, is maternally imprinted. *Eur J Hum Genet* 11:138–144
  58. Ono R, Shiura H, Aburatani H, Kohda T, Kaneko-Ishino T, Ishino F (2003) Identification of a large novel imprinted gene cluster on mouse proximal chromosome 6. *Genome Res* 13:1696–1705
  59. Neitzel H (1986) A routine method for the establishment of permanent growing lymphoblastoid cell lines. *Hum Genet* 73:320–326
  60. Hannula K, Lipsanen-Nyman M, Scherer SW, Holmberg C, Hoglund P, Kere J (2001) Maternal and paternal chromosomes 7 show differential methylation of many genes in lymphoblast DNA. *Genomics* 73:1–9
  61. McRae AF, Matigian NA, Vadlamudi L, Mulley JC, Mowry B, Martin NG, Berkovic SF, Hayward NK, Visscher PM (2007) Replicated effects of sex and genotype on gene expression in human lymphoblastoid cell lines. *Hum Mol Genet* 16:364–373
  62. Cowles CR, Hirschhorn JN, Altshuler D, Lander ES (2002) Detection of regulatory variation in mouse genes. *Nat Genet* 32:432–437
  63. Yan H, Yuan W, Velculescu VE, Vogelstein B, Kinzler KW (2002) Allelic variation in human gene expression. *Science* 297:1143
  64. Lo HS, Wang Z, Hu Y, Yang HH, Gere S, Buetow KH, Lee MP (2003) Allelic variation in gene expression is common in the human genome. *Genome Res* 13:1855–1862
  65. Bray NJ, Buckland PR, Owen MJ, O'Donovan MC (2003) Cis-acting variation in the expression of a high proportion of genes in human brain. *Hum Genet* 113:149–153
  66. Pastinen T, Sladek R, Gurd S, Sammak A, Ge B, Lepage P, Lavergne K, Villeneuve A, Gaudin T, Brandstrom H, et al (2004) A survey of genetic and epigenetic variation affecting human gene expression. *Physiol Genomics* 16:184–193
  67. Doss S, Schadt EE, Drake TA, Lusis AJ (2005) Cis-acting expression quantitative trait loci in mice. *Genome Res* 15:681–691
  68. Heard E, Disteche CM (2006) Dosage compensation in mammals: fine-tuning the expression of the X chromosome. *Genes Dev* 20:1848–1867