The Story of Rett Syndrome: From Clinic to Neurobiology

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The postnatal neurodevelopmental disorder Rett syndrome (RTT) is caused by mutations in the gene encoding methyl-CpG binding protein 2 (MeCP2), a transcriptional repressor involved in chromatin remodeling and the modulation of RNA splicing. MECP2 aberrations result in a constellation of neuropsychiatric abnormalities, whereby both loss of function and gain in MECP2 dosage lead to similar neurological phenotypes. Recent studies demonstrate disease reversibility in RTT mouse models, suggesting that the neurological defects in MECP2 disorders are not permanent. To investigate the potential for restoring neuronal function in RTT patients, it is essential to identify MeCP2 targets or modifiers of the phenotype that can be therapeutically modulated. Moreover, deciphering the molecular underpinnings of RTT is likely to contribute to the understanding of the pathogenesis of a broader class of neuropsychiatric disorders.

Introduction

Two severely disabled young girls were relentlessly wringing their hands as they sat in the laps of their respective mothers in the waiting room of a Vienna pediatric clinic. This coincidental occurrence prompted Dr. Andreas Rett to search for more patients with the same unusual behavior. A year later, in 1966, Dr. Rett described similar findings in 22 patients, reporting for the first time a unique clinical entity that now bears his name (Rett, 1966). But it would not be until 17 years later that Rett syndrome (RTT) became recognized in the medical community when Dr. Bengt Hagberg, a Swedish neurologist, and his colleagues reported 35 cases of RTT in the English language (Hagberg et al., 1983), and recognized this clinical entity as a condition “similar to a virtually overlooked syndrome described by Rett in the German literature.”

In addition to the worldwide recognition of RTT, the 1980s witnessed major strides in another field, namely DNA methylation. For the first time, a connection was established between DNA methylation and heritable changes in gene expression. Scientists identified the CpG dinucleotide to be the site of almost all DNA methylation in mammalian genomes and began to explore the effects of this modification on gene activity. CpG islands were found to be unmethylated in the native state (with the exception of sequences on the inactive X chromosome), and methylation of CpGs was associated with gene silencing and alterations in chromatin structure. Two mechanisms have been demonstrated for DNA methylation-mediated gene repression. The first suggests that methylation of CpG sites within gene promoters will inhibit sequence-specific binding of transcription factors. In the second, more prevalent mechanism, the repression is mediated by proteins that specifically bind to methylated CpGs (methyl-CpG binding proteins) and alter chromatin structure, rendering it inaccessible to members of the transcription machinery. In 1992, Dr. Adrian Bird and his colleagues identified a novel mammalian protein that binds methylated CpGs, methyl-CpG binding protein 2 (MeCP2) (Lewis et al., 1992). The gene encoding MeCP2 was localized to the X chromosome in the mouse (Quaderi et al., 1994), and the protein repressed transcription in vitro (Nan et al., 1997). Soon afterwards, additional members of the methyl-CpG binding protein family were identified and included methyl-CpG binding domain proteins (MBD) 1–4 (Hendrich and Bird, 1998).

Meanwhile, as the DNA methylation field was deciphering repression mechanisms mediated by methyl-CpG binding proteins, the RTT community was trying to understand the pathophysiology of this puzzling condition. The turning point for RTT research came with the discovery of its genetic basis in 1999. The surprising finding that mutations in the widely expressed MECP2 are at the root of RTT (Amir et al., 1999) brought together the fields of epigenetics and neurobiology.

Clinical Features of RTT

RTT (MIM 312750) is a postnatal progressive neurodevelopmental disorder that manifests in girls during early childhood; however, not all the symptoms are prominent initially, but rather appear over stages (Figure 1). Patients with RTT appear to develop normally up to 6–18 months
of age. The child with RTT seemingly achieves appropriate milestones, including the ability to walk, and some patients even say a few words. One early indicator of neurological involvement is deceleration of head growth, leading to microcephaly by the second year of life. With the onset of developmental stagnation, the acquired microcephaly is accompanied by general growth retardation, weight loss, and a weak posture brought on by muscle hypotonia. As the syndrome progresses, patients lose purposeful use of their hands and instead develop stereotypic hand wringing or washing movements, and in some cases clapping, flapping, and mouthing of the hands. Social withdrawal and loss of language become apparent in addition to irritability and self-abusive behavior. Other autistic features also manifest, including expressionless face, hypersensitivity to sound, lack of eye-to-eye contact, indifference to the surrounding environment, and unresponsiveness to social cues (Nomura, 2005). The onset of mental deterioration is accompanied by loss of motor coordination and the development of ataxia and gait apraxia. The earliest autonomic perturbation is hyperventilation during wakefulness. Most girls with RTT suffer additional breathing anomalies, including breath-holding, aerophagia, forced expulsion of air and saliva, and apnea. One of the most arduous features of RTT is the occurrence of seizures, which range from easily controlled to intractable epilepsy, with the most common types being partial complex and tonic-clonic seizures (Jian et al., 2006). The seizures tend to decrease in severity after the teenage years and into adulthood, presenting minor problems after the age of forty. Amelioration of the social component of the autistic-like behavior occurs sometime between 5 to 10 years of age. Despite having a normal appetite, patients continue to lose weight and many suffer from osteopenia, scoliosis, and rigidity as they age. Behavioral abnormalities during this postregression phase include teeth grinding, night laughing or crying, screaming fits, low mood, and anxiety episodes elicited by distressful external events (Mount et al., 2001). Patients suffer devastating motor deterioration, generalized rigidity, dystonia, and worsening of scoliosis. Most girls with RTT lose mobility, and are often wheelchair-bound during the teenage years. Additional autonomic abnormalities include hypertrophic, cold blue feet, severe constipation, oropharyngeal dysfunction, and cardiac abnormalities, including tachycardia, prolonged corrected QT intervals, and sinus bradycardia. As patients get older they often develop Parkinsonian features (Hagberg, 2005; Roze et al., 2007). The condition reaches a plateau and some patients survive up to the sixth or seventh decade of life in a severely debilitated physical condition.

**Genetic Basis of RTT**

Given that the vast majority of patients with RTT are females, early reports postulated an X-linked dominant mode of inheritance with fateful consequences in hemizygous males. RTT has an incidence of \( \sim 1/10,000 \) female live births. However, since more than 99% of RTT cases are sporadic, it was very hard to map the disease locus by traditional linkage analysis. Using information from rare familial cases, exclusion mapping identified the Xq28 candidate region, and subsequent screening of candidate genes in RTT patients revealed mutations in MECP2 (Amir et al., 1999). Mutations in MECP2 are found in more than 95% of classic RTT cases; most arise de novo in the paternal germline and often involve a C to T transition at CpG dinucleotides (Trappe et al., 2001; Wan et al., 1999). The spectrum of mutation types includes

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**Figure 1. Onset and Progression of RTT Clinical Phenotypes**

After a period of normal development, a healthy-looking baby girl falls into developmental stagnation, followed by rapid deterioration, loss of acquired speech, and the replacement of purposeful use of the hands with incessant stereotypies, a characteristic of the syndrome. Patients also develop social behavior abnormalities and are often misdiagnosed as having autism. The condition worsens with loss of motor skills and profound cognitive impairment. In addition, patients suffer from anxiety, seizures, and a host of autonomic abnormalities.
missense, nonsense, and frameshift mutations, with over 300 unique pathogenic nucleotide changes described (Christodoulou et al., 2003), as well as deletions encompassing whole exons (Archer et al., 2006b; Pan et al., 2006; Ravn et al., 2005). Eight missense and nonsense mutations account for ~70% of all mutations, while small C-terminal deletions account for another ~10%, and complex rearrangements constitute ~5%. Several phenotype-genotype correlation studies have been reported and some general conclusions can be made. Mutations affecting the NLS of MeCP2 or early truncating mutations tend to cause more severe phenotypes than missense mutations, whereas C-terminal deletions are associated with milder phenotypes (Smeets et al., 2005). In addition, the R133C mutation causes an overall milder phenotype (Kerr et al., 2006; Leonard et al., 2003; Neul et al., 2007), while the R270X mutation is associated with increased mortality (Bienvuu and Chelly, 2006). Because several mutations introduce premature stop codons throughout the gene and are predicted to result in a null allele, RTT results from loss of MeCP2 function. However, hypomorphic alleles may also occur and result, for instance, in truncated forms of MeCP2 that retain partial function.

**Phenotypic Variability in RTT**

Atypical forms of RTT that deviate from the classical clinical presentation occur. These variants range from milder forms with a later age of onset to more severe manifestations. Of the milder variants, the “forme fruste” (or “worn-down form”) has a later age of onset compared with the classical form, with regression occurring between 1 to 3 years of age; hand use is sometimes preserved with minimal stereotypic movements. The preserved speech variant is another benign form of RTT characterized by the ability of patients to speak a few words, although not necessarily in context. Patients with this variant have a normal karyotype, suggesting that genetic modifiers suppress the infantile lethality typically seen with loss-of-function MECP2 mutations (Budden et al., 2005; Dayer et al., 2007; Maiwald et al., 2002; Masuyama et al., 2005). Lastly, some MECP2 mutations that do not cause RTT in females can cause moderate, nonspecific to profound mental retardation or psychiatric disorders in males. Deficits in language and motor skills, obesity, autistic features, and epilepsy are also common in this category of mutations.

**CDKL5 Mutations Explain a Congenital RTT-like Disorder**

Mutations in the X-linked gene cyclin-dependent kinase-like 5 (CDKL5) were identified in patients with a rare congenital disorder with intractable early-onset seizures often accompanied by RTT-like features. Alterations in this gene were originally found to cause early-onset epilepsy and infantile spasms with severe mental retardation (Grosso et al., 2007). Although the majority of patients with CDKL5 mutations are female, there are rare occurrences in males suffering from severe mental retardation, infantile spasms, or early-onset epilepsy. The protein product of CDKL5, previously known as serine/threonine kinase 9 (STK9), autophosphorylates itself and phosphorylates MeCP2 in vitro, and this latter activity is eliminated in pathogenic CDKL5 mutants (Bertani et al., 2006). Disruption of N7NG1, which encodes the axon guiding molecule Netrin G1, was described in one female patient with atypical RTT and early-onset seizures (Borg et al., 2005). However, this depending on the extent of such favorable carriers, some patients can be mildly affected or are even asymptomatic carriers of MECP2 mutations. The latter cases are usually identified because of the occurrence of RTT in their offspring. The best examples for illustrating the dramatic effects of XCI patterns in RTT are monozygotic twins who manifest very different phenotypes (Drapin et al., 2000). In addition, skewed XCI patterns occur in brain regions of female mice heterozygous for a mutant MECP2 allele, where phenotypic severity correlates with the degree of skewing (Young and Zoghbi, 2004). Finally, somatic mosaicism in females with MECP2 mutations is another rare but important source of phenotypic variability (Bourdon et al., 2001).
Table 1. Phenotypes of MeCP2 Dysfunction

<table>
<thead>
<tr>
<th>MeCP2 State</th>
<th>Sex-Associated Syndromes and Symptoms</th>
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<tr>
<td>Female</td>
<td>Male</td>
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<tr>
<td>Loss of Function</td>
<td>Classic RTT                                                           Infantile encephalopathy</td>
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<td></td>
<td>Atypical RTT                                                             Classic RTT (47,XXY or somatic mosaic)</td>
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<td></td>
<td>Angelman-like syndrome                                                   Mental retardation with motor deficits</td>
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<td></td>
<td>Mental retardation with seizures                                         Bipolar disease, mental retardation, and tremors</td>
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<td></td>
<td>Mild mental retardation                                                  Juvenile-onset schizophrenia, mental retardation, and tremors</td>
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<tr>
<td></td>
<td>Learning disability                                                      Mental retardation, psychosis, pyramidal signs, and macroorchidism</td>
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<td>Autism</td>
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<td></td>
<td>Normal</td>
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<tr>
<td>Overexpression</td>
<td>Preserved speech variant of RTT                                          Severe mental retardation and RTT features</td>
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<td></td>
<td>Non-specific X-linked mental retardation</td>
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The MECP2 Duplication Disorder

Perhaps one of the more intriguing findings regarding RTT and MeCP2 is the fact that loss of function and gain in MECP2 dosage result in clinically similar neurological disorders. In fact, duplications of Xq28 that span the MECP2 locus have been reported in males with progressive neurodevelopmental phenotypes. The patients suffer from mental retardation with facial and axial hypotonia, progressive spasticity, seizures, recurrent respiratory infections, and often premature death. In addition, autistic features and RTT phenotypes, including head growth deceleration, motor delay, ataxia, hand stereotypes, teeth grinding, and absence of speech, have been reported in these boys (Friez et al., 2006; Lugtenberg et al., 2006; Meins et al., 2005; Van Esch et al., 2005). A male patient with a triplication of the locus was also described with a worse early-onset neurological phenotype (del Gaudio et al., 2006). Only one duplication case has been identified in females, in a patient with the preserved speech variant of RTT (Ariani et al., 2004). Studies of transgenic mice that express wild-type MECP2 at twice the normal level reveal that doubling the dosage of this protein leads to a progressive neurological phenotype similar to that observed in human patients (Collins et al., 2004). These observations, together with the finding that MECP2 is the only common gene shared among all patients with the duplication syndrome, give credence to the notion that MECP2 is the gene within the ~400 kb duplicated region that is responsible for these phenotypes.

MECP2 Mutations in Other Neuropsychiatric Disorders

Disruptions in MECP2 result in a host of neuropsychiatric disorders, of which classic and variant forms of RTT represent only one chapter (Table 1). On the milder side of the scale are females with mild mental retardation, learning disabilities, and autism spectrum disorders (Carney et al., 2003; Lam et al., 2000). MECP2 mutations can also cause severe mental retardation with epilepsy and Angelman-like syndrome in females (Milani et al., 2005; Watson et al., 2001). The spectrum of MECP2 disorders worsens with complex forms of severe mental retardation in males that are associated with epilepsy, ataxia, tremor, hyperactivity, autism, and bipolar disease (Klauck et al., 2002) or juvenile-onset schizophrenia (Cohen et al., 2003; Lam et al., 2000).

The full spectrum of phenotypes in MECP2 disorders is far from being understood. Mutations that are predicted to result in a null allele suggest that the neurodevelopmental abnormalities are a result of MeCP2 loss of function. However, the MECP2 duplication disorder demonstrates that an increase in protein levels can be equally detrimental to the nervous system. Studies in loss-of-function and duplication models are beginning to provide insight on how variations in the level of this protein lead to neuronal dysfunction (see below).

MeCP2 Expression and Functions

MECP2 consists of four exons that code for two different isoforms of the protein, due to alternative splicing of exon 2 (Figure 2). The MeCP2 splice variants differ only in their N-termini; the more abundant MeCP2-e1 isoform (encoded by MECP2α) contains 24 amino acids encoded by exon 1 and lacks the 9 amino acids encoded by exon 2, whereas the start site for the MeCP2-e2 isoform (encoded by MECP2β) is in exon 2 (Dragich et al., 2007; Kriaucionis and Bird, 2004; Mnatzakanian et al., 2004). In addition, MECP2 has a large, highly conserved 3′-untranslated region that contains multiple polyadenylation sites, which can be alternatively used to generate four different transcripts. Expression studies in mice showed that the longest transcript is the most abundant in brain, with
higher expression during embryonic development, followed by postnatal decline, and subsequent increase in expression levels later in adult life (Pelka et al., 2005; Shahbazian et al., 2002b). These different MECP2 transcripts could be subject to tissue-specific and developmentally stage-specific regulation. Although the MeCP2 protein is widely expressed, it is relatively more abundant in the brain, primarily in mature postmitotagory neurons (Jung et al., 2003). MeCP2 protein levels are low during embryogenesis and increase progressively during the postnatal period of neuronal maturation (Balmer et al., 2003; Cohen et al., 2003; Kishi and Macklis, 2004; Mulaney et al., 2004; Shahbazian et al., 2002b). The pattern of increasing expression in the cortex follows an inner-to-outer sequence akin to that of cortical development. In the olfactory epithelium, MeCP2 expression coincides with maturation of the olfactory receptor neurons (ORNs) and precedes the onset of synaptogenesis (Cohen et al., 2003). Both MeCP2 isoforms are nuclear and colocalize with methylated heterochromatic foci in mouse cells. A recent report suggests that MeCP2 translocates to the nucleus upon neuronal differentiation (Miyake and Nagai, 2007). Since MeCP2 is expressed in mature neurons and its levels increase during postnatal development, MeCP2 may play a role in modulating the activity or plasticity of mature neurons. Consistent with this, MECP2 mutations do not seem to affect the proliferation or differentiation of neuronal precursors. Although the mechanisms that regulate the complex MECP2 expression patterns are unknown, a recent study identified the core promoter and several cis-regulatory elements that drive MECP2 expression (Liu and Francke, 2006). These regulatory sequences may dictate the spatial and temporal patterns of MECP2 expression.

MeCP2 is a member of the methyl-CpG binding protein family (Hendrich and Bird, 1998), and is composed of three domains: the MBD, the transcriptional repression domain (TRD), and a C-terminal domain (Cohen et al., 2003; Harikrishnan et al., 2005; Kokura et al., 2001; Nan et al., 1998, 2003; Kaludov and Wolffe, 2000; Kimura and Shiota, 2003; Kokura et al., 2001; Nan et al., 2007). However, the precise functional consequences of these protein-protein interactions remain unknown. To add to the complexity, MeCP2 also interacts with the RNA-binding protein Y box-binding protein 1 (YB1) to regulate splicing of reporter constructs (Figure 3B). The finding of aberrant RNA splicing patterns in a Mecp2 mutant mouse model of RTT (Young et al., 2005) together with the fact that MeCP2 can form complexes with RNA in vitro independently of its MBD (Jeffery and Nakielny, 2004) suggest that MeCP2 might modulate RNA splicing in vivo. Given that MeCP2 interacts with other proteins, chromatin, DNA, and RNA, it is clearly a multifunctional protein, with roles in chromatin remodeling and RNA splicing.

Modeling RTT and Related Disorders in Mice

To uncover the molecular changes that underlie RTT, three mouse models with different MeCP2 mutations were generated (Table 2). Mecp2 conditional knockout
mice, lacking either exon 3 or both exons 3 and 4 (Chen et al., 2001; Guy et al., 2001), undergo a period of normal development, followed by severe progressive neurological dysfunction, leading to death at 8–10 weeks of age. Female Mecp2+/C0 mice have behavioral abnormalities as well, but with a later age of onset. Moreover, an embryonic MeCP2 deletion only in neurons, using a nestin-Cre transgene, results in a phenotype resembling the ubiquitous knockout, demonstrating that MeCP2 dysfunction in the brain is sufficient to cause the disease (Guy et al., 2001).
Mecp2 null mice display no initial phenotype until 3–6 weeks of age, when male mutant mice develop a stiff, uncoordinated gait, hypoaactivity, tremor, hindlimb clamping, and irregular breathing. Symptoms worsen and ultimately lead to weight loss and death by 10 weeks. The respiratory rhythm disturbances observed in the Mecp2 null mice have been attributed to alterations in bulbar postinspiratory discharges and to malfunction of the Kolliker-Fuse region of the pons that enhances postinspiratory activity and leads to the development of respiratory dysrhythmia and apnea (Stettner et al., 2007). The brains of Mecp2 null mice are smaller in size and weight than brains of wild-type littermates, but have no detectable structural abnormalities, except for smaller, more densely packed neurons. In addition, the olfactory neurons of Mecp2 null mice demonstrate abnormalities of axonal targeting in the olfactory bulb, suggesting a function for MeCP2 in terminal neuronal differentiation (Matarazzo et al., 2004). When Mecp2 is deleted in postmitotic neurons using a calcium-calmodulin-dependent protein kinase II (CaMKII)-Cre transgene, similar but less severe neurological phenotypes are observed with a later age of onset, confirming a critical role for MeCP2 in mature neurons. These mice display gait ataxia, increased anxiety, and impaired social behavior (Chen et al., 2001; Gemelli et al., 2006). In further support of this idea, when Mecp2 is expressed in postmitotic neurons of Mecp2 null mice under the control of the endogenous tau promoter, the neurological phenotypes of the knockout mice are rescued (Luikenhuis et al., 2004). These studies not only confirmed that neuronal MeCP2 dysfunction is responsible for the neurological phenotype, but also that MeCP2 is not very essential to early brain development. Recently, another Mecp2 null mouse model was reported in which exon 3 and part of exon 4 were deleted. The mice are hypoaactive and display forepaw clamping, aggressive behavior, kyphosis, seizures, and motor abnormalities, and die by 1 year of age. In addition, higher levels of MeCP2 expression in other transgenic lines are associated with more severe phenotypes (Collins et al., 2004). Introducing a copy of the MECP2 transgene into Mecp2 null mice restores protein levels to normal and rescues the neurological abnormalities of both mouse models, indicating that phenotypes of the transgenic mice are indeed modulated by MeCP2 levels. Overexpression of MECP2 in adult neurons under control of the tau promoter also results in a progressive neurological syndrome. Studies of these mouse models suggest that MeCP2 levels must be tightly regulated even postnatally and that the slightest perturbation results in deleterious neurological consequences (Collins et al., 2004; Luikenhuis et al., 2004). The best illustration of the clinical relevance of such observations is the fact that duplication of the MECP2 locus in human male patients causes profound mental retardation and progressive neurodevelopmental disorders.

Effects of MeCP2 Dysfunction on the Brain

Neuropathological Changes

Despite the profound neurological phenotype in RTT, the major morphological abnormalities detected in the central nervous system (CNS) are an overall decrease in the size of the brain and of individual neurons. Autopsy studies show a 12%–34% reduction in brain weight and volume in patients with RTT, the effect most pronounced in the prefrontal, posterior frontal, and anterior temporal regions (Armstrong, 2005). The RTT brain shows no obvious degeneration, atrophy, or inflammation, and there are no signs of gliosis or neuronal migration defects (Jellinger et al., 1988; Reiss et al., 1993). These observations indicate that RTT is a disorder of postnatal neurodevelopment rather than a neurodegenerative process. There is a decrease in the size of cortical minicolumns (Casanova et al., 2003), which could correlate with observations of reduced dendritic branching of layers III and V pyramidal neurons in the frontal, temporal and motor regions, and of layers II and IV of the subiculum (Armstrong, 2005). Neurons of hippocampal CA1 and occipital regions are relatively preserved (Armstrong et al., 1995). In addition, dendritic spines of the RTT frontal cortex are sparse and short, with no other apparent abnormalities (Belichenko et al., 1994). Although neuronal size is reduced in the cortex, thalamus, basal ganglia, amygdala, and hippocampus, there is an increase in neuronal cell packing in the hippocampus (Kaufmann and Moser, 2000). Hypopigmentation of the zona compacta of the substantia nigra and reduction in cell number in the nucleus basalis of Meynert...
have been reported (Kitt and Wilcox, 1995). Olfactory biopsies from RTT patients reveal that the mature ORNs are dysmorphic, and that there is a marked increase in the ratio of immature to mature ORNs (Ronnert et al., 2003). Delayed neuronal maturation and synaptogenesis were also detected in the 
Mecp2^{-/-} cerebral cortex (Fukuda et al., 2005). Neuropathological studies in both humans and mice were all performed well after the onset of symptoms. It would be interesting to determine if alterations exist in hippocampal or cortical neurons prior to the onset of symptoms, at least in the existing mouse models. Nonetheless, the results of neuropathological studies suggest a role for MeCP2 in neuronal maturation and maintenance.

Additionally, a variety of neurochemical changes are documented in the cerebrospinal fluid and brain tissue of patients with RTT (Blue et al., 1999; Calamandrei et al., 2001; Guideri et al., 2004; Lipani et al., 2000; Paterson et al., 2005; Riikonen, 2003; Saito et al., 2001; Viola et al., 2007; Wenk, 1997; Zoghbi et al., 1989). However, it should be noted that the majority of these data result from small samples of postmortem tissue and none of these changes are diagnostic or occur in every patient.

Neurophysiological Abnormalities

Neurophysiological studies of patients with RTT suggest that both the CNS and the autonomic nervous system contribute to the pathophysiology of the disease. In RTT, the peripheral auditory and visual systems are normal, as demonstrated from evoked potential studies. Altered somatosensory evoked potentials and abnormal electroencephalogram (EEG) findings of focal, multifocal, and generalized epileptiform discharges, and the occurrence of rhythmic slow theta activity, all suggest altered cortical excitability in the RTT brain. However, the EEG patterns are not diagnostic of RTT, and they vary between patients and at different stages of the disorder (Moser et al., 2007). Electrocardiographic recordings demonstrate long corrected QT intervals and suggest perturbation of the autonomic nervous system (Glaze, 2005). Studies of MeCP2 mouse models reveal abnormalities in long-term potentiation (LTP) and impaired synaptic plasticity; LTP is reduced in 
Mecp2^{-/-} cortical slices (Asaka et al., 2006) and in 
Mecp2^{308/Y} cortical and hippocampal slices (Moretti et al., 2006). In contrast, LTP is enhanced with doubling of MeCP2 in 
Mecp2^{tgr} hippocampal slices (Collins et al., 2004). In addition, loss of MeCP2 in mice results in reduced spontaneous activity in cortical slices due to a decrease in the total excitatory synaptic drive and an increase in the total inhibitory drive (Dani et al., 2005). Cultured hippocampal neurons from 
Mecp2 null mice have decreased frequency of spontaneous excitatory synaptic transmission, and an increase in short-term synaptic depression rate (Nelson et al., 2006). Chao et al. (2007) investigated the physiological consequences of either loss or doubling of MeCP2 on an individual neuron using autaptic cultured hippocampal neurons from 
Mecp2^{-/-} and 
MECP2^{tgr} mice, and they found an approximately 50% reduction and 100% enhancement of synaptic outputs in glutamatergic 
Mecp2^{-/-} and 
MECP2^{tgr} neurons, respectively. They went on to demonstrate that these physiological changes predominantly result from an altered number of glutamatergic synapses rather than dysfunction of the presynapse or postsynapse. Neurons from 
Mecp2^{-/-} mice have an approximately 40% reduction in VGLUT1-PSD95-positive puncta, whereas those from 
MECP2^{tgr} mice have a 60% increase in such puncta in comparison with wild-type mice (Chao et al., 2007).

The alterations in glutamatergic synapse number were observed both at the single-neuron level and in vivo during the early or “presymptomatic” disease stage (Chao et al., 2007). This finding is noteworthy in that it suggests that homeostatic compensatory changes must occur in response to altered synapse number. Altogether, these findings indicate that MeCP2 is essential in modulating synaptic function and plasticity, and that MeCP2 function is critical in regulating the number of excitatory synapses during early postnatal development.

MeCP2 Target Genes and Relevance to RTT Pathogenesis

Since RTT results from dysfunction of a putative transcriptional repressor, it is logical to pursue the transcriptional targets of MeCP2 to gain insight into disease pathogenesis. Transcriptional profiling studies using brain tissue from 
Mecp2 null mice did not reveal major gene expression changes, suggesting that MeCP2 may not be a global transcriptional repressor as previously thought, and that loss of MeCP2 must lead to subtle gene expression changes (Tudor et al., 2002). Alternatively, if MeCP2 regulates the expression of a select number of genes in different subsets of neurons, such transcriptional changes would be missed in samples of brain tissue containing a host of vastly different neuronal types. Several studies have used candidate gene approaches or samples from both human and mouse tissues and identified putative MeCP2 targets that might be relevant to the pathogenesis of RTT. Some of these targets have been confirmed while others yielded different results in different laboratories. A list of the targets that have been studied more extensively is shown in Table 3. In this section we will limit the discussion to a handful of targets that might provide insight about the function of MeCP2, RTT pathogenesis, or both.

MeCP2 Regulates Gene Expression in an Activity-Dependent Manner

Brain-derived neurotrophic factor (BDNF) was identified as a target of MeCP2 through a candidate gene approach (Chen et al., 2003; Martinowich et al., 2003). Rat 
Bdnf is under the control of four promoters, one of which, promoter III, is activated by calcium influx through L-type voltage-sensitive calcium channels (Tao et al., 1998). Chen et al. (2003) found that in the absence of neuronal activity in wild-type cultured neurons, MeCP2 is bound to the rat 
Bdnf promoter III and mediates its transcriptional repression. Membrane depolarization triggers the phosphorylation of MeCP2 at serine 421 (S421) through a CaMKII-dependent mechanism, and releases it from the promoter,
Deletion of campal neuronal sizes. Furthermore, forebrain-specific observed in totic neurons of mice mimicked some of the phenotypes mice. Moreover, conditional deletion of thus allowing transcription (Chen et al., 2003; Zhou et al., Jordan and Francke, 2006; Makedonski et al., 2005; Samaco not reproduced in independent studies (Hogart et al., 2007; Table 3. MeCP2 Target Genes

<table>
<thead>
<tr>
<th>Gene</th>
<th>Function</th>
<th>References</th>
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<tbody>
<tr>
<td>Bdnf</td>
<td>neuronal development and survival</td>
<td>Chen et al., Martinowich et al.</td>
</tr>
<tr>
<td>xHairy2a</td>
<td>neuronal repressor</td>
<td>Stancheva et al.</td>
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<tr>
<td>DLX5/ Dlx5</td>
<td>neuronal transcription factor</td>
<td>Horike et al.</td>
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<tr>
<td>Sgk1</td>
<td>hormone signaling</td>
<td>Nuber et al.</td>
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<tr>
<td>Fkbp5</td>
<td>hormone signaling</td>
<td>Nuber et al.</td>
</tr>
<tr>
<td>Uqrc1</td>
<td>mitochondrial respiratory chain</td>
<td>Kriaucionis et al.</td>
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<tr>
<td>ID1-3/ id1-3</td>
<td>neuronal transcription factors</td>
<td>Peddada et al.</td>
</tr>
<tr>
<td>FXYD1/ Fxyd1</td>
<td>ion channel regulator</td>
<td>Deng et al.</td>
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<tr>
<td>IGFBP3/ Igfbp3</td>
<td>hormone signaling</td>
<td>Itoh et al.</td>
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<tr>
<td>GABRB3</td>
<td>GABA-A receptor</td>
<td>Samaco et al.</td>
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For some of these targets (DLX5 and UBE3A), the data were not reproduced in independent studies (Hogart et al., 2007; Jordan and Francke, 2006; Makedonski et al., 2005; Samaco et al., 2005; Schule et al., 2007).

thus allowing transcription (Chen et al., 2003; Zhou et al., 2006) (Figure 3A). In neurons expressing an S421A mutant form of MeCP2, the neuronal activity-dependent transcription of the mouse Bdnf promoter IV (which corresponds to rat promoter III) was impaired, suggesting that the unphosphorylatable form of MeCP2 cannot be released from the Bdnf promoter in response to membrane depolarization (Zhou et al., 2006). Another study found that the depolarization-induced increase in BDNF expression in mouse cortical cultures correlates with a decrease in methylation of CpGs in the Bdnf promoter IV region. Moreover, the increase in Bdnf transcription involved dissociation of MeCP2 and its associated corepressor complex from Bdnf promoter IV (Martinowich et al., 2003). So while these cortical culture studies provide evidence that MeCP2 binds the Bdnf promoter and regulates transcription of Bdnf in an activity-dependent manner, in vivo studies provide intriguing findings that question the role of MeCP2 as a repressor of Bdnf expression. Chang and colleagues reported that BDNF protein levels are decreased rather than increased in brains of symptomatic Mecp2−/− mice. Moreover, conditional deletion of Bdnf in postmitotic neurons of mice mimicked some of the phenotypes observed in Mecp2 null mice, including hindlimb claspings, reduced brain weight, and reduced olfactory and hippocampal neuronal sizes. Furthermore, forebrain-specific deletion of Bdnf in Mecp2−/− mice resulted in earlier onset of locomotor dysfunction and reduced lifespan, while forebrain-specific overexpression of Bdnf in Mecp2−/− mice improved locomotor function and extended their lifespan (Chang et al., 2006). These results demonstrate in vivo interaction between Mecp2 and Bdnf, and a correlation between altered BDNF levels and neurologic impairment in Mecp2 null mice. One possible explanation for the discrepancy in observed BDNF levels between the in vivo and cell culture data is that neuronal activity was experimentally controlled in isolated cortical cultures (Chen et al., 2003), whereas in brains the neurons are inherently at different activity states (Chang et al., 2006). Since Bdnf is known to be upregulated in response to neuronal activity, the reduced cortical activity in Mecp2 null mice is expected to negatively affect Bdnf expression, hence masking the expected upregulation that would normally result from loss of repression in resting cortical neurons that lack MeCP2 (Chang et al., 2006). A recent study found that although Mecp2−/− nodose ganglia and cortical neurons express less Bdnf mRNA and contain lower levels of the protein compared with wild-type neurons, they secrete a greater percentage of their total BDNF content. This enhanced secretion is due to an increase in BDNF stores available for release. Catecholamine release from Mecp2−/− adrenal chromaffin cells is similarly affected, indicating general neurosecretory alterations in Mecp2 null mice (Wang et al., 2006). The fact that Bdnf null mice display a subset of phenotypes observed in Mecp2 null mice raises the possibility that the two proteins have certain overlapping effects. It is possible that MeCP2 dysfunction reduces overall neuronal activity and indirectly results in decreased BDNF. Alternatively, the alleviation of some of the Mecp2−/− phenotypes upon BDNF overexpression may be a consequence of the general benefits of this neurotrophic factor. More studies are needed to determine the effects of MeCP2 on Bdnf expression in vivo and the role of BDNF in the initial stages of RTT pathogenesis. Irrespective of the effects of MeCP2 on Bdnf expression, there is compelling evidence that the phosphorylation of MeCP2 at S421 occurs in response to activity and that this phosphorylation leads to displacement of MeCP2 from the Bdnf promoter (Chen et al., 2003; Zhou et al., 2006). The finding that MeCP2 is phosphorylated at S421 in the suprachiasmatic nucleus in response to light exposure in mice after they are placed in 48 hr of darkness is an elegant demonstration of a role for this protein in activity-dependent modulation of gene expression.

**MeCP2 as a Modulator of Gene Expression**

The role of MeCP2 in regulating the expression of some genes that are involved in the stress response is consistent with its critical role in the context of altered neuronal activity. Stress is clearly an altered physiologic state that calls for dynamic changes in the expression of a variety of genes that control the activity of the hypothalamic-pituitary-adrenal axis, and the effector genes that are needed to modulate the activity of steroids and their receptors. Gene expression studies using RNA from Mecp2 null mouse brains reveal upregulation of the glucocorticoid-regulated genes, serum glucocorticoid-inducible kinase 1 (Sgk1) and FK506-binding protein 5 (Fkbp5). The reported expression changes were observed in
presymptomatic, early symptomatic, and late symptomatic MeCP2 null mice. It is interesting that although these genes are induced in response to stress, the basal plasma glucocorticoid levels in these animals were not elevated, suggesting that the induced differences are not a result of high hormone levels. In addition, ChIP analysis showed that MeCP2 binds to the Fkbp5 and Sgk1 genes in wild-type brain. These findings raised the possibility that at least some RTT symptoms arise from the disruption of MeCP2 regulation of stress-responsive genes (Nuber et al., 2005).

Following a phenotype-driven approach, McGill et al. identified a MeCP2 target gene that is potentially relevant to a specific RTT feature (McGill et al., 2006). The RTT mouse model, Mecp2<sup>308/Y</sup>, carrying a truncated MeCP2 allele, displays increased anxiety-like behavior (McGill et al., 2006; Shahbazian et al., 2002a). This is reminiscent of clinical reports of frequent high-anxiety episodes in RTT patients (Mount et al., 2001). To determine if stress underlies the anxiety in Mecp2<sup>308/Y</sup> mice, McGill and colleagues evaluated the physiological stress response in these animals and found that Mecp2<sup>308/Y</sup> mice have an enhanced physiologic response to stress manifested as increased corticosterone levels. This finding led to the evaluation of Crh as a target gene given that it encodes the neuropeptide corticotropin-releasing hormone (CRH), which modulates behavioral and physiologic responses to stress (Bale and Vale, 2004; Bruhn et al., 1984). RNA in situ hybridization and quantitative real-time RT-PCR on the paraventricular nucleus of the hypothalamus, the central amygdala, and the bed nucleus of the stria terminalis, three brain regions critical to the stress response, revealed increased Crh levels in Mecp2<sup>308/Y</sup> mice. ChIP analysis revealed that MeCP2 binds the Crh promoter, which is enriched for methylated CpGs. The enhanced Crh expression detected in Mecp2<sup>308/Y</sup> mice is due to a functional defect in the truncated MeCP2<sup>308</sup> protein itself, since there were no differences in Crh promoter methylation patterns between the wild-type and mutant mice (McGill et al., 2006). This study suggests that overexpression of a specific MeCP2 target might explain a specific RTT phenotype. Crh is an attractive candidate in the investigation of therapeutic intervention strategies in RTT given that Crh receptor 1 (Crh1) antagonists have been shown to reduce anxiety (Bale and Vale, 2004). It is noteworthy that the increase in Crh RNA levels results from upregulation of expression specifically where it is normally expressed, rather than from ectopic expression in neurons where it is normally repressed. These data argue that MeCP2 is not a silencer that turns off its targets where they are not normally expressed, but rather functions as a modulator of gene expression depending on the physiologic state of the organism. Metaphorically speaking, MeCP2 may be best thought of as the dimmer that regulates the amount of light rather than the switch that turns the lamp on and off. The facts that MeCP2 undergoes phosphorylation upon light exposure (a new physiologic state) and that it regulates RNA levels of a neuropeptide whose levels fluctuate according to the level of stress are consistent with this model.

Rescuing RTT in Mouse Models
The absence of neuronal degeneration in MeCP2 disorders begs the question of whether restoring MECP2 expression would restore normal neuronal function and reverse the resulting disease phenotypes. Recent work by Guy et al. (2007) and Giacometti et al. (2007) provides evidence supporting the feasibility of disease reversibility in mouse models of RTT. Guy et al. created a mouse in which endogenous MeCP2 is silenced by insertion of a Lox-Stop cassette and can be conditionally activated through Cre-mediated deletion of the cassette. The Mecp2<sup>lox-Stop</sup> allele behaved as a null mutation, and its activation was controlled by a tamoxifen-inducible (TM-inducible) Cre transgene. Acute TM injections caused sudden activation of MeCP2 and led to either rapid death or complete phenotypic rescue of the null mice. The associated toxicity resembled that caused by MeCP2 overexpression and was not due to the TM injections. A more gradual approach for induction using smaller and repeated doses of TM eliminated the toxicity and reversed the late-onset neurological phenotype of adult Mecp2<sup>lox-Stop</sup>/+-; cre heterozygotes. The results indicate that MeCP2-deficient neurons are not permanently damaged, since MeCP2 activation leads to robust abrogation of advanced neurological defects in both young and adult animals. The authors propose a model in which neuronal MeCP2 target sites are defined by DNA methylation patterns that are preserved in its absence and that guide newly synthesized MeCP2 to its correct chromosomal positions. MeCP2 is normally repressed. These data argue that MeCP2 is not a silencer that turns off its targets where they are not normally expressed, but rather functions as a modulator of gene expression depending on the physiologic state of the organism. Metaphorically speaking, MeCP2 may be best thought of as the dimmer that regulates the amount of light rather than the switch that turns the lamp on and off. The facts that MeCP2 undergoes phosphorylation upon light exposure (a new physiologic state) and that it regulates RNA levels of a neuropeptide whose levels fluctuate according to the level of stress are consistent with this model.

In an independent study, Giacometti et al. (2007) demonstrated partial disease rescue by postnatal reactivation of MeCP2 in mutant animals. The investigators targeted a transgene carrying the mouse Mecp2-e2 cDNA downstream of a LoxP-Stop-LoxP (LSL) cassette to the Col1a1 locus, which provides a strong ubiquitous promoter. The LSL-Mecp2 transgene was then placed on a Mecp2 null background to determine if it could rescue upon excision of the Stop signal. Four different Cre transgenes, as follows, were tested to activate Mecp2: Nestin-Cre in neuronal and glial precursors; Tau-Cre in postmitotic neurons during embryogenesis; CaMKII-Cre 93 (C93) in the forebrain, hippocampus, midbrain, and brainstem at ~postnatal day (P) 0–P15; or CaMKII-Cre 159 (C159) in the forebrain at ~P15–P30. Although activation of the LSL-Mecp2 transgene prolonged the lifespan and delayed motor deterioration of Mecp2<sup>+/Y</sup> mice, the extent of rescue directly correlated with the time, level, and site of...
Cre expression. The most efficient symptomatic rescue was obtained in lines that provided early and wide Cre expression in most neurons (Nestin-Cre and Tau-Cre), extending the lifespan of mutant mice to 8 months. Postnatal MeCP2 activation in C93 and C159 lines extended the lifespan by 4 weeks, with the earlier C93 expression giving a more efficient rescue than the later C159 expression (Giacometti et al., 2007). Inappropriate Mecp2 expression levels resulting from use of nonendogenous promoters could account for the partial disease rescue obtained in this study, since this was not seen by Guy et al. when Mecp2 was activated under the control of its endogenous promoter. In fact, MeCP2 levels were much lower than the expected wild-type levels when induced by the C159 line, and neither line expressed the protein in the cerebellum. The findings from the C93 and C159 lines argue that it is critical that MeCP2 levels are restored to the expected wild-type levels and done so in all neurons, or at least in neurons outside of CaMKII domains, to achieve rescue. This conclusion is bolstered by another study in which induction of MeCP2 expression using a CaMKII promoter failed to rescue the lethality in MeCP2 null mice (Alvarez-Saavedra et al., 2007). Altogether, these data underscore the importance of proper expression of MeCP2, probably throughout the CNS or at least beyond the CaMKII expression domain, to achieve rescue. What is exciting, however, is that once proper expression of MeCP2 is achieved, symptoms can be reversed even in adult animals, implying that neurons might not be permanently damaged.

New Questions and Challenges
The past 8 years have witnessed many exciting discoveries that are relevant to MeCP2 function and RTT, yet many of these discoveries have inspired new questions and unveiled unexpected challenges. In the following section we will pose some of the questions that are worthy of consideration and potential new areas of investigation.

What Is the Full Spectrum of MECP2 Disorders?
The findings that MECP2 mutations cause RTT as well as a spectrum of neuropsychiatric phenotypes and that either loss or doubling of MeCP2 causes such phenotypes raise the possibility that regulatory mutations or posttranscriptional alterations that slightly alter MeCP2 levels might cause more restricted phenotypes such as autism, seizures, Parkinsonian features, or cognitive impairment (Coutinho et al., 2007). Given the importance of MeCP2 for postnatal neuronal function, it is conceivable that hypomorphic mutations might compromise neuronal plasticity such that overt phenotypes will become apparent only upon aging. It will take coordinated and large-scale efforts to establish if variations in MECP2 contribute to late-onset neurological disorders involving cognition and motor control.

What Are the Functions of MeCP2 and How Are They Affected by the Various Mutations?
Based on various in vivo studies, a few conclusions can be safely made about the function of MeCP2. It binds DNA at specific sites and this binding is affected by its phosphorylation state at S421, which in turn depends on neuronal activity. The finding that the expression changes in some of its targets are small argues that the protein modulates expression in response to activity and that such modulation is critical for normal neuronal function. Such modulatory effects would be easily missed when profiling RNA from heterogeneous neuronal populations. This is particularly true for targets that are expressed in specific neurons.

The finding that either loss or doubling of MeCP2 results in progressive postnatal neurological disorders that have overlapping features is quite intriguing. The recent discovery that such loss and doubling result in decreases and increases (respectively) in excitatory synapse numbers argues that MeCP2 plays an important role in determining excitatory synapse number in early development. Whether this is achieved by modulating expression or splicing of factors critical for synapse formation (or both) remains to be determined. The finding, however, that loss and doubling of MeCP2 have opposing effects on synapses argues that certain expression or splicing changes might go in opposite directions in Mecp2 null versus duplication mice. Thus, these two models might provide an excellent resource to uncover some of the primary targets of MeCP2, which in turn would be an excellent framework to evaluate the functional consequences of some of the other RTT-causing MECP2 mutations. There are several alleles that alter one amino acid outside of the MBΔ or cause late truncations, raising the possibility that some of these might lead to a gain-of-function or hypermorphic effect as observed for the duplication.

What Is the Anatomical Origin of Various RTT Phenotypes and What Are the Associated Molecular Changes?
Given the diversity of phenotypes associated with MeCP2 dysfunction, a key question is whether individual phenotypes result from dysfunction of the protein in specific neurons, whether the phenotypes result from dysfunction of the entire neuronal network (the more compromised the network, the more phenotypes become apparent), or a combination of both (Figure 4). The identification of isolated partial phenotypes that are not always the same among patients with favorable XCI supports a model whereby dysfunction in specific neurons could explain one or more clinical features. The starting point of the disease mechanism could be cell-autonomous changes occurring during postnatal development that subsequently cause specific symptoms. Such select neuronal changes together with altered experiences might eventually lead to malfunction of an entire network, which is then manifested as additional neurobehavioral abnormalities. The molecular corollary of such a model is that the neuropsychiatric features of RTT and related disorders result from misregulation of some genes expressed in a specific set of neurons. Studies of large numbers of patients with essentially any one of the RTT phenotypes might identify females with partial symptoms and favorable XCI. Eventually, the contributions of specific neurons versus the
networks of neurons to the RTT phenotype could be addressed in the mouse using neuron-specific deletions of Mecp2. The finding that anatomical changes, such as the decrease in glutamatergic synapses, are only detected in the presymptomatic stage (Chao et al., 2007) argues that compensatory changes might mask the primary consequences of MeCP2 dysfunction. Therefore, to get at some of the primary pathogenic events, one needs to study presymptomatic as well as early symptomatic animals.

The Challenge of Developing Therapies for MECP2 Disorders

The recent studies demonstrating that neurological deficits resulting from loss of MeCP2 can be reversed upon restoration of gene function are quite exciting. The next phase of research needs to assess how complete the recovery is. Clearly, lethality, level of activity, and hippocampal plasticity are rescued, but are the animals free of any other RTT symptoms such as social behavior deficits, anxiety, and cognitive impairments? Since postnatal rescue results in viability, it will be important to evaluate if even the subtler phenotypes of RTT and MECP2 disorders are rescued when protein function is restored postnatally. This is particularly important given emerging data about early neonatal experiences and their long-term effects on behavior in adults (Plotsky et al., 2005; Sullivan et al., 2006). The genetic rescue data are promising because they show that neurons that have suffered the consequences of loss of MeCP2 function are poised to regain functionality once MeCP2 is provided gradually and in the correct spatial distribution. This provides hope for restoring neuronal function in patients with RTT. However, the strategy in humans will require providing the critical factors that function downstream of MeCP2 because of the challenges in delivering the correct MeCP2 dosage only to neurons that lack it, given that the slightest perturbation in MeCP2 level is deleterious. Thus, therapeutic strategies necessitate the identification of the molecular mechanisms underlying individual RTT phenotypes and picking out the candidates that can be therapeutically targeted. Although the data are minimal, it is quite likely that there will be many genes whose expression is sufficiently altered to cause neuronal dysfunction, probably due to loss of normal homeostatic responses. While it is conceivable that some of these molecules could be investigated for potential therapeutics, it might prove challenging to restore levels of tens of targets.

An alternative approach will be to identify proteins or pathways that suppress MeCP2 dysfunction phenotypes, which might prove easier to target therapeutically. The fact that there are human patients with milder phenotypes in spite of severe mutations (Dayer et al., 2007) argues that some variant in another protein or proteins might subdue the disease. Identifying such modifiers using various mouse models might prove very helpful. Lastly, given that loss and gain of MeCP2 have opposing effects on neurons, it will be important to establish whether certain mutant MECP2 alleles are functionally null or hyperactive when contemplating therapeutic trials.

In closing, the RTT story started in the clinic, but today has inspired many exciting basic science studies in neurobiology and epigenetics. It is anticipated that the next chapter in this story will involve translating some of the discoveries back to the clinic to benefit patients with RTT and patients with related neurological disorders.

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