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Beyond Widespread *Mecp2* Deletions to Model Rett Syndrome: Conditional Spatio-Temporal Knockout, Single-Point Mutations and Transgenic Rescue Mice

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Abstract

Rett syndrome (RTT) is one of the leading causes of intellectual disabilities in women. In addition to a few autistic features, characteristic symptoms that distinguish from classical autism include stereotypic hand movements, motor coordination deficits, breathing abnormalities, seizures and loss of acquired speech as well as purposeful hand use. RTT is highly associated with *MECP2*, the gene encoding for the transcription factor that binds methylated Cytosine in C-p-G islands in DNA, controlling gene expression and chromatin remodeling. In this review, we will briefly discuss current perspectives on *MeCP2* function, and then will describe in detail novel mouse models of RTT based on loss-of-function of *Mecp2* and their use for establishing rescue models, wherein we pay close attention to behavioral and morphological phenotypes.

Introduction

The autism spectrum disorder Rett syndrome (RTT; Online Mendelian Inheritance in Man #312750) is the leading cause of intellectual disabilities in girls with an incidence of 1:10,000 worldwide [1,2]. RTT cases occur sporadically with an extremely low familial rate [3,4]. Individuals with RTT develop typically until 6-18 months when a constellation of neurological hallmarks appears. Besides autistic features such as aberrant visual and aural contacts, characteristic symptoms that distinguish from classical autism include stereotypic hand movements, motor coordination deficits, breathing abnormalities, seizures and loss of acquired speech as well as purposeful hand use [5,6]. Underlying these traits are mutations of the gene *MECP2* located in chromosome Xq28 that encodes *MeCP2* (methyl-CpG-binding protein 2) in its two isoforms (e1 and e2) following alternative splicing of its four exons [7-9]. In this review, we will briefly discuss current perspectives on *MeCP2* function, and then will describe in detail several mouse models of RTT based on loss-of-function of *Mecp2* and their use for establishing rescue models (Figure 1, Table 1), wherein we pay close attention to behavioural and morphological phenotypes. As to electrophysiological manifestations, we refer the readers to a recent review [10].

MeCP2 Function

MeCP2 is a transcription factor that binds methylated Cytosine in C-p-G islands in DNA, controlling gene expression and chromatin remodeling [11-13]. MeCP2 dysfunction as a result of pathogenic mutations (i.e. missense, nonsense, truncations due to premature STOP codons) accounts for most RTT cases. As an X chromosome-linked disorder, complete absence of functional MeCP2 causes early mortality in boys, who hardly survive over 2 years. Females live much longer owing to the presence of a considerable amount of functional MeCP2 produced by the allele in the non-inactivated X chromosome [14], albeit reduced by approximately 50% from typical levels (i.e. mosaicism of X chromosome inactivation).

Normal MeCP2 function requires two major domains, the methyl-CpG binding domain (MBD) and the transcriptional repression domain (TRD). MeCP2 was initially thought to primarily play an inhibitory role in gene expression by recruiting chromatin remodeling protein complexes and transcriptional co-repressors like mSin3a and Histone Deacetylases (HDACs) [15]. This initial view of MeCP2 function as a transcriptional repressor has been extended recently to include an activating function, as one of the significant findings from a microarray study demonstrated that the majority of affected genes are activated in *MECP2* overexpressing mice and down-regulated in *Mecp2* null mice [16]. However, only a small number of genes were selected in that study to confirm MeCP2 binding specificity, and the possibility that MeCP2 might indirectly promote gene expression via regulation of gene silencers such as REST/CoREST [17] or microRNAs [18,19] has not been formally excluded. Furthermore, the changes observed in the Chahrour et al. [16], microarray study and others [20,21] are usually small in magnitude, which suggests that even though directly bound to targeted gene, MeCP2 might not act as a classical transcriptional factor but rather as a fine-tuning gene regulator.

Research on RTT has been mostly focused on neuronal abnormalities, such as reduced cell body size, delayed dendritic maturation and synaptogenesis, reduced dendritic branching and axonal arborization, altered spine and presynaptic bouton density (see detailed discussion below for each mouse model). These findings
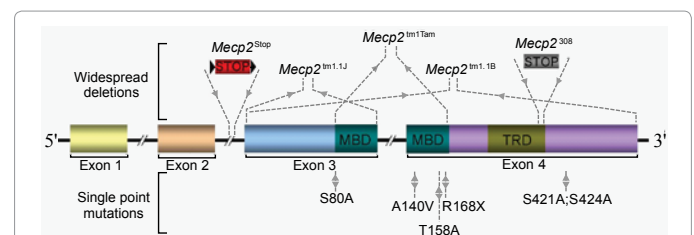

Figure 1: Schematic representation of the mouse *Mecp2* gene indicating the different regions manipulated to generate loss-of-function deletions and single-point mutations.

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Mouse Model	Regions targted	Onset	Life span	Body weight	Brain weight	Behavioral phenotype							Learning/memory			References
						Motility	Locomotor coordination	Hindlimb activity	Axiety	Social interaction	Agression	Regular breathing	Cue-	Context-	Spatial-	
Mecp2[tm1.1B]	Whole body	4-6w	7-8w	↓	↓	↓	↓	↓	↓	↓	–	↓	–	–	–	28,45
Mecp2[tm1.1J]	Whole body	5-7w	10-12w	↓←*	↓	↓	↓	↓	↓	↑	–	↓	↓	←	–	34, 43-45
Mecp2[tm1Tam]	Whole body	4-5w	8-12w	↓	–	↓	↓	↓	↓	–	–	↓	↓	↓	–	48
Mecp2[Stop]	Whole body	~6w	~11w	↓	–	↓	↓	↓	–	–	–	↓	–	–	–	26,49
Mecp2[308]	Whole body	~16w	1yr	←	←	↓←*	↓←*	↓	↑	↓↓↑&	–	↓	←	←↓#	←↓#	51, #53, *54, 55, *58, *59
Mecp2[2loxJ] CamK-Cre93	Postnatal forebrain	~12w	–	↑	↓	↓←*	↓	←	↑	←	–	↓	↓	←	–	43, *60
Mecp2[2loxB] Sim1-Cre	Hypothalamus	16-20w	Normal	↑	–	←	←	↓	↑	↓	↑	–	←	←	–	61
Mecp2[2loxB] TH-Cre	Dopaminergic/noradernergic neurons	–	Normal	–	–	↓	↓	←	←	←	←	←	←	←	–	62
Mecp2[2loxB] PET1-Cre	Serotonergic neurons	–	Normal	–	–	←	←	←	←	↑	←	←	←	–	62
Mecp2[2loxB] Viaat-Cre	Interneurons	~5w	~26w	↓	–	↓	↓	↓	↑	←	–	–	–	↓	63
Mecp2[2loxB] Dlx5/6-Cre	Forebrain interneurons	~5w	~80w	↓	–	←	↓	↓	←	↑	–	←	←	–	63
Mecp2[2loxJ] hGFAP-CreT2	Astrocytes	–	Normal	↓	–	←	←	↓	←	←	–	←	←	–	23
Mecp2[R168X]	Whole body	5-6w	~12w	Varying	–	↓	–	↓	–	–	–	↓	–	–	–	73
Mecp2[A140V]	Whole body	–	Normal	←	–	←	←	←	–	–	–	←	–	–	–	75
Mecp2[S421A]	Whole body	–	Normal	–	–	←	↓	–	←	–	–	–	–	–	←	79
Mecp2[S421A;S424A]	Whole body	–	Normal	←	←	←↓*	←	←	←	–	–	←	←	↑	↑	80, *81
Mecp2[S80A]	Whole body	–	Normal	←	←	↓	←	←	–	–	–	←	–	–	–	81
Mecp2[T158A]	Whole body	~5w	~16w	↑↓	↓	↓	↓	↓	↓	–	–	↓	↓	–	56

←,no change; ↓,decrease; ↑,increase; −,not determined.

Table 1: Summary of all mouse lines with loss-of-function deletions and single-point mutations in the Mecp2 gene, listing major differences in behavioural assessments.

led to the assumption that RTT originates exclusively from neuronal dysfunction. However, this traditional view has been questioned by recent observations of impaired morphology in wildtype neurons when co-cultured with MeCP2-deficient glial cells [22]. Consistently, restoration of MeCP2 levels in glial cells *in vivo* significantly alleviated several RTT-like behaviors in *Mecp2* null mice, as well as improving neuronal morphology [23]. Such non cell-autonomous effects on neurons are also displayed by MeCP2-deficient microglia, which release high levels of glutamate that are deleterious to co-cultured neurons [24]. Consistently, transplants of wildtype bone marrow to *Mecp2* null mice arrested the progression of RTT-like features due to the generation of myeloid cells that differentiated into a microglial phenotype [25].

Another classical notion is that MeCP2 is indispensable for normal brain development, which infers that irreversible neurological dysfunction would occur in the absence of MeCP2 from an early age. However, recent studies found that re-activating the endogenous *Mecp2* gene at normal levels in mature fully symptomatic *Mecp2* null mice reverts several RTT-like phenotypes [26]. Such observations indicate that MeCP2's function goes beyond early brain development and may include additional roles in neuronal maintenance throughout the whole life [27]. More provocative findings on MeCP2 function continue to come out; however, none of those discoveries would take place without the use of current experimental mouse models, as discussed below, and those to come in the future.

Widespread *Mecp2* Deletions

Mecp2[tm1.1B] mice (a.k.a. Bird nulls)

The *Mecp2*[tm1.1B] mouse line was generated in the laboratory of

Adrian Bird by crossing females with *Mecp2* exons 3 and 4 flanked by loxP sites (*Mecp2*[2loxB]) with males ubiquitously expressing the *Cre* transgene to delete the loxP-flanked exons [28]. It should be noted that *Mecp2*[2loxB] mice already have ~50% lower levels of *MeCP2* than wildtype controls (before Cre-mediated recombination), which cause significant neurological symptoms similar to *Mecp2* null mice, albeit less severe; thus they are considered a hypomorph *Mecp2* allele [29,30]. The progeny after Cre-mediated recombination and deletion of loxP flanked exons 3 and 4, however, completely lacks *Mecp2* mRNA and protein expression [28,31]. Hemizygous null mice (i.e. *Mecp2-/y* males without an X chromosome) do not show any overt phenotypes until 4-6 weeks of age, but rapidly develop several neurological deficits and typically die at 7-8 weeks. Neurological deficits are usually homogenous, including inertia, ataxia, hindlimb clasping when suspended by the tail, irregular breathing, and reduced body weight. It should be noted that these features were observed in mice with a C57BL/6 genetic background. When these mice were crossed to the 129 strain, pronounced weight changes were not observed, but the remaining phenotypes remained unaffected. Heterozygous female mice (i.e. with a mosaic expression of wildtype and null alleles) develop similar RTT-like symptoms, but they have a conspicuously slower and milder disease progression.

Brain specific *Mecp2* deletion was obtained by breeding *Mecp2*[2loxB] mice with *Nestin-Cre* transgenic mice, which deleted *Mecp2* exons 3 and 4 in the neuronal and glial cell lineage. These mice displayed a phenotype similar to the widespread MeCP2 deficiency, which supports the notion that RTT originates from its impaired function in the brain. Neuropathological examination of *Mecp2*[tm1.1B] mice in

the symptomatic stage did not reveal any major abnormalities such as disorganized cortical lamination and ectopias. However, more detailed analyses showed that cortical growth is stalled as symptoms appear, and that neurons are smaller and more packed at a higher density in layer II/III of somatosensory and motor cortices [32]. In these cortical layers, the length of apical dendrites and dendritic spine density are lower compared to wildtype mice. These impairments are accompanied by the occurrence of immature dendrites and dendritic spines also in layers V and VI of the motor cortex [33]. In motor cortex layer V, however, diverse synaptic phenotypes were found: spine numbers are largely decreased in apical dendrites but not basal dendrites [34,35]. The immunoreactivity levels of the presynaptic marker VGLUT1 (vesicular glutamate transporter-1) and the postsynaptic marker PSD95 (postsynaptic density-95) are lower in hippocampal neurons grown in autaptic microisland cultures [36]. Intriguingly, the density of VGLUT1 puncta is lower in hippocampal area CA1 of perfusion-fixed *Mecp2*tm1.1B mice than in wildtypes, but only at 2 weeks of age (presymptomatic stage), and not 5 weeks when overt symptoms start to appear [36], which again suggest that *Mecp2*tm1.1B mice have diverse synaptic phenotypes in different brain regions.

In the olfactory system, primary sensory neurons of *Mecp2*tm1.1B mice have impairments in axonal fasciculation, path-finding, and terminal differentiation [37,38], but on the postsynaptic side, no abnormalities in the mitral cells were observed [39]. The consequences of MeCP2 deficiency in *Mecp2*tm1.1B mice are also manifested in postnatal neurogenesis processes, including delayed maturation of newly-born neurons, altered expression of presynaptic proteins, and reduced formation of dendritic spines [40]. However, it does not affect the generation of immature neurons, which is in agreement with an early conclusion that MeCP2 is not involved in neuronal differentiation but only in neuronal maturation and maintenance in a cell-autonomous manner [41,42].

*Mecp2*tm1.1J mice (a.k.a. Jaenisch mutants)

Unlike the complete loss of MeCP2 protein expression in *Mecp2*tm1.1B mice, the deletion of only exon 3 in *Mecp2*tm1.1J mice leads to the expression a protein fragment lacking part of the MBD but with an intact but seemingly non-functional C-terminus [43]. Indeed, these mice (in either 129, C57BL/6, or BALB/c backgrounds) show a very similar neurological phenotype to *Mecp2*tm1.1B mice, although slightly milder and with a longer lifespan (10-12 weeks). Brain-specific *Mecp2* exon 3 deletion by recombination of floxed *Mecp2* (*Mecp2*2loxB) with Nestin-Cre also results in phenotypical features identical to that of widespread whole-body deletion, as in *Mecp2*tm1.1J mice. At the neuropathological level, *Mecp2*tm1.1J mice also have reduced brain weight and neuronal size [43].

More detailed analyses of different brain regions at the cellular level revealed that both mice have similar characteristics, but that *Mecp2*tm1.1B mice generally exhibit a more severe phenotype [44,45]. For example, dendritic spine density of all principal neurons of the hippocampus is lower in *Mecp2*tm1.1B mice than in wildtype controls, while this feature is only apparent in area CA1 of *Mecp2*tm1.1J mice [46]. However, a recent study showed that CA1 pyramidal neurons in the hippocampus of *Mecp2*tm1.1J mice have lower dendritic spine density and smaller asymmetric spine synapses than wildtype controls, but only in the presymptomatic stage [47].

*Mecp2*tm1Tam mice

*Mecp2*tm1Tam mice lack the entire MBD sequence, and carry a non-functional splicing site that prevents splicing and transcription of the downstream *Mecp2* sequence, leading to the complete absence of MeCP2 protein in the brain [48]. Male mutant mice stop to grow at 4-5 weeks of age and die at 8-12 weeks (mice in the 129 background typically die earlier than those in the mixed 129/C57BL/6 background), while heterozygous females have the same growth profile as wildtype littermates. These mice showed cerebellumdependent motor ability deficits, and impaired hippocampus/amygdala-dependent behavioral tasks, such as cued and contextual fear learning [48].

*Mecp2*Stop mice

To allow a more controlled deletion of *Mecp2*, its endogenous transcription was disrupted by inserting a *lox-STOP-Lox* cassette before exon 3 (*Mecp2*Stop) that can be removed by Cre-mediated recombination [26,49]. Similar to *Mecp2*tm1.1B mice, these mice (C57BL/6 background) do not show any signs of *MeCP2* protein expression, and develop identical RTT-like neurological phenotypes. Male *Mecp2*Stop mice typically start to show progressive symptoms at ~6 weeks of age with death occurring at ~11 weeks, while heterozygous females are fertile and display neurological phenotype only after 4-12 months. A recent study described that these mice show thinner neocortex and *corpus callosum*, smaller cell body size, and shorter length of basal dendrites in layer II/III pyramidal cells of the motor cortex than their wildtype controls [50]. At the synaptic level, spine density in hippocampal area CA1 is lower compared to wildtype controls. They also show impaired long-term potentiation (LTP) at excitatory synapses in the hippocampus, which may lead to learning and memory deficits [49].

*Mecp2*308 mice

*Mecp2*308 mice (either C57BL/6J or 129/SvEv backgrounds) were generated by introducing a premature *STOP* codon in the *Mecp2* gene, which leads to the expression of a protein truncated at amino acid 308 containing the part of the MBD, TRD, and nuclear localization signal [51]. Under careful examination, male hemizygous *Mecp2*308 mice display more intense curling, and more pivoting and head rising behaviors as early as postnatal day 3 [52]. Symptoms become evident at 6 weeks of age, which include tremors, hypoactivity, kyphosis, myoclonic seizure, and stereotypic forelimb motion. Overall, these symptoms are much milder than those observed in *Mecp2*tm1.1B and *Mecp2*tm1.1J mice. Also different from those mice, male *Mecp2*308 mice have normal body and brain weight, and can survive over 1 year. No obvious histological abnormalities were found in the brain. Detailed examination of neurons and glial cells using several markers, including microtubule-associated protein 2 (MAP2) and glial fibrillary acidic proteins (GFAP), did not reveal any significant differences with age-matched wildtype littermates [51]. Consistent with those findings, the same group reported that except for subtle changes of PSD length, neither the dendritic arborization in layer III and V pyramidal neurons of the frontal cortex nor the density and distribution of synaptic vesicles are affected in male *Mecp2*308 mice more than 1 year old [53].

Intriguingly, *Mecp2*308 mice exhibit upregulation of corticotrophin-releasing hormone (CRH) and its associated heightened anxiety [51,54,55], which differs from other *Mecp2* mutant mice [44,48,56], and hypomorph floxed mice [29,30]. The cellular bases for increased anxiety have been related to the amygdala; consistently, both synaptic maturation and developmental elimination are irregularly enhanced in principal neurons of the lateral nucleus of *Mecp2*308 mice [57]. Again owing to the potentially aberrant function of truncated *MeCP2* protein, behavioral changes are often manifested in a subtle way and giving rise

to some conflicting observations, such as for fear learning [51,53] and social interaction tasks [54,58,59].

Specific Spatio-Temporal Deletions

Deletion in forebrain postmitotic neurons: *Mecp2*²loxJ; CamK-Cre93 mice

To generate mice with *Mecp2* deletion restricted to forebrain postmitotic neurons, *Mecp2*exon3-floxed (*Mecp2*²loxJ, Jaenisch floxed) males were crossed with females expressing Cre under control of the *CamK* promoter [43]. *MeCP2* protein reduction in this conditional mouse line starts at the perinatal phase and reaches a maximal level at postnatal day 21. These mice have a delayed and milder phenotype than those resulting from germline or mediated deletions [43]. Symptoms include hindlimb clasping, impaired diurnal activity, and cue-sensitive *Nestin-Cre*-fear conditioning; however, context dependent fear conditioning is unaffected [60]. Weight gain, elevated anxiety, and abnormal social interaction were also pronounced in these mice [60]. Histological analyses showed smaller neuronal somata in the cortex and hippocampus, but not in the hindbrain such as cerebellum [43]. One of the interesting conclusions we can draw from these studies is that the cerebellum, a brain region critical for motor control, may not contribute to the manifestation of impaired motor coordination. Loss of *Mecp2* in broad forebrain regions and in some regions of the basal ganglia is sufficient to cause the observed motor deficits.

Deletion in hypothalamic neurons: *Mecp2*²loxB; *Sim1-Cre* mice

Breeding heterozygous *Mecp2*²loxB (Bird floxed) females with males (FVB background) expressing Cre under control of the *Sim1* promoter generated hypothalamus-specific *Mecp2* conditional knockout mice [61]. Immunohistochemical staining demonstrated the absence of *MeCP2* protein in several primary regions of the hypothalamus, including the paraventricular (PVN) and supraoptic nuclei (SON). As expected from such cell-specific *Mecp2* deletion, these mice recapitulate a subset of the phenotypes observed in patients with typical or atypical RTT. These mice show stereotypical movement, kyphosis, and increased body weight, but lack tremor, impaired motor coordination, and learning and memory deficits. Furthermore, hypothalamus-mediated behaviors are impaired, resulting in enhanced anxiety, and increased aggression in response to stress [61]. On the basis of this study, we can conclude that proper *MeCP2* function in the hypothalamus is critical for homeostatic responses to a variety of physiological stimuli, which agrees with heightened anxiety and altered stress in RTT patients, symptoms indicative of hypothalamic dysfunction.

Deletion in dopaminergic and noradrenergic neurons (*Mecp2*²loxB; *TH-Cre* mice) and serotonergic neurons (*Mecp2*²loxB; *PET1-Cre* mice)

To specifically remove *Mecp2* from dopaminergic and noradrenergic neurons, *Mecp2*²loxB (Bird floxed) females were bred with males (FVB/N background) expressing Cre under control of *tyrosine hydroxylase* (*TH*) promoter [62]. Deletion of *Mecp2* from serotonergic neurons was achieved by Cre-mediated recombination in *PC12 ets factor* 1(PET1)-expressing neurons (C57BL/6 background) [62]. In *Mecp2*²loxB;*TH-Cre* mice, *MeCP2* is significantly decreased in neurons of *substantia nigra* or ventral tegmental area of *locusceruleus*. *Mecp2*2loxB;*PET1-Cre* mice show *MeCP2* deficient neurons in the dorsal and medial raphe nuclei. By comparing the phenotype between *Mecp2*²loxB;*PET1-Cre*knockouts, their wildtype counterparts, and *Mecp2*²loxB Bird floxed hypomorph mice, Samaco et al. [62] found no obvious differences in several features,

including breathing pattern, repetitive behaviors, anxiety levels, motor learning and social interaction. However, conditional knockout mice with *Mecp2* deletions in dopaminergic and noradrenergic neurons showed subtle but characteristic phenotypes, such as hypoactivity and aggression, respectively. This study provides strong evidence for the importance of *MeCP2* in the regulation of aminergic transmitters, and contributes to our understanding of the role of different transmitters in specific phenotypes.

Deletion in GABAergic neurons: *Mecp2*²loxB; *Viaat-Cre* and *Mecp2*²loxB; *Dlx5/6-Cre*mice

Deletion of *Mecp2* from GABAergic interneurons was accomplished by crossing *Mecp2*²loxB (Bird floxed) females with males (FVB background) expressing Cre under control of the *Viaat* promoter [63]. *Mecp2*²loxB;*Viaat-Cre* mice exhibit the stereotypic neurological phenotype observed in *Mecp2*tm1.1B and *Mecp2*tm1.1J mice, developing symptoms at 5 weeks of age and having a half-survival of 26 weeks. An additional number of features distinct from Bird nulls and Jaenisch mutants include impaired sensorimotor gating and arouse (assessed with acoustic startle response and pre-pulse inhibition tasks) and enhanced social interests, but without altered anxiety and aggression. Interestingly, the expression levels of the GABA synthetic enzymes glutamic acid decarboxylase 65 (*Gad65*) and *Gad67* are reduced in *Mecp2*²loxB;*Viaat-Cre* mice, with a concomitant reduction in vesicular GABA content and impaired synaptic inhibition. To further dissect *MeCP2* function in forebrain interneurons, male mice (FVB background) expressing Cre under the *Dlx5/6* promoter were crossed with *Mecp2*2loxB females [63]. It is worth noting that the *Dlx5/6* promoter is only expressed at high levels in parvalbumin-positive forebrain interneurons that innervate pyramidal cell somata, but is not expressed in interneurons that target pyramidal cell dendrites, such as calretinin positive and somatostatin-positive interneurons [64]. The phenotype observed in *Mecp2*²loxB;*Dlx5/6*-Cre mice thus resulted from *MeCP2* deficiency only in a subset of forebrain interneurons with a specific functional role. Compared to the more widespread *Mecp2* deletion in all interneurons in the *Mecp2*²loxB;Viaat-Cre mice, *Mecp2*²loxB;*Dlx5/6*-Cre mice show milder symptoms and longer life-span, surviving to over 80 weeks. Except for decreased motility, *Mecp2*²loxB;*Dlx5/6*-Cre mice show similar phenotypic features presented by *Mecp2*²loxB;Viaat-Cre, including hindlimb clasping, reduced locomotor activity, kyphosis, breathing dysfunction. [63]. It is surprising that unlike other conditional mutants, deletions of *Mecp2* in all interneurons or even in a specific subset of interneurons result in a multitude of RTT-like symptoms usually observed in widespread *Mecp2* deletions. A tenable conjecture will be that perturbation of *MeCP2* function even in small-scale territory of GABAergic interneurons is enough to ruffle the neuronal network and ultimately result in a phenotype similar to that seen in mice with a widespread loss of *MeCP2* function.

Deletion in glial cells: *Mecp2*2loxJ; *hGFAP-CreT2* mice

Neuronal dysfunction was initially considered the sole basis for RTT etiology, but accumulating evidence indicates that glial cells may also have an important role in disease pathophysiology [12]. Contrary to an initial belief, *MeCP2* is expressed in significant levels by glial cells, including astrocytes and oligodendrocytes [22]. Furthermore, astrocytes from *Mecp2*tm1.1J mice had detrimental consequences on either wildtype or *Mecp2*-deficient neurons, an effect also observed by astrocyte-conditioned medium [22]. Consistently, cytokine production altered astrocytic glutamate clearance in *Mecp2*tm1.1B mice [65,66]. In addition to astrocytes, microglia also shows impaired function in

*Mecp2*tm1.1B mice, as they release excitotoxic levels of glutamate [24], and do not respond properly to immunological stimuli [25].

To confirm these unexpected roles of *MeCP2* role in glial cells, *Mecp2*2loxJ (Jaenisch floxed) mice were crossed with mice (mixed FVB/N/C57BL/6 background) harboring a tamoxifen-inducible *Cre* transgene driven by the human *GFAP* regulatory element (*Mecp2*2loxJ;*hGFAP*-CreT2). The specific *Mecp2* deletion in astrocytes after postnatal day-21 results in a number of classical RTT-like symptoms, including decreased body weight, hindlimb clasping, and irregular breathing, while longevity, locomotion, and anxiety were not affected [23]. The observation of normal dendritic complexity in neurons of these astrocyte-specific *Mecp2* null mice appears to contrast with earlier reports in widespread *Mecp2* mutant mice; however, it is conceivable that *Mecp2* deletion in astrocytes at this later age (i.e. P21) does not seriously compromise already established neuronal morphology. These studies demonstrate that functional loss of glia, an integral component critical for proper brain function, would breach the intimate relationship with neurons and lead to the development of the RTT phenotype.

Viral-mediated *Mecp2* deletion: Cre- and shRNA-expressing viruses

To achieve *Mecp2* deletion in a spatial as well as temporal manner, viral-mediated approaches have been successfully employed. Targeted *Mecp2* deletion achieved by injections of Adeno-Associated Virus (AAV) expressing Cre recombinase in the basolateral amygdala of *Mecp2*2loxJ mice (Jaenisch floxed) resulted in increased anxiety and deficits in cue-dependent fear conditioning, without affecting motor coordination, locomotor activity and social behavior [67]. Also, MeCP2 protein expression was knockdown with short hairpin RNAs (shRNAs) designed to target *Mecp2* mRNAs. Rat pups that received intraventricular injections of MeCP2 shRNA show altered sensory-motor reflexes and neurobehavioral abnormalities during early development, but only transiently [68]. Even though *MeCP2* knockdown by shRNA injections into the dorsal striatum resulted in decreased intake of a drug-of-abuse, RTT-like phenotypes were not apparent [18]. Infusions of MeCP2 shRNA-expressing lentivirus into the *Nucleus Accumbens* (NAc) had a converse effect (increased drug reward response), but also without occurrence of RTT like symptoms [69]. It is undoubted that new findings would emerge by using these vial mediated knockdown techniques, which can achieve local ablation of gene expression inaccessible to DNA promoter-driven knockout otherwise.

Tamoxifen-driven expression of Cre recombinase: *Mecp2*2loxB; *CreER* mice

Through its interaction with the estrogen receptor (ER), tamoxifen triggers the nuclear translocation of an engineered Cre-ER complex in CAGGS-CreER mice, allowing the Cre recombinase to interact with loxP sites flanking gene regulatory regions of interest and excise them. Systemic tamoxifen injections allowed *Mecp2* deletion at postnatal-day 60 or older *Mecp2*2loxB; CAGGS-CreER mice (C57BL/6J background), which resulted in characteristic RTT-like behavioral deficits and death 13 weeks after treatment onset [27]. A follow-up study revealed that conditional *Mecp2* deletion either at 5 weeks or at 11 weeks resulted in similar disease progression and lifespan (16-17 weeks after onset of tamoxifen treatment) [70]. Intriguingly, despite the RTT-like disease progression and fatality, no differences were found at the cellular and molecular levels, including neuronal density and soma size, dendritic complexity, and expression of several synaptic proteins. Another study

extending the age of tamoxifen onset to even earlier and later found that, although all mice eventually reached the same disease severity by 10 weeks after tamoxifen onset, mice treated at 3 weeks-of-age develop symptoms more stagnantly in the first week compared to mice that received tamoxifen starting at 11- and 20-weeksof-age [71]. It is also interesting to note that, irrespective of ages for tamoxifen treatments, all mice die at ~40 weeks of age. These studies suggest that MeCP2 levels are critical during an early age (8-14 weeks) when synaptic refinement occurs, as well as later (30-45 weeks) when aging processes initiate. Even though this hypothesis awaits further examination, it is increasingly clear that proper MeCP2 levels are of great importance in the adult brain. The use of tamoxifen-induced *Cre* recombination has produced fruitful results regarding the required timing of MeCP2 function. In combination with several models of local *Cre-ER* expression, it has enabled us to map the neuroanatomic origins of phenotypes observed in RTT mouse models, shedding light on the sources of clinical presentations in RTT individuals.

Single Point Mutations

Missense or nonsense *MECP2* mutations, including R106W, R133C, T158M, R168X, R255X, R270X, R294X, and R306C, account for more than half of the RTT cases [72]. Thus, mice carrying a single nucleotide change in *Mecp2* would represent the closest experimental model of patients with RTT.

Truncated *MeCP2*: *Mecp2*R168X mice

*Mecp2*R168X mice (mixed C57BL/6, 129S6/SvEv Tac background) carry a single point mutation, where the codon for arginine at position 168 is replaced with a premature *STOP* codon [73]. These mice express a truncated *MeCP2* protein at ~50% wildtype *MeCP2* levels. As seen in other *Mecp2* mouse lines, male mutant mice are more severely affected than female heterozygotes. Male *Mecp2* R168X mice have a short lifespan (~12 weeks) with neurological features starting as early as 5-6 weeks, while female heterozygotes show symptoms only after six months of age and live longer than 1 year. Symptoms in males consist of forelimb stereotypes, hindlimb clasping and atrophy, hypoactivity, and breathing irregularities, while females have similar but milder signs. Compared with two main mutant mice in phenotypic severity, they are milder than *Mecp2* tm1.1B and close to *Mecp2*tm1.1J mice. A note added in proof in the Lawson-Yuen et al. [73] paper warned about mistakenly introduced mutations after the STOP codon, which preclude the use of these mice to test read-through compounds that skip STOP codons, such as aminoglycoside antibiotics (i.e. the full-length protein will express several mutations of unpredictable consequences).

MECP2 mutation in males: *Mecp2*A140V mice

The *Mecp2*A140V mouse model is unique because it mimics atypical RTT cases occurring mostly in males. The *Mecp2*A140V mutation is known to retain the methyl-CpG binding function while losing its ability to be associated with the mental disability-related protein ATRX [74]. *Mecp2*A140 mice (mixed 129X1/S1, C57BL/6 background) show normal levels of *Mecp2* mRNA and protein, which may allow normal lifespan, weight, and motor coordination, as well as breathing rhythms. However, they do show some RTT-like features, such as increased neuronal cell density, and decreased complexity of dendritic branching in layer II/III pyramidal neurons of the somatosensory cortex [75].

Phosphorylation mutants: *Mecp2*S421A and *Mecp2*S421A;S424A mice

Post translational modifications of MeCP2 are known to have a critical role in its binding to target genes and their ensuing expression

[12]. Phosphorylation of MeCP2 sites, especially at serine 421, has been under intense investigation. Several studies suggested a de-repression model in which phosphorylation of MeCP2 following neuronal activity prevents it from binding to target genes and results in expression of genes and the ensuing modulation of dendritic patterning and dendritic spine morphogenesis [76-78]. This view has been revised by a recent study using MeCP2 S421A point mutation mice (mixed sv129, C57BL/6 background) and ChIP-Seq [79]. These mutant mice exhibit some cellular phenotype including an increase in the number of dendritic branches in cortical pyramidal neurons, and a shift towards synaptic inhibition in the excitation/inhibition. At the behavioral level, responses to a novel mouse were significantly impaired, but motor activity, social interaction, spatial learning and memory were unaffected. It is surprising that, in this study using ChIP-Seq analysis for genome wide DNA-protein association and ChIP-qPCR for specific association, they did not find any significant changes in *MeCP2* binding to target genes. These findings suggest phosphorylation of MeCP2 at serine 421 does not regulate specific genes, but instead plays a role in fine-tuning global gene expression. However, the authors also pointed out that global histone-like manipulation of MeCP2 at serine 421 does not exclude the possibility of facilitating discrete gene expression in the presence of other locus-specific modifications of MeCP2. This notion, although not been tested, could be true. Interestingly, a recent study showed that the additional substitution of serine for alanine at position 424 in *Mecp2*$^{S421A;S424A}$ mice (C57BL/6 background) resulted in enhanced contextual fear learning, spatial memory, hippocampal synaptic plasticity (i.e. LTP at CA3-CA1 and mossy fiber-CA3 synapses), and synaptogenesis, while motor activity, coordination and nervousness were unaffected [80]. However, an earlier study using mice (C57BL/6 background) with similar mutations had shown increased locomotor activity [81]. The conflicting observations in these studies suggest complex and diverse consequences of phosphorylation at different MeCP2 sites. Phosphorylation at S421, for example, was revealed to result in completely different outcomes as for phosphorylation at S80, as discussed next.

Phosphorylation mutants: *Mecp2*^{S80A} mice

Mice (C57BL/6 background) with serine 80 replaced by alanine in MeCP2 (*Mecp2*^{S80A}) to prevent phosphorylation at this site did not exhibit any RTT-like phenotypes, except for decreased locomotor activity [81]. In striking contrast to phosphorylation at S421, S80 phosphorylation was negatively regulated by neuronal activity. The finding of an opposing regulation of S421 and S80 phosphorylation by neuronal activity raises the intriguing possibility that *MeCP2* phosphorylation is engaged differentially under resting and active states.

Mecp2 hypomorphs: *Mecp2*T158A mice

Threonine 158 in *MeCP2* is known to play a crucial role in the stabilization of the MBD and the binding of *MeCP2* to its target genes [82]. Since a threonine 158 mutation is common in RTT individuals, *Mecp2*^{T158A} mice (sv129, C57BL/6 background) were generated to investigate its role in RTT pathogenesis [56]. Male *Mecp2*^{T158A} mice develop RTT-like symptoms at 5 weeks of age and die at ~16 weeks, a similar or slightly milder disease than that seen in *Mecp2*^{R168X} mice. Similar to all other mouse models, and likely due to the mosaicism of an X-link mutant allele, female *Mecp2*^{T158A} heterozygous mice do not present with symptoms until 17 weeks of age, and live a normal lifespan. In addition to the characteristic RTT-like phenotype, which includes hypoactivity, hindlimb clasping, impaired motor coordination, and learning & memory deficits, *Mecp2*^{T158A} mice have an initial body weight loss that later subsides, a different pattern of weight variability than that presented by *Mecp2*^{R168X} male mice. Unlike the complete absence of *MeCP2* in *Mecp2*^{R168X} mice and the normal levels of *MeCP2* protein in *Mecp2*^{A140V}, *Mecp2*^{S421A}/*Mecp2*$^{S421A;S424A}$, and *Mecp2*^{S80A} mice, symptomatic *Mecp2*^{T158A} mice show ~60% lower levels of *MeCP2* protein compared to age-matched littermate controls. This reduction in *MeCP2* levels is thought to underlie impaired expression of target genes (e.g. *Bdnf*).

Transgene Rescue of *Mecp2* expression

Ever since the discovery that *MECP2* mutations cause RTT, despite the recognized challenge to only target the mutant allele in a X-linked disorder, prevention or reversal of RTT phenotypes has been attempted by means of transgene expression in experimental mouse models. An unexpected corollary of these studies is that elevated levels of MeCP2 are as detrimental to neurological function as their reduction [83].

Neuronal *Mecp2* overexpression: Tau-*Mecp2*; *Mecp2*tm1.1J mice

An additional copy of the mouse *Mecp2* gene was inserted into the *Tau* locus (encoding the neuron-specific microtubule associated protein Tau) to test for the rescue of RTT-like phenotypes by increasing *MeCP2* protein levels in neurons [84]. However, it was found that homozygous *Tau-Mecp2* mice (FVB background) exhibit similar symptoms as *Mecp2*tm1.1J mice (Jaenisch mutants), likely due to excessive levels *MeCP2* protein under control of the *Tau* promoter. Incidentally, a similar neurological phenotype was observed in mice that overexpressed the human *MECP2* gene from a P1-derived artificial chromosome containing all regulatory elements [85], demonstrating that *MeCP2* overexpression is detrimental to brain function. When *Tau-Mecp2* mice (mixed 129, C57BL/6 background) were bred with *Mecp2*$^{tm1.1J}$ (Jaenisch mutants), their progeny showed a significant reduction of all RTT-like phenotypes examined [84], suggesting that the mild increase in *MeCP2* levels from one copy of Tau locus was sufficient to prevent disease onset but not too high to be deleterious.

Cre-mediated *Mecp2* reactivation by cell-specific promoters: *CAGGS*Mecp2^{Stop}; Mecp2$^{tm1.1J}$; *Nestin-Cre/Tau-Cre/CamK-Cre* mice

A construct containing the synthetic CAGGS promoter, a loxP flanked STOP cassette, and mouse *Mecp2* cDNA was targeted to the *Col1a1* locus and subsequently transferred into ES cell to generate CAGGS-*Mecp2*^{stop} mice (C57BL/6 background) [86]. The lack of *Mecp2* expression due to a STOP codon results in the characteristic RTT-like phenotypes in CAGGS-*Mecp2*^{stop} mice. These mice were crossed with *Mecp2*$^{tm1.1J}$ mice (Jaenisch mutants), and the resulting offspring were bred with different *Cre*-expressing transgenic mice. Removal of the STOP codon by *Cre*-mediated recombination leads to reactivation of the CAGCS-*Mecp2* transgene and significant *MeCP2* protein expression, which was higher in *Nestin-Cre* (neural lineage) and *Tau-Cre* (neuron-specific microtubule associated protein) mice than in *CamK-Cre* mice (specific to forebrain neurons). Mice with higher *Mecp2* transgene expression showed longer lifespan than *Mecp2*$^{tm1.1J}$ mice without overt RTT-like phenotypes, while mice with lower *Mecp2* transgene expression showed slight improvements compared to *Mecp2*$^{tm1.1J}$ mice. For example, *CamK-Cre* rescue mice lacked the characteristic low brain weight and small neuronal soma size observed in *Mecp2* mutant mice [86].

CreER-mediated *Mecp2* reactivation by systemic tamoxifen treatment: *Mecp2*StopB; *CreER* mice

As above, lack of *Mecp2* expression due to a *STOP* codon results in characteristic RTT like phenotypes in *Mecp2*StopB;*CreER* mice (C57BL/6 background). Excision of the *STOP* cassette by tamoxifen-induced nuclear translocation of the CreER complex results in the re-expression of *Mecp2* under control of native regulatory elements [26]. Compared to the rapid progression of RTT-like phenotypes in male *Mecp2StopB;CreER* mice, mice receiving tamoxifen starting at 3-4 weeks after birth showed a significant delay in symptom progression and a longer lifespan of up to 17 weeks of age. Similar delayed onset symptoms and extended lifespan were observed in female *Mecp2*StopB;*CreER* mice after tamoxifen treatment.

Tet-On-mediated human *MECP2* expression in neurons: *MECP2-TREEGFP*; *CamK-tTA/Eno2-tTA*; *Mecp2*308 mice

A *MECP2-TRE-EGFP* construct was designed to allow for bi-directional regulation of both human *MECP2* and EGFP (for identification of expressing cells) under the control of a central *TRE* (tetracycline-responsive element) [87]. TRE activates gene transcription once its *tetO* recognition sequence is bound by tTA (tetracycline transactivator), which is given to mice orally in the drinking water. Progenies resulting from the crossbreeding of mice containing the *MECP2-TRE-EGPF* transgene with those containing the *CamK-tTA* or *Eno2-tTA* transgenes express *MeCP2* in neurons of the forebrain and the striatum and cerebellum, respectively. These inducible mice (C57BL/6 background) were crossed to *Mecp2*308 mice to test for the prevention of RTT-like symptoms. Surprisingly, cell specific expression of *Mecp2* failed to prevent most of the phenotypes characteristic of *Mecp2*308 mice, except for a marginal increase in motor activity in *CamK* rescue mice (forebrain neurons). However, earlier studies described that specific manipulations of *Mecp2* expression in forebrain neurons resulted in detectable, albeit mild, behavioural consequences [43,60,86]. The expression of a partially functional truncated protein in *Mecp2*308 mice could contribute to this discrepancy. Also, normal levels of *Mecp2* transgene expression may increase *MeCP2* protein dosage too much, and thus compromise any potential beneficial effects. Indeed, using a similar strategy, female heterozygous *Mecp2*tm1.1B mice with optimal *Mecp2* transgene expression showed improved rearing and locomotor activity [88].

CreER-mediated *Mecp2* reactivation in glial cells by systemic tamoxifen treatment: *Mecp2*Stop; *hGFAP-Cre* mice

Glial-specific reactivation of endogenous *Mecp2* silenced by a loxP-flanked STOP codon was achieved by excising it with a tamoxifen-inducible *CreER* placed under control of the human *hGFAP* promoter [23]. Despite lack of *Mecp2* expression in neurons, *Mecp2* reactivation exclusively in glial cells extended the lifespan to 7.5 months compared with only 3 months in oil-treated *Mecp2*Stop;*hGFAP-Cre* mice (C57BL/6 background). Tamoxifen-treated *Mecp2*Stop;*hGFAP-Cre* mice show increased activity levels, decreased anxiety, while the characteristic irregular breathing (apnea) frequency is also improved compared to oil-injected controls. Consistent with these improvements in behavioural features, the size of neuronal somata dendritic arborization, and VGLUT1 protein expression were all significantly increased in tamoxifen-treated *Mecp2*Stop;*hGFAP-Cre* mice.

Introduction of wildtype microglia into *Mecp2* null brain: bone marrow transplants in *Mecp2*Stop; *Lysm-Cre* mice

Since *Mecp2*-deficient microglia has adverse effect on wildtype neurons in co-cultures [24], it is reasonable to speculate that repopulation of *Mecp2*-containing microglia would improve RTT-like symptoms in *Mecp2* deficient hosts. Indeed, a recent study demonstrated that transplantation of wildtype bone marrow into presymptomatic *Mecp2* null mice (postnatal day 28) after whole-body irradiation-mediated immune ablation resulted in engraftment of microglia-like myeloid cells into the brain parenchyma and arrested the progression of neuropathology including recovery of body and brain size, enhancement of lifespan, improvement of gait and locomotor activity (but not hindlimb clasping), and reduction of apneas and breathing irregularities [25]. However, no significant rescue was observed when bone marrow transplants were performed during the time RTT-like phenotypes begin to appear (postnatal day 40 or 45). Phenotypic prevention also failed if irradiation did not include the brain, which leads to reconstitution of only peripheral but not brain microglia-like myeloid cells. To further support this strategy, *Lysm-Cre* mice (mixed 129, C57BL/6 background), which express Cre under control of the *Lysm* promoter, were crossed with *Mecp2*stop mice to generate progeny with normal *Mecp2* expression by removal of STOP codon only in myeloid cells, granulocytes, and microglia. Such cell-specific *Mecp2* reactivation was sufficient to improve breathing patterns, prolong lifespan, and increase both body weight and locomotor activity. Furthermore, long-term treatment of Mecp2*Stop;Lysm-Cre* mice with annexin-V, a pharmacological manipulation that blocks phagocytic activity of microglia, failed to prevent RTT-like disease progression, strongly suggesting that micrglia dysfunction contributes to RTT pathophysiology.

Concluding Remarks

The various experimental mouse models described above have enabled researchers to discover novel mechanisms at the molecular, cellular, and circuit levels that contribute to *MeCP2* dysfunction in RTT, providing remarkable platforms for testing a wide variety of genetic, pharmacological, and physiological interventions to improve the quality of life in this debilitating disease. Notwithstanding, and considering the significant differences between research rodents and humans at all levels, there is great need to establish consistent and effective human-based models. Undoubtedly, the use of patient-specific human induced pluripotent stem stem cells (iPSCs) is by far the closest model experimental research and manipulations [89]. Neuronal differentiation of human iPSCs derived from RTT individuals has been successfully achieved [89-94], which one day should allow high-throughput screening of pharmacological libraries. With RTT research evolving into full-fledged endeavors in an ever-growing number of research laboratories, we hope that the scientific knowledge gained from these and new animal models will be translated into rational therapies available for RTT individuals in not too distant future.

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