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Rett syndrome: a surprising result of mutation in *MECP2*

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The identification of mutations in the gene encoding methyl CpG binding protein 2 (MeCP2) in Rett syndrome represents a major advance in the field. The current model predicts that MeCP2 represses transcription by binding methylated CpG residues and mediating chromatin remodeling. A physical interaction between MeCP2, histone deacetylases and the transcriptional co-repressor Sin3A has been demonstrated, as well as an association of MeCP2 with the basal transcription apparatus. It is unclear, however, whether MeCP2-mediated chromatin remodeling is necessary for transcriptional repression *in vivo*. Eight recurrent missense and nonsense mutations account for >65% of the mutations identified in Rett syndrome probands, and as predicted from the sporadic nature of the disorder, most mutations are *de novo*. The severity of the phenotype is likely to reflect the pattern of X chromosome inactivation in relevant tissues, although the type and position of the mutation may also play a role. Although much is known about the biochemical function of MeCP2, the phenotype of Rett syndrome suggests that it plays an unexplored but critical role in development and maintenance of the nervous system.

INTRODUCTION

The genetic basis and mode of inheritance of Rett syndrome (RTT; MIM 312750) were debated in the years since the original descriptions by Andreas Rett and Bengt Hagberg of a severe childhood neurological disorder associated with developmental regression and acquisition of stereotypic hand movements (1,2). In October 1999, however, the identification of mutations in the gene encoding methyl CpG binding protein 2 (MeCP2) confirmed the initial hypotheses that RTT is an X-linked dominant disorder and that it usually results from new mutations in the relevant gene (3). Although the identification of the gene that causes RTT represents a major advance in our understanding of this enigmatic disorder, there is no obvious link between loss of function of MeCP2 and the pathogenesis of RTT.

MAPPING THE RTT LOCUS

The earliest hypothesis on the inheritance of RTT was founded on the seemingly exclusive appearance of the disorder in females. It was thought that it must be an X-linked dominant disorder, perhaps lethal to males (2,4). However, proving this hypothesis was confounded by that fact that classic RTT is almost always sporadic and is associated with decreased reproductive fitness of the affected females (due to significant cognitive deficits). Thus, familial recurrences were rare but were absolutely requisite for defining the inheritance pattern and mapping the locus. The first progress in mapping came from studies of two maternally related half-sister pairs and an aunt–niece pair (5–7). If the gene were X-linked, then the causative gene should lie on the shared region of the X chromosome. Regions that were inherited concordantly or discordantly by the probands were identified by genotyping and the locus was excluded from regions that were discordantly inherited. This process of exclusion mapping was exceptionally useful in the context of an X-linked dominant trait because it allowed significant mapping information from relatively few meioses, but required pedigrees where the parent of origin of the mutation was clear. Since most recurrences were full-sister pairs, this approach could not be used to narrow further the region with certainty because of the possibility of a paternal germline mosaicism for the mutation. Nonetheless, a portion of the long arm was tentatively excluded based on data from several pairs of full sisters (8). Subsequently, two additional families were identified that allowed further narrowing of the candidate region. Based on allele sharing between an affected aunt, niece and nephew and three sisters with classic RTT whose mother showed strongly skewed X chromosome inactivation, the locus was mapped to Xq27.3 qter, a particularly gene-rich part of the genome (9–12). In many disorders, genetic mapping is followed by intensive efforts to identify transcripts within the critical region. As each new transcript is identified, it is carefully screened for mutations in probands with the disorder. Because of the extensive mapping of the region, primarily due to the efforts of the Human Genome Project, the difficulty was not in identifying a transcript to screen, but in choosing which transcripts to screen first.

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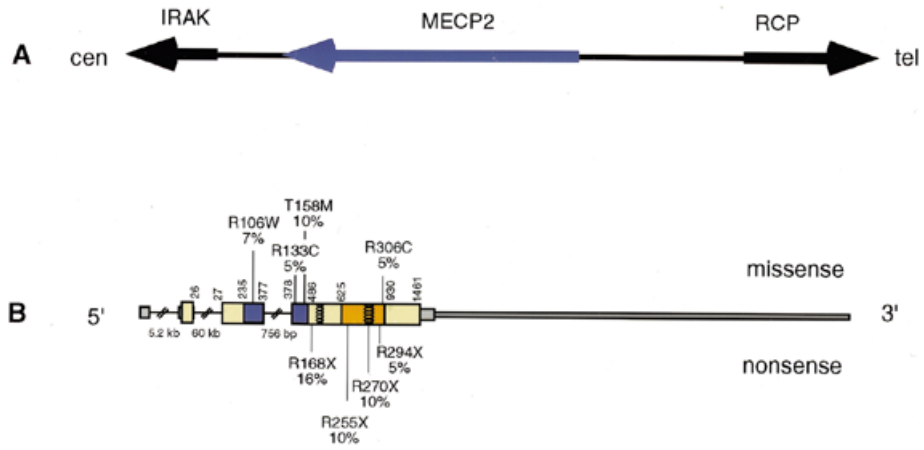

Figure 1. (**A**) The *MECP2* gene in Xq28 is flanked by the IRAK and RCP loci in humans (40). Orientation of transcription is indicated by the direction of the arrows. (**B**) The genomic organization of the *MECP2* gene. It is comprised of four exons, and nucleotide positions relative to the ATG codon are indicated. The untranslated regions are shown in gray. The 5′-UTR is relatively short (167 nucleotides), and contained in exon 1 (69 nucleotides) and part of exon 2 (98 nucleotides). The coding sequence for the methyl-binding domain is indicated in blue, and is split between exons 3 and 4. The region encoding the transcription repression domain (orange) lies in the fourth exon (59,60,62,78). There are two nuclear localization signals (hatched), one encoded by the region between nucleotides 173 and 193 (63) and the other lying within the transcription repression domain. The long 3′-UTR contains two polyadenylation signals that are differentially utilized. The thicker segment indicates the region encoding the short transcript (1.8 kb) and the long (~10 kb) transcript is indicated by the thinner gray segment. Positions of the common mutations and their relative frequencies are indicated above (missense) and below (nonsense).

CANDIDATE GENE SCREENING

Studies of RTT kindreds were used not only to map the locus but also to give clues about the gene's identity. The more severe phenotype in males confirmed X-linked dominant inheritance, whereas the finding that three unaffected or minimally affected carrier females had strongly skewed X-inactivation patterns indicated that the causative gene was likely to undergo X-inactivation (11,13–15). This was also supported by variable phenotypes in affected monozygotic twin pairs (16,17). A small rearrangement in Xq28 was also considered, particularly after a rare child with both RTT and incontinentia pigment was identified (18). Although either a microdeletion or duplication would be consistent with an X-linked dominant condition, no rearrangements were detected by high-resolution genotyping, fluorescence *in situ* hybridization (FISH) or Southern analysis (19, and our unpublished data).

Numerous laboratories were also involved in mutation screening of candidate genes from the 'unexcluded' portions of the X chromosome. In order to choose candidate genes, the RTT phenotype was scrutinized in the hope of detecting clues as to the gene's identity. The most obvious pathology in RTT is limited to the nervous system where there is evidence of abnormal development or maintenance of neurons in cortical layers 3 and 5 (20). Of note, mosaicism for the mutation is not apparent at the cellular level. All neurons in affected regions appear to have similar abnormalities in dendritic organization, suggesting that the effects of mutation are not cell autonomous. Initially, attention was directed largely toward genes expressed either predominantly or solely in the central nervous system (CNS), and genes for neurotransmitter receptors, neural-specific proteins, were excluded (12,21–30). Closer examination of the phenotype in females with RTT and insight gained from the more severe phenotype manifested by the affected males suggested that the causative gene was important for the function of tissues outside the CNS. For example, probands generally have some degree of growth failure, even with adequate nutrition (31,32). This was strikingly apparent in the affected boys, who displayed severe postnatal growth failure (14). Osteopenia is also common, despite apparently normal homeostasis of calcium, phosphate and the endocrine systems that regulate bone density (33). In addition, about half of girls with RTT have prolonged QT intervals, possibly related to the sudden death seen in children with RTT (34,35).

Thus, the face of the culprit gene evolved. It lay in distal Xq and was subject to X-inactivation. It was likely to be expressed not only in the brain; neuropathological findings indicated that it played a role in neuronal development and maintenance of normal connectivity, particularly within the cortex, basal ganglia and probably brainstem. Mutations did not have cell autonomous effects but globally altered neurons in susceptible regions of the brain. Thus, in the fall of 1999, using the positional candidacy strategy, Amir *et al*. (3) identified mutations in the *MECP2* gene in several probands with RTT. The gene had been mapped previously into the candidate region and the full genomic sequence was available (36); missense and nonsense mutations were found that were predicted to lead to loss or decreased function of the protein. In addition, mutations were found in most of the families that had been used for mapping the gene as well as a number of sporadic cases of mixed ethnic backgrounds (3,37).

MECP2 GENE ORGANIZATION AND EXPRESSION

The *MECP2* gene spans 76 kb in Xq28 and lies between the interleukin I receptor-associated kinase (IRAK) and the red opsin (RCP) loci, a region of conserved synteny with mouse Chr XC (38–40). The gene is composed of four exons that are transcribed from telomere to centromere (Fig. 1A), with the 1461 nucleotide coding sequence in exons 2–4 (Fig. 1B). The 3′-untranslated region (3′-UTR) in exon 4 is unusually long (8.5 kb) and well-conserved between human and mouse,

although its functional role has not been elucidated (40,41). The *MECP2* gene is considered a housekeeping gene because it has a CpG island associated with the 5′ end and is ubiquitously expressed (40). There are several potential Sp1-binding sites in the CpG island and indirect evidence that Sp1 at least partially regulates its expression (42). The gene is subject to X-inactivation in mouse and human cells (39,43).

In mammals, *MECP2* is widely expressed in embryonic and adult tissues, although expression is at low levels early in development (44). There are three transcripts detected in most tissues in humans which vary in length (1.8, ~7.5 and ~10 kb) and arise from differential use of polyadenylation signals in the 3′-UTR. In human and rodent tissues, short (1.8 kb) and long (10 kb) transcripts are present in most tissues although there appears to be tissue-specific variation in expression of the two transcripts. By northern blot of adult tissues, brain and spinal cord have higher expression of the long transcript, relative to the shorter one. They are expressed at similar levels in lung, kidney, tissues from the gastrointestinal tract, thyroid and adrenal glands, whereas the smaller transcript is more abundant in cardiac and skeletal muscle, lymphoid tissues, liver and placenta (39,40). By *in situ* hybridization in mouse, there is low level expression of the long transcript in the developing nervous system, with increased expression in postnatal hippocampus and olfactory bulb (41). The two transcripts have similarly short half-lives (3–4 h); therefore, the functional significance for the differential expression is unknown (40).

MeCP2 STRUCTURE AND FUNCTION

CpG dinucleotides are not randomly distributed in the genome but densely populate much of the heterochromatic regions of chromosomes as well as the promoter regions of many genes. Of the cytosine residues found in these CpG dinucleotides in mammalian genomes 60–90% are modified by methylation at the carbon 5 position. Methylation of the cytosine residues of CpG dinucleotides is an important mechanism of gene silencing, both in terms of stable silencing of heterochromatin and in the reversible regulation of gene expression (for reviews see refs 45,46). This epigenetic mechanisms of transcriptional repression are clearly important for processes such as X chromosome inactivation (47), imprinting (48) and the silencing of endogenous retroviruses (49), but is also used in the tissue-specific and developmental regulation of transcription (50). Gene silencing mediated by DNA methylation is thought to occur predominately through an indirect mechanism in which repressor proteins are recruited to methylated sites (51), although methylation alone can sometimes directly repress transcription (52). MeCP2, a protein that couples DNA methylation to the silencing machinery is probably a critical mediator of methylation-dependent repression.

Initial attempts to identify protein-mediated CpG methylation-dependent repression led to the identification of MeCP1 (53,54) and MeCP2 (55). Both MeCP family members bind symmetrically methylated CpG dinucleotides (44) and do so in a sequence-independent manner (54). Unlike MeCP1, which has a higher affinity for densely methylated DNA (56), MeCP2 can bind singly methylated CpG pairs (55). Nan *et al.* (57) demonstrated that the degree of MeCP2-mediated repression of methylated templates plateaus at 0.5–1.0 mCpG/100 bp and remains at a constant level for higher methylation densities.

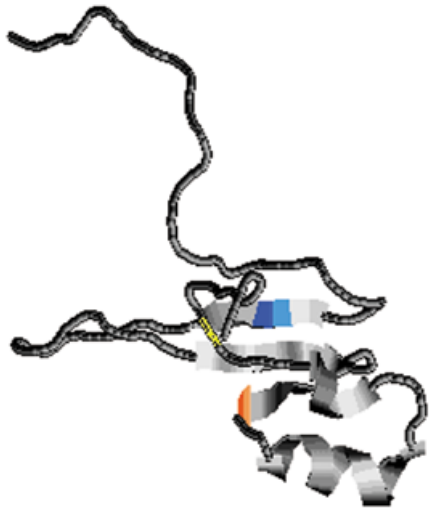

Figure 2. The predicted structure of the methyl CpG binding domain elucidated by Wakefield *et al.* (65) assembled in RasMac v2.6 using the coordinates placed in the Brookhaven Protein Data Bank (http://www.rcsb.org/pdb/) for MeCp2 (PDB ID 1qk9). Three of the four β-sheets are seen and are predicted to interact with the mCpG in the major groove. Positions of recurrent mutations are shown: R106W (blue), R133C (orange) and T158M (yellow) (65,108).

Immunofluorescence studies have shown strong localization of MeCP2 to pericentric heterochromatin in the mouse (55), whereas more diffuse staining is seen in rat (55) and human (58) chromosomes. Little MeCP2 expression is seen during embryonic development but is detected at a higher abundance than MeCP1 in all rat adult somatic tissues, with the highest expression in brain (44). Although MeCP1 and MeCP2 share some functional homology, differences in binding specificity and expression patterns suggest that MeCP1 does not compensate for loss of MeCP2 function.

The MeCP2 polypeptide contains a methyl-binding domain (MBD) (59), a transcriptional repression domain (TRD) (60,61) and two putative nuclear localization signals (NLS) (62,63). The MBD consists of an 85 amino acid stretch at the N-terminal end of the protein, which is both necessary and sufficient to bind DNA in the presence or absence of assembled chromatin (59). Nuclear magnetic resonance solution data of the MBD revealed a novel wedge-shaped structure consisting of four antiparallel β-sheets and a loop with a hydrophobic pocket on the face of the domain that are predicted to bind mCpG base pairs in the major groove of DNA (Fig. 2) (64,65). In *Drosophila* cells, which do not modify DNA by methylation, MeCP2 is able to repress SP1 transactivation of a methylated promoter and a C-terminal deletion mutant containing the MBD and the NLS is sufficient to confer this repression (63). The TRD was delineated by Nan *et al.* (57), who mapped the domain to amino acids 207–310 by replacing the MBD with the GAL4 DNA-binding domain fused to various MeCP2 deletions and assaying for repression of a reporter gene containing the GAL4 binding element (57). A co-repressor complex containing the transcriptional co-repressor

Table 1. Summary of mutations identified in *MECP2*

Nucleotide change	Amino acid change	Domain	Frequency	References
258–259delCA	fs	M	0.42	82
291C→A	D97E	M	0.42	12
301C→A	P101T	M	0.42	76
302C→A	P101H	M	0.42	76
302C→T	P101L	M	0.42	76
316C→T	R106W	M	7.50	3,12,37,74,76,82,ms[a]
317G→A	R106Q	M	0.42	75
378-2ag splice site	fs	M	0.83	74,82
397C→T	R133C	M	3.75	3,12,37,76,82,ms[a]
398G→A	R133H	M	0.42	ms[a]
401C→G	S134C	M	0.83	76,82
407del507+insGCTTTTAG	fs	M	0.42	76
411delG	L138X	M	0.42	37
NP[b]	Y141X	M	0.42	74
455C→G	P152R	M	3.33	74,76,82,ms[a]
463T→A	F155I	M	0.42	12
464T→C	F155S	M	0.42	3
NP[b]	D156E	M	0.42	74
473C→T	T158M	M	10.42	12,74–76,82,ms[a,c]
502C→T	R168X	I	15.42	12,74–76,82,ms[a,c]
NP[b]	Q170X	I	0.42	74
592A→T	R198X	I	0.42	75
611C→T	S204X	I	0.42	[a]
620insT	fs	I	0.42	3
654delGAAG	fs	T	0.42	76
674C→G	P225R	T	1.25	74,76
677insA	fs	T	0.42	75
706delG	fs	T	0.42	74
710insT	fs	T	0.42	[a]
730C→T	Q244X	T	0.42	[a]
747insC	fs	T	0.42	[a]
763C→T	R225X	T	10.42	12,37,74–76,82,ms[a,c]
NP[b]	K256X	T	0.42	74
803delG	fs	T	2.08	37,76,ms[c]
808C→T	R270X	T	7.92	82,74–76,ms[a]
855del4	fs	T	0.42	ms[a]
880C→T	R294X	T	5.42	74–76,82,ms[a]
904C→G	P302A	T	0.42	82
905C→G	P302R	T	0.42	75
905C→T	P302L	T	0.42	76
914A→G	K305R	T	0.42	ms[a]
916C→T	R306C	T	5.00	12,37,74–76,82,ms[a,c]
917G→A	R306H	T	0.83	76
965C→T	P322L	C	0.83	82,[a]

Continued over.

Table 1. *Continued.*

Nucleotide change	Amino acid change	Domain	Frequency	References
965C→G	P322A	C	0.42	75
1098del70	fs	C	0.42	12
1011del191	fs	C	0.42	ms[a]
1038del82	fs	C	0.42	ms[a]
1053ins10	fs	C	0.42	12
1096del101	fs	C	0.42	12
1116del84	fs	C	0.42	74
1120del69	fs	C	0.42	12
1141del55	fs	C	0.42	12
1147del170ins3	fs	C	0.42	74
1154del32	fs	C	0.42	ms[a]
1156del17	fs	C	0.42	75
1156del44	fs	C	0.42	76
1157del41	fs	C	0.42	76
1157del43	fs	C	0.42	82
1158del10	fs	C	0.42	75
1159del43	fs	C	0.42	12
1160del26	fs	C	0.83	ms[a]
1163del26	fs	C	0.42	75
1163del34	fs	C	0.42	82
1163del43	fs	C	0.42	ms[a]
1165del26	fs	C	0.42	75
1182del7	fs	C	0.42	12
1193insT	fs	C	0.42	74
1194insT	fs	C	0.42	75
1364–1365insC	fs	C	0.42	82
1451del4	fs	C	0.42	82
1461A→C	X487C	Terminus	0.42	75

M, MBD; I, intermediate region; T, TRDm; C, C-terminal region.
[a]K. Hoffbuhr *et al.*, manuscript in preparation.
[b]Nucleotide change not provided.
[c]I. Houwink-Manville *et al.*, manuscript in preparation.

Sin3A and histone deacetylases 1 and 2 (HDAC1 and -2) has been shown to associate with MeCP2 within the TRD by co-immunoprecipitation (60,61). The current model predicts that MeCP2 represses transcription through a mechanism that involves binding to mCpG residues and recruitment of HDACs to modify chromatin structure. The deacetylation of histones allows DNA to wind more tightly around the histone, preventing transcription machinery access to the promoters (for reviews see refs 46,58,66,67). There is evidence to suggest that the process may be more complicated than this model since transcriptional repression by MeCP2 does not always require deacetylase activity (60,68,69). Furthermore, MeCP2 has been demonstrated to inhibit the assembly of the basal transcriptional machinery onto methylated promoters in the absence of chromatin assembly and to associate with TFIIB (68) and E2F (70). An interaction between MeCP2 and the transcriptional machinery provides evidence of a mechanism through which MeCP2 is able to repress transcription at a distance (>500 bp) (57,60,68). Finally, MeCP2 may act not only as a dynamic transcriptional repressor but may also play a role in the architecture of silenced chromatin by binding matrix attachment sites (71–73). Thus, the role of MeCP2 in the nucleus is complex and it is likely to mediate transcriptional silencing through several overlapping mechanisms.

THE SPECTRUM OF MUTATIONS IN *MECP2* IN RETT SYNDROME

Because of the length of the untranslated portions of the gene (particularly introns 1 and 2, and the 3′-UTR), the mutation analysis thus far has focused on the coding sequence. There are mutation data reported for >200 probands and overall most laboratories are detecting mutations in up to 80% of classic RTT probands as well as some patients who are considered to have variant forms of RTT (3,37,74–77; K. Hoffbuhr *et al.*, manuscript in preparation; I. Houwink-Manville *et al.*, manuscript in preparation). Curiously, despite the fact that almost all mutations are *de novo*, there are eight common point mutations resulting from C→T transitions that account for ~65% of mutations in probands with classic RTT (Fig. 1; Table 1) (3,12,37,74–76; K. Hoffbuhr *et al.*, manuscript in preparation; I. Houwink-Manville *et al.*, manuscript in preparation). There is also a propensity for small deletions (accounting for another ~10% of known mutations) in the 3′ end of exon 4 where there are a series of pentanucleotide (CCACC) repeats. This region does not encode a known protein domain, although there is evidence to suggest that the region may be involved in interaction of MeCP2 and the nucleosome core (78). More than 60 distinct mutations have been identified; the majority are nonsense or frameshift mutations although missense mutations affecting highly conserved amino acid residues are also seen. The other RTT mutations have been identified in only one or two probands to date; however, since many of these are also C→T transitions (or A→G, representing a C→T change on the antisense strand), it is expected that recurrences will be seen with continued mutation analysis.

In ~20–25% of classic sporadic RTT probands, no *MECP2* mutation has been detected using polymerase chain reaction (PCR)-based genomic screening approaches or direct DNA sequencing. These methods would not be expected to detect large deletions or intragenic inversions, as seen in the factor VIII gene (79). Limited studies using FISH and Southern blots have not detected any rearrangements to date (our own unpublished data). Several laboratories are now systematically screening the untranslated portions of the gene and the newly identified exon 1 in this sample. Of note, the rate of mutation detection in sister pairs is considerably lower than in the sporadic cases (29%), although the sample size is small (74). Since sibling recurrences are a relatively rare event, does this implicate a second disease locus, possibly autosomal? Mutations have been found in the families that targeted the screening to Xq28 (except in one pair of half-sisters whose mutation has not yet been identified), therefore a putative second RTT locus is essentially unmapped. The absence of sister–brother pairs (or brothers with RTT) would implicate a second X-linked gene if indeed there were a second locus.

In the familial cases used for mapping, skewed inactivation was seen in asymptomatic or mildly affected carriers of *MECP2* mutations (9,11,13,37). Although it is possible that the skewed inactivation results from selection against the mutant allele, it should be noted that in each of the known cases of asymptomatic carriers with non-random X-inactivation, an affected family member with the same mutation has a random pattern (9,11,37). Somewhat surprisingly, in the course of screening the sporadic cases of RTT, two normal mothers were found to carry potentially disease-causing mutations but escaped their effects because of non-random skewing of X-inactivation (74,75). This finding has important implications in recurrence risk counseling, since carriers have a 50% risk of transmitting the mutant allele. In addition, mutation analysis of several of the familial cases indicates recurrence resulting from germline or somatic mosaicism of one parent (37). It had been hypothesized that an excess of paternally derived mutations might account for the nearly exclusive occurrence of RTT in females (80): for the small number of mutations for which parental origin of the disease allele has been distinguished, both maternally and paternally derived mutational events contribute (37,74).

EFFECTS OF MUTATION ON FUNCTION OF MECP2

Nonsense and frameshift mutations, which lead to the creation of premature termination codons in the 5′ end of the coding sequence, are likely to lead to unstable transcripts through the process of nonsense-mediated decay (NMD). In contrast, similar mutations within the last exon may by-pass this pathway and result in the production of a truncated protein (81). Thus, the two RTT frameshift mutations reported in exon 3 (74,82) are predicted to be null alleles because they should undergo NMD, although this has not been demonstrated experimentally. The relative paucity of truncating mutations identified in the 5′ exons may result from absence of phenotype (and thus no mutation analysis) in carrier females because of selection against a true null allele leading to secondary skewing of X-inactivation during development. The majority of the RTT mutations are nonsense or frameshift mutations that lie in the last exon and that would be expected to escape the NMD pathway.

If truncated proteins are being made, the question as to the level of residual function remains and could be important in understanding the mechanisms involved in the cellular dysfunction in RTT. To address this, there are available data from the numerous deleted and truncated mutants that were generated during the elucidation of the functional domains of MeCP2 (44,57,59–61,63,78). Comparison of the human mutations with these experimental mutants supports the possibility that RTT mutations are actually hypomorphic alleles with some retained function, although the truncated proteins have not been identified to date from RTT cell lines.

The functional consequences of the missense mutations on MeCP2 function are just beginning to be addressed experimentally. Two common MBD mutations observed in RTT (R106W and R133C) are both located on the β-sheets comprising the wedge-shaped domain (Fig. 2) (64,65) and have a greatly reduced (>100-fold) affinity for methylated DNA in unassembled chromatin (83). Differential binding of these mutant forms is seen in native mouse heterochromatin with markedly reduced binding of the R106W mutant, whereas the R133C mutant resembles wild type (S. Kudo *et al.*, manuscript in preparation). The other common MBD mutation (T158M), which replaces the threonine residue on the loop structure outside of the DNA-binding domain with a methionine, shows only a small reduction in binding affinity to methylated DNA (2-fold) (83) and intermediary binding to native heterochromatin (S. Kudo *et al.*, manuscript in preparation). The T158 in MeCP2 is conserved from *Xenopus* to human;

however, it is not present in any other MBD family members, which suggests that this residue has a critical role in the precise functioning of MeCP2 unrelated to its methyl-binding activity.

If the mutations lead to partial loss of function of MeCP2, how does this affect the cell? Loss of a transcriptional silencer has been proposed to lead to transcriptional 'noise' resulting from low-level illegitimate transcription (84,85). Even low-level dysregulation of transcription may be particularly important in postmitotic cells that are no longer globally reassembling their chromatin after dividing. The potential role of MeCP2 in heterochromatin assembly and silencing of pseudogenes and 'junk' DNA may also be important and there is experimental evidence to suggest that there may be observable differences in heterochromatin in Rett probands. Prior to the identification of mutations in MECP2, Vorsanova *et al.* (86,87) reported an unusual configuration of the inactive X chromosome in a portion of lymphocytes from RTT probands. The chromosome showed an alteration in replication timing that they termed a C-type inactive X chromosome. Subsequently, they also noted decreased compaction of the heterochromatic regions on chromosome 9 in cells from the same RTT probands (86,87). These alterations are intriguing given the role of MeCP2 in chromatin remodeling and may indicate that heterochromatic regions are particularly affected by MeCP2 mutation. Furthermore, recent data indicating that the subterminal regions of chromosomes are densely methylated may suggest that these regions are also particularly important targets for MeCP2 (88).

MENTHYLATION AND CHROMATIN REMODELING IN NEURONAL DEVELOPMENT

The epigenetic mechanism of gene silencing through methylation is believed to be of considerable importance for development of the differentiated cellular phenotype. Global demethylation of the genome occurs immediately after fertilization, and *de novo* remethylation follows implantation (89) and is maintained through mitosis through the activities of DNA hemimethyltransferases. Modification of genomic DNA by methylation provides a mechanism by which the fate of a differentiated cell is established and can be stably passed on through mitosis (leading to clonal expansion of cells expressing similar repertoires of genes). The role of methylation and chromatin remodeling in development has been partially explored in several model systems. Complete loss of function of the DNA methyltransferase 1 (Dnmt1) enzyme in pluripotent embryonic stem (ES) cells did not appear to have a deleterious effect on their viability in culture, but when incorporated into embryos loss of the enzyme led to embryonic lethality and developmental abnormalities (49). Similarly, treatment of zebrafish embryos with a chemical inhibitor of cytosine methylation, 5-aza-cytidine, resulted in an abnormal phenotype suggesting that methylation is required for normal gastrulation and the patterning of somites (90). Expression of MTase is highest in the CNS and somites of developing zebrafish embryos (90), and expression remains elevated in adult nervous tissues in the mouse (91). This finding is curious because the reason underlying a continued requirement for Dnmt activity in postmitotic cells is not obvious. It is possible that modification of chromatin prevents access of active transcriptional machinery to non-essential genes and is necessary for efficient transcriptional regulation required of specialized cells. Thus, neurons with defects in epigenetic silencing may no longer be able to carry out molecular responses in an appropriate time scale due either to a dilution out of transcriptional regulators because of an abundance of available binding sites or to excess transcriptional noise (for discussion see ref. 84). It can be imagined that defects in gene regulation can translate into altered electrochemical activity in the nervous system and thus disrupt the establishment of synaptic connections. Although no direct experimental evidence exists to date, a role for DNA methylation in long-term learning and memory has also been proposed (92). It has been established that the cyclic AMP-responsive element binding protein (CREB) is required for long-term potentiation (LTP) in the hippocampus (93) and that methylation has been implicated as a regulator of CREB function (94). It will be interesting to see whether *MeCP2* knockout mice have any deficiencies in the generation of LTP or neuronal connectivity.

Chromatin reorganization through the concerted action of mSin3A and HDACs has been implicated in mediating gene silencing through an association with various transcriptional regulators (95,96, and references therein). Unliganded retinoic acid and thyroid hormone receptors and antagonist-bound steroid receptors appear to mediate repression through the interaction with a co-repressor complex (NCoR and SMRT) which attracts Sin3A and HDACs (97,98). Activation of transcription by nuclear receptors in the agonist-bound state is proposed to involve a mechanism through which the HDAC-containing complex is replaced with a complex containing histone acetyltransferase (HAT) activity (for review see ref. 96). The transcriptional repression of >20 neuron-specific genes in non-neuronal cell types is mediated by the neural restrictive silencer factor (NRSF) encoded by *REST*, which also associates with mSin3A and HDAC1 (95,99). Homozygous *REST* knockout mice are embryonic lethal and show ectopic expression of neuronal genes in non-neuronal tissues; however, NRSF does not appear to be involved in cell-fate determination since neuronal patterning appears relatively normal (100). Treatment of non-neuronal cells with trichostatin A, a potent inhibitor of histone deacetylases, induces expression of neuron-specific genes silenced by NRSF and suggests that histone deacetylation is involved in the repression mechanism mediated by NRSF (95). The *GluR2* gene, which encodes a subunit of the AMPA receptor, is regulated by both NRSF and SP1, and has been shown to be differentially methylated at a CpG island in certain tissues (101). The role of methylation in the tissue-specific expression of the *GluR2* is currently unknown; however, it is possible that CpG methylation has an unexplored role in the regulation of other neuron-specific genes with similarly CG-rich promoters. Studies by Saluz and Jost (102,103) in an avian model have demonstrated that the steroid estradiol stimulates demethylation of the estrogen response element and decreased activity of the mCpG-binding protein MDBP-2. These studies suggest that the methylation status of specific genes can be regulated by environmental stimuli that could further serve to regulate gene expression by altering the DNA binding affinity of mCpG-binding proteins such as MeCP2. Thus, over the next several years it will be of great importance for our understanding of the pathology of Rett syndrome to identify

classes of genes that are under the direct regulatory control of MeCP2.

ARE THERE GENOTYPE–PHENOTYPE CORRELATIONS IN RETT SYNDROME?

The use of clinical criteria to define RTT limited the amount of variability that was accepted under the umbrella of the diagnosis; however, there has always been an issue regarding the phenotypic range of the disorder. This led to the classification of new RTT variant groups from the milder end of the spectrum (e.g. forme fruste and preserved speech variant) to the more severe end (congenital or early seizure onset) (104,105). Male cases were always particularly problematic in that, as an X-linked dominant disorder, it was difficult to rectify karyotypically normal males whose symptoms were the same as females. Mutation analysis of *MECP2* indicates that the clinically defined syndrome does not always coincide with an identifiable mutation in the gene and the corollary is also true: mutation in the gene does not always lead to RTT (3,12,37,74–77,82). The effect on phenotype is likely to represent a combined effect of X-inactivation although the type of mutation may also have some impact (37,74,76). Mutations that lead to complete loss of MeCP2 may actually lead to milder phenotypes in females through selection against cells that express the mutant allele. Cheadle *et al*. (76) reported increased phenotypic severity with nonsense mutations compared with missense mutations; however, the results of the genotype–phenotype data have been inconsistent across groups (3,12,37,74–76,82). The problem lies in difficulties in the classification of severity of individual features in a disorder that evolves over time. Particularly problematic are scoring features such as ambulation, which may begin late and be lost in adolescence, and scoliosis, which may develop late in childhood or after ambulation is lost. In reviewing the phenotypes reported for a given mutation, there appears to be wide variability in each of the domains examined, which probably reflects differences in X-inactivation patterns at the cellular level (3,12,37,74–76,82). The phenotype associated with *MECP2* mutations ranges from normal to mild learning disabilities to classic RTT, the variant forms to a severe congenital encephalopathy (37). The encephalopathy may be seen in females with *MECP2* mutations and unfavorable X-inactivation as well as males with *MECP2* mutations may result in unexplained neonatal deaths or sudden infant death syndrome.

FUTURE DIRECTIONS

Although the initial attempt to knock out *MECP2* in mice led to early lethality, recent efforts look promising (A. Bird, personal communication). Microarrays and chromatin immunoprecipitation strategies are likely to prove exceptionally valuable in determining the transcriptional consequences of *MECP2* mutation. Correlation between model systems and available patient samples is crucial for elucidating the pathogenesis of the phenotype in RTT. Somewhat surprisingly, mutations in a methyl transferase, DNMT3B, in humans leads to immunodeficiency–centromeric instability–facial anomalies (ICF) syndrome, a recessively inherited disorder characterized by combined immunodeficiency, dysmorphic features and bizarre radial multiradial chromosome formations due to abnormalities in heterochromatin condensation (106,107). The relationship between ICF and RTT will need to be explored.

When such intensive efforts have been directed toward searching for a disease gene, there is often a sense that the work is done when the gene is identified, particularly when it encodes a protein of known function. Although it had been hoped that identification of the causative gene would lead to immediate insight into the pathogenesis of this enigmatic disorder, this has not proved to be the case. Now the focus is on answering the real questions that underlie these efforts. Why do mutations in *MECP2* cause this phenotype? Are *MECP2* mutations associated with other autism spectrum disorders such as autism with regression? Are *MECP2* mutations lethal embryonically to most males or are they simply being missed because of the severe, but relatively non-specific phenotype? How do mutations in *MECP2* disrupt neuronal maturation, development and maintenance of synapses and dendritic arbors? What genes are being dysregulated? Is there an early marker that will identify these children before regression begins? Can we target therapies? The real work lies ahead.

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