Rett syndrome: a complex disorder with simple roots

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Abstract | Rett syndrome (RTT) is a severe neurological disorder caused by mutations in the X-linked gene *MECP2* (methyl-CpG-binding protein 2). Two decades of research have fostered the view that MeCP2 is a multifunctional chromatin protein that integrates diverse aspects of neuronal biology. More recently, studies have focused on specific RTT-associated mutations within the protein. This work has yielded molecular insights into the critical functions of MeCP2 that promise to simplify our understanding of RTT pathology.

AT-hook
A DNA-binding motif first identified in the high-mobility group (HMG) chromatin proteins. It specifically recognizes the minor groove of AT-rich DNA.

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Since the recognition of Rett syndrome (RTT) as a discrete disorder[1,2], the clinical criteria for its diagnosis have been progressively refined. Classical RTT affects around 1 in 10,000 live female births and is characterized by an approximately 6-month period of overtly normal development. This is followed by the onset of symptoms, which include deceleration of head growth, gait abnormalities, loss of speech, breathing disturbances and the replacement of purposeful hand movements with repetitive stereotypies[3,4]. At a cellular level, neurons in patients with RTT are smaller and more densely packed, and reductions in the length, complexity and number of dendritic spines are observed[5]. Moreover, despite its regressive nature, studies of brain pathology suggest that RTT is not a disorder of neurodegeneration[5]. The disease is rarely observed in males, as mutations in methyl-CpG-binding protein 2 (*MECP2*) are generally paternally rather than maternally derived[6], and inactivation of the sole X-linked copy of *MECP2* leads to severe neonatal encephalopathy and early lethality. A diverse set of neurological impairments are seen in males with mutations in *MECP2* that do not give rise to a complete loss of function[7]. Classical RTT can be observed in boys in rare circumstances, such as somatic mosaicism[8] or co-occurrence with Klinefelter syndrome[9].

The discovery that RTT is a monogenic disorder caused by mutations in the *MECP2* gene[10], which encodes the two known isoforms of MeCP2 (REFS 11,12), offered the opportunity to link downstream pathology to a defined molecular lesion. In particular, this knowledge has enabled the development of *Mecp2*-mutant mice[13,14] and cell lines[15–17], which are invaluable model systems for the study of RTT (TABLE 1).

In this Review, we discuss what the past two decades of research on MeCP2 have taught us about the biology of RTT. We start by outlining how Cre–*lox*P technology has been used to define the roles of MeCP2 in diverse cell types throughout the brain (TABLE 1). Next, we consider MeCP2 as a chromatin-associated protein and describe the various DNA-binding modules within the molecule that have been reported. We then discuss the evidence that MeCP2 might have a role in the regulation of gene expression by activating or repressing transcription, or by functioning at a post-transcriptional level. We also assess reports concerning how MeCP2 function is itself regulated, for example, by microRNAs (miRNAs) and activity-dependent phosphorylation (BOX 1). Finally, we describe how models of MeCP2 function can be evaluated in light of the RTT mutation spectrum. This approach has highlighted two crucial regions of the protein: the methyl-CpG-binding domain (MBD) and the NCOR–SMRT interaction domain (NID[18]; also referred to as a 'basic cluster' (REF. 19)) (FIG. 1). An AT-hook-like domain has also been shown genetically to contribute to MeCP2 function[20]. Precisely defining the roles of these regions holds promise for more clearly elucidating the molecular basis of RTT pathology.

MeCP2 functions throughout the brain

Much work on RTT has relied on mice carrying mutations in the *Mecp2* gene[13,14]. However, when interpreting observations made using these animals, some potential caveats should be kept in mind. First, hemizygous *Mecp2*-null male mice are frequently used when, in fact, the most direct representation of patients with RTT would be *Mecp2*-heterozygous

Table 1 | **A table of mouse and cell models of Rett syndrome and information on their phenotypes**

MeCP2 status	Phenotype	Refs
Mouse models		
Deletion from SIM1-expressing neurons in the hypothalamus	Heightened stress response, increased aggressive behaviour, hyperphagia and obesity	130
Deletion from the POMC neurons in the arcuate nucleus of the hypothalamus	Over-eating and obesity	131
Removal from dopaminergic and noradrenergic neurons	Motor abnormalities	132
Removal from serotonergic neurons	Aggressive behaviour	132
Removal from forebrain GABAergic neurons	Seizures	133, 134
Deletion in the brainstem and spinal cord	Abnormal breathing responses and early lethality	135
Viral-mediated deletion in the basolateral amygdala	Defects in cue-dependent fear conditioning	136
Removal from GABA-releasing neurons	Repetitive behaviours, progressive motor dysfunction, breathing defects and early lethality	36
Deletion in postnatal forebrain neurons	Similar to MeCP2-null animals but milder and delayed	14,35
MECP2-*mutant cell lines*		
Neurons from human iPSCs (patient-derived or using TALENs to delete MeCP2)	Reduced nuclear size, transcription and translation; impaired mitochondrial function, fewer synapses and reduced spine density	15,16
Neurons from mouse ESCs	Reduced nuclear size and transcription	17

ESC, embryonic stem cell; GABA, γ-aminobutyric acid; iPSC, induced pluripotent stem cell; MeCP2, methyl-CpG-binding protein 2; POMC, pro-opiomelanocartin; TALEN, transcription activator-like effector nuclease.

female mice. Second, mice develop overt symptoms of *Mecp2* deficiency at a much later stage in development than is the case in humans. Nevertheless, many of the neurological features of RTT — including an abnormal gait, breathing disturbances and premature lethality in males — are recapitulated in *Mecp2*-mutant mice[13,14]. Underscoring the importance of this protein in the brain, specific deletion of MeCP2 in this organ results in an extremely similar phenotype to that observed in the whole-body knockout[13,14]. Moreover, expression of MeCP2 from the *Tau* locus, which is active in post-mitotic neurons and normally encodes a microtubule-associated protein, broadly rescues the phenotype of *Mecp2*-null animals, emphasizing the importance of MeCP2 in these cells[21]. Consistent with these observations, the MeCP2 protein is most highly expressed specifically in post-mitotic neurons[22–26]. The reversibility of the RTT-like phenotype upon restoration of MeCP2 (REF. 27) and the catastrophic consequences of MeCP2 removal in adult mice[28–30] argue that MeCP2 is not a neurodevelopmental regulator. Rather, MeCP2 seems to be required for the maintenance of normal neuronal function throughout life.

MeCP2 functions in neuronal and non-neuronal cells in the brain. Deletion of MeCP2 from glial cells has mild phenotypic consequences, which is consistent with the idea that its primary function is in neurons[31]. However, co-culture experiments have revealed that MeCP2 in glia has a non-cell-autonomous role in supporting the normal dendritic morphology of neurons[32]. Furthermore, re-expression of MeCP2 in astrocytes completely rescued the breathing abnormalities observed in *Mecp2*-null male mice, leading to greatly increased survival rates[31]. A slight increase in lifespan is also observed when MeCP2 is expressed specifically in oligodendrocytes of *Mecp2*-null mice[33]. MeCP2 function is therefore not restricted to neuronal cells. Interestingly, wild-type microglia introduced by transplantation of bone marrow were reported to ameliorate some of the deficits observed in *Mecp2*-null mice[34].

Does MeCP2 function vary in different cell types? MeCP2 has been genetically removed from specific brain regions and neuronal subtypes in mice, and the resulting phenotypes were examined (TABLE 1). Loss of MeCP2 in a specific domain compromises the function of that brain region. For example, deletion of the *Mecp2* gene exclusively in the forebrain causes behavioural abnormalities — including limb clasping, impaired motor coordination, increased anxiety and abnormal social behaviour — but does not affect locomotor activity or context-dependent fear conditioning, the control of which resides elsewhere in the brain[35]. Similarly, loss of MeCP2 from all inhibitory GABA-releasing neurons leads to a severe RTT-like phenotype[36], whereas a smaller subset of MeCP2-negative neurons has much milder consequences (TABLE 1). It seems that the variable mouse phenotypes caused by removing MeCP2 in specific brain areas may simply reflect impairment of the normal functions of these regions. Support for the idea that MeCP2 performs a related function in different cell types

SIM1-expressing neurons
Neurons expressing the transcription factor SIM1 (single-minded homologue 1) found in tissues such as the hypothalamus, which is involved in regulating body weight homeostasis.

GABA-releasing neurons
Neurons that produce γ-aminobutyric acid (GABA), the main inhibitory neurotransmitter in the brain.

Box 1 | **Regulation of MeCP2 function**

Levels of methyl-CpG-binding protein 2 (MeCP2) must be precisely controlled in order to prevent the neurological deficits associated with overexpression[21,115–117] or underexpression[118,119] of this protein. Two mechanisms that have been proposed to regulate MeCP2 function include microRNAs[120,121] and activity-dependent phosphorylation. Neuronal activity induces phosphorylation of many proteins, including transcriptional regulators, which then facilitate appropriate responses to stimuli[122]. The possibility that MeCP2 is controlled in this manner has been extensively examined. Initially, MeCP2 was found to be phosphorylated in response to calcium influx induced by membrane depolarization[123,124], and several phosphorylation sites have since been identified[125–127] (FIG. 1). Different sites are preferentially modified in response to different inputs. For example, MeCP2 is preferentially phosphorylated on Thr308 and Ser421 in response to membrane depolarization, whereas Ser86 and Ser274 are preferentially phosphorylated sites after stimulation with brain-derived neurotrophic factor (BDNF) or forskolin[127]. However, Ser80 is phosphorylated in resting cultured neurons and in the brain but becomes dephosphorylated upon stimulation with potassium chloride or kainic acid[126].

The consequences of MeCP2 phosphorylation on particular residues have been explored by expressing mutated forms of MeCP2 in cultured rodent neurons and also by generating mouse strains in which these sites have been mutated. Mice with the S80A mutation display decreased locomotor activity[126]. Overexpression of wild-type MeCP2 in organotypic hippocampal slices results in a simplification of the dendritic arbour, and this effect is reversed by the S421A mutation[125]. However, mice with this mutation have only a subtle neurological phenotype; furthermore, no changes in either the association of MeCP2 with chromatin or gene expression patterns were detected in *Mecp2*^{S421A} mice compared with wild-type animals[128]. Mice with both S421A and S424A mutations show enhanced long-term potentiation and improved performance in hippocampus-dependent memory tests compared to wild-type animals[129]. This phenotype resembles that of mice overexpressing MeCP2 (REF. 117) and is consistent with the notion that phosphorylation of these residues could negatively regulate MeCP2 function.

Molecular evidence also supports the idea that neuronal activity negatively regulates MeCP2 through a phosphorylation-dependent mechanism. Southwestern blots indicate that phosphorylated forms of MeCP2 have reduced affinity for DNA[123], and chromatin immunoprecipitation analysis suggests that MeCP2 is more tightly associated with chromatin in *Mecp2*$^{S421A;S424A/y}$ mice than in wild-type animals[129]. A reduced association of MeCP2 with chromatin is observed in *Mecp2*$^{S80A/y}$ mice and, because phosphorylation of Ser80 is reduced by membrane depolarization, this is also consistent with MeCP2 being negatively regulated by neuronal activity. Interestingly, modification of Thr308 abolishes the interaction of MeCP2 with the NCOR (nuclear receptor co-repressor)–SMRT (silencing mediator of retinoic acid and thyroid hormone receptor) complex[127], which is in line with the idea that activity-dependent phosphorylation negatively regulates MeCP2 function. One observation suggesting that activity-dependent phosphorylation of MeCP2 might be relevant to Rett syndrome (RTT) pathology is that modification of Thr308 is lost in mice bearing the RTT-associated R306C mutation. However, mutations of this or other phosphorylation sites have not been reported in patients with RTT, and T308A-mutant mice are relatively mildly affected[127]. Activity-dependent modulation of MeCP2 function might therefore be involved in the ability of neurons to integrate and appropriately respond to diverse sets of stimuli, but the role of this function in RTT pathology remains to be fully established.

comes from the observation that gene expression profiles are similarly altered in different regions of the brain when *Mecp2* is absent[37]. Regional deletion of *Mecp2* nevertheless provides a useful method for deducing the contribution of specific brain regions to RTT pathology.

A different interpretation has recently come from studies of manually sorted subpopulations of discrete neuronal subtypes. According to this study, MeCP2 deficiency alters the expression of different sets of genes in different neuronal cell types[38]. For example, the *Doc2b* (double C2 beta) gene is upregulated in noradrenergic locus coeruleus neurons of *Mecp2*-knockout mice compared with wild-type mice, whereas expression of this gene is decreased in L5 pyramidal neurons when *Mecp2* is absent. Furthermore, in *Mecp2*-null mice, expression of the immediate early gene *Fos* is decreased in certain structures of the forebrain, whereas *Fos* expression is increased in the hindbrain[39]. These studies are compatible with the idea that MeCP2 function differs between cell types, and they focus attention on its mechanism of action at a molecular level.

MeCP2 binds to DNA

The MBD of MeCP2 binds to modified cytosines. MeCP2 was originally identified biochemically as a factor that interacts specifically with DNA bearing methylated cytosines in the context of CpG dinucleotides[40]. The amino-terminal MBD[41] (FIG. 1) mediates the association of MeCP2 with densely methylated heterochromatic foci in mouse fibroblasts, suggesting that it is a functional protein domain *in vivo*[42]. Moreover, mutations in this domain of MeCP2 result in decreased residence time at heterochromatic foci, as assessed by fluorescence recovery after photobleaching (FRAP)[43,44]. This suggests that binding to methylated DNA is impaired by mutations in the MBD in living cells. Further evidence that MeCP2 binds to methylated CpG comes from chromatin immunoprecipitation assays, which reveal a preferential association of MeCP2 with the methylated alleles of imprinted genes in mouse embryonic stem cells[45] and in the mouse brain[24]. Recently, MeCP2 was also reported to interact with methylated CpA dinucleotides *in vitro* and in the mouse brain[46,149]. CpA methylation is prevalent in neurons[46,47] and accumulates at a time in development

CpG dinucleotides
Cytosine bases connected to adjacent guanine bases in the same strand of DNA. This sequence is symmetrical and is therefore base-paired with CpG on the complementary DNA strand.

Imprinted genes
Genes that are expressed in a parent-of-origin-specific manner. They frequently show DNA methylation specific to the parent of origin.

Matrix attachment regions
AT-rich DNA elements defined by their ability to interact with the nuclear matrix *in vitro*. They are thought to organize chromatin into a series of loops or domains.

when MeCP2 expression levels are increasing[22–24], which indicates that it may have a role in the recruitment of MeCP2 to chromatin.

At the atomic level, the solution structure of the MBD from the related protein, MBD1, complexed with methylated DNA suggests that specific binding is mediated by a patch of hydrophobic residues within the MBD[48]. However, the co-crystal structure of the MeCP2 MBD bound to DNA suggests that binding specificity is conferred via recognition of methylation-dependent hydration of the major groove of DNA[49].

The MBD of MeCP2 does not exclusively interact with 5-methylcytosine (5mC)-containing DNA. It has also been shown to bind to 5-hydroxymethylcytosine (5hmC)-containing DNA *in vitro*[50,51], although this interaction appears to be weaker than that between MeCP2 and 5mC[52–55]. Nuclease accessibility, which assays open chromatin structure, is slightly reduced in *Mecp2*-null nuclei at 5hmC sites, but not at 5mC sites, implying a connection between MeCP2 and 5hmC *in vivo*[50]. The fact that 5hmC is enriched in the brain[47,56], together with the slight alterations in the distribution of 5hmC in the absence of MeCP2 (REF. 57), suggests that this mode of binding might be physiologically relevant. Other interactions between the MBD and the genome might also have a functional role; for example, the chicken orthologue of MeCP2, attachment region binding protein (ARBP), was identified owing to the affinity of the MBD for AT-rich DNA associated with 'matrix attachment regions' (REFS 58,59).

Collectively, the evidence suggests that the primary mode of MeCP2 interaction with the genome is between modified cytosine and the MBD. The MBD is the only domain of MeCP2 shown to bind to DNA *in vitro* with sequence specificity[60,61]. Accordingly, chromatin immunoprecipitation analyses have revealed that MeCP2 becomes profoundly redistributed if the MBD is mutated or if DNA methylation is removed[62]. This demonstrates that the MBD is a sequence-specific DNA-binding domain that facilitates the recruitment of MeCP2 to chromatin *in vivo*. Missense mutations causing RTT in patients greatly reduce the binding of MeCP2 to methylated DNA *in vitro*[60,61] and *in vivo*[62], suggesting that interactions with methylated DNA are necessary for MeCP2 function. The RTT-causing R133C mutation in the MBD has recently been reported to specifically abolish the interaction of MeCP2 with 5hmC *in vitro*, implying that this mode of binding could be highly relevant to the pathology of RTT[50]. However, whether this mutation also affects binding to 5mC is unclear, as some previous reports suggest that this interaction is impaired, whereas others suggest that it is not[61,63,64]. Testing the effect of the R133C mutation on binding to different modified forms of cytosine throughout the genome in the brain will be an interesting avenue of future research.

Levels and patterns of 5mC and 5hmC vary between cell types in the brain[47,50]. As MeCP2 binding seems to depend on DNA modification profiles, this suggests a potential mechanism by which MeCP2 could exert distinct effects in different brain regions and cell types. The stage is therefore set for a detailed examination of the hypothesis that reading of 5hmC and/or 5mC profiles by MeCP2 constitutes a cell type-specific epigenetic mechanism.

Other modes of interaction between MeCP2 and the genome. Consistent with the abundance of MeCP2, which approaches that of the histone octamer in postmitotic neurons[24], MeCP2 binds globally across the genome in the brain. Its binding profile broadly tracks the density of methylated cytosine, but regions devoid of DNA methylation also show significant binding[24]. Furthermore, although absence of DNA methylation leads to redistribution of MeCP2 along the genome, the protein remains chromatin-associated after crosslinking[62]. Light microscopy reveals an interesting discrepancy between MeCP2 localization in paraformaldehyde-fixed cells versus live cells[44]. In fixed mouse cells, localization of MeCP2 to heterochromatin is dependent on an intact MBD, as mutants cause dispersal throughout the nucleus. However, in live cells, these same mutants appear correctly localized despite the absence of a fully functional MBD. Although the MBD is dominant in determining MeCP2 localization, these

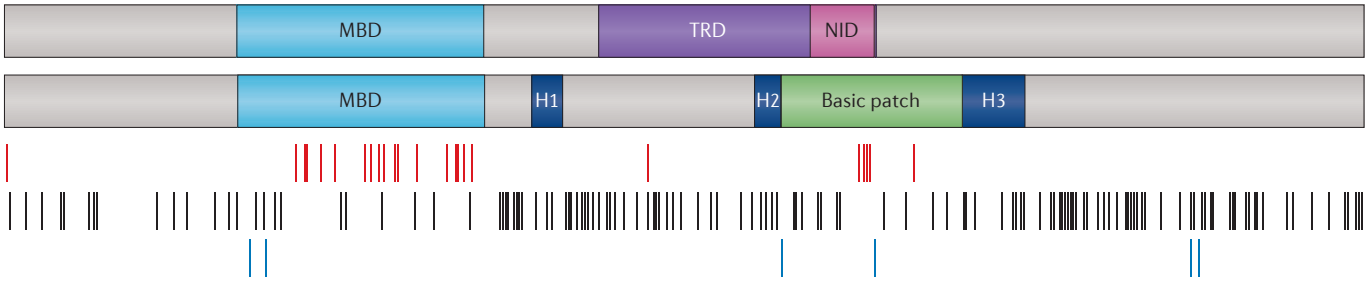

Figure 1 | **The primary structure of MeCP2 illustrating domains implicated in the pathology of Rett syndrome.** The methyl-CpG-binding domain (MBD), transcriptional repression domain (TRD), three AT-hooks (H1–H3) and NCOR–SMRT interaction domain (NID; also known as a 'basic cluster') are depicted. The missense mutations causing Rett syndrome are plotted in red and highlight the importance of the MBD and the NID. Shown in black are the neutral variants from the Exome Aggregation Consortium (ExAC), which tend to lie outside these domains. Phosphorylation sites, which respond to neuronal activity, are shown in blue (BOX 1). MeCP2, methyl-CpG-binding protein2; NCOR, nuclear receptor co-repressor; SMRT, silencing mediator of retinoic acid and thyroid hormone receptor.

observations raise the possibility that other domains also influence its interaction with the genome.

In this connection, three AT-hook motifs have been reported in MeCP2 (REFS 20,40) (FIG. 1). Mutating one of these resulted in reduced association of MeCP2 with major satellite repeat elements in the mouse brain as assessed by chromatin immunoprecipitation[20]. Thus, a region outside the MBD appears to contribute to the recruitment of MeCP2 to chromatin *in vivo*. Other regions beyond the MBD are reported to bind to DNA *in vitro* in a manner that is independent of DNA methylation[65–67]. In this vein, a recent report identified a 'basic cluster' in MeCP2 that was shown to bind to DNA *in vitro* and to enhance binding to the major satellite repeat elements *in vivo*[19]. This region of the protein is rich in positively charged amino acids and includes a residue that is frequently mutated in RTT (R306C). Finally, *in vitro* crosslinking experiments suggest that contact occurs between MeCP2 and nucleosomal histone H3 (REF. 68), and it has also been reported that MeCP2 can bind to an isolated N-terminal histone H3 tail[69]. Whether binding to histones represents a mechanism for recruiting MeCP2 to chromatin *in vivo* has yet to be assessed.

Future work will be required to resolve the binding profiles of MeCP2 *in vivo* and also to uncover their determinants. It will be of crucial importance to examine the molecular and phenotypic consequences of mutations in individual DNA-binding domains of MeCP2 in patients (see below) and in model systems[18–20,70]. It will also be necessary to genetically manipulate the various modifications of DNA *in vivo* and to assess their effect on MeCP2 binding.

MeCP2 regulates gene expression

MeCP2 as a transcriptional repressor. In view of evidence that MeCP2 interacts with methylated DNA, a modification that is linked to gene silencing[71], early work on MeCP2 examined its potential role in transcriptional repression. In support of this scenario, a transcriptional repression domain (TRD) (FIG. 1) was mapped in experiments involving recruitment of MeCP2 fragments to a reporter gene using a heterologous DNA-binding module[72]. Moreover, MeCP2 has been reported to interact with the histone deacetylase (HDAC)-containing co-repressor complexes SIN3A[73,74], NCOR (nuclear receptor co-repressor) and SMRT (silencing mediator of retinoic acid and thyroid hormone receptor; also known as NCOR2)[75,76]. Although the SIN3A-binding site on MeCP2 has not been precisely defined, the NCOR–SMRT interaction domain (NID) (FIG. 1) was located within the TRD, and it was shown that recruitment of the NCOR–SMRT complex is responsible for MeCP2-mediated repression[18] (FIG. 2). Also consistent with a gene silencing model, intact MeCP2 was found to specifically repress methylated reporter genes[13,77]. In view of the sensitivity of this repression to deacetylase inhibitors, a model was proposed whereby MeCP2-mediated silencing involves the modulation of chromatin structure through the removal of acetyl groups from histone lysine residues[18,73,74]. Recently, it has been reported that transcriptional repression by MeCP2 in the brain is preferentially targeted to long genes enriched in methylated CpA dinucleotides[38,149]. Although the effects on individual genes are small, the number of genes involved is large, and so this phenomenon could be highly relevant to RTT pathology.

Silencing of repetitive elements and retrotransposons. Repetitive sequences such as L1 retrotransposons, the major satellite and intracisternal A particle elements are heavily methylated in the mouse brain, and MeCP2 associates with these regions as determined by chromatin immunoprecipitation[24,78]. Transcripts from these elements are moderately more abundant in nuclei from *Mecp2*-null brains than in those from the wild type[24], which suggests that MeCP2 acts to repress their expression. This raises the possibility that increased transposition of repetitive DNA might be a feature of MeCP2 deficiency; consistent with this hypothesis, increased L1 retrotransposition has also been documented in the brains of RTT patients, as well as from *Mecp2*-null mice compared with wild-type controls[78]. Do repetitive transcripts leading to transposition have a role in the aetiology of RTT? This is unlikely because the neurological defects seen in *Mecp2*-null mice can be robustly reversed by re-expression of MeCP2 (REFS 27,79,80). Insertional mutagenesis would not be reversible in this way; therefore, retrotransposition is unlikely to underlie the RTT phenotype.

MeCP2 as a transcriptional activator. Global expression profiling, using microarrays to measure the activity of individual genes throughout the genome, initially failed to reveal striking differences in gene expression patterns in brains from *Mecp2*-null mice compared with wild-type mice[81]. Subsequently, analyses of specific brain regions in *Mecp2*-null mice and from mice overexpressing MeCP2 have shown that numerous genes are consistently and reciprocally dysregulated, although changes in gene expression are subtle[37,82]. The data indicate that more genes are downregulated than upregulated in the absence of MeCP2 — a bias that suggests that the majority of genes are positively regulated by MeCP2. Mechanistically, evidence has been presented that MeCP2 activates transcription through recruitment of cyclic AMP-responsive element-binding protein 1 (CREB1) to target gene promoters[82] (FIG. 2). Consistent with this idea, reduced transcription is observed in run-on assays using nuclei from neurons derived from *Mecp2*-null mouse embryonic stem cells[17]. Additionally, gene expression analysis normalizing to cell number rather than total RNA revealed that transcripts, including ribosomal RNA, are globally under-represented in neurons derived from *MECP2*-deficient human induced pluripotent stem cells (iPSCs)[16]. These results have been interpreted in the light of the finding that MeCP2 can bind to 5hmC (as discussed above), which is often associated with gene activity[50,83,84]. For example, 5hmC is enriched in active genes in neurons[50].

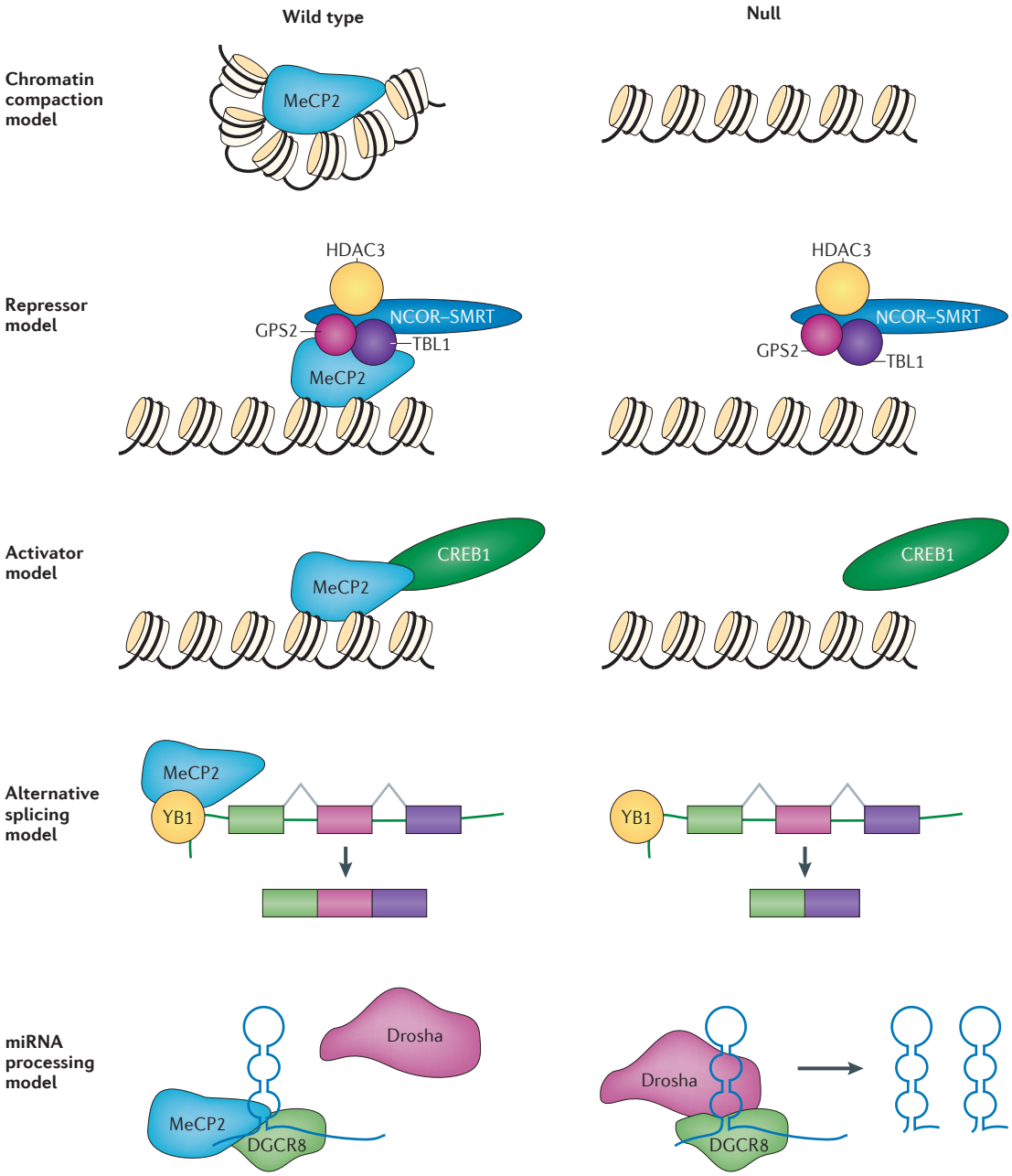

Figure 2 | **Summary of proposed molecular functions for MeCP2.** Methyl-CpG-binding protein 2 (MeCP2) has been proposed to compact chromatin structure, to repress transcription by recruiting the nuclear receptor co-repressor (NCOR)–SMRT (silencing mediator of retinoic acid and thyroid hormone receptor) co-repressor complex, or to activate transcription by recruiting the co-activator cyclic AMP-responsive element-binding protein 1 (CREB1). It has also been proposed that MeCP2 regulates alternative splicing via an interaction with YB1, a Y-box transcription factor, or that it regulates microRNA (miRNA) processing by interacting with DGCR8 to prevent the formation of the Drosha–DGCR8 complex. GPS2, G protein pathway suppressor 2; HDAC3, histone deacetylase 3; TBL1, transducin β-like protein 1.

Remaining controversies. The evidence is not watertight to support either an activator or a repressor model of MeCP2 function. On the one hand, much of the evidence for the repressor function is derived from the use of artificial reporter genes, which might not reflect the *in vivo* function of the protein. The associated deacetylase activities that mediate this repression are also reported to be frequently present at active promoters[85]. On the other hand, the reduction in RNA levels observed in MeCP2-deficient neurons could be a secondary consequence of unhealthy neurons producing less protein[86] and becoming smaller[14,87]. Importantly, models in which MeCP2 functions as a transcriptional activator or repressor

are not mutually exclusive. An intriguing possibility is that MeCP2 could function differently at different loci. This seems especially pertinent in the light of data suggesting that multiple different interactions might be involved in the recruitment of MeCP2 to chromatin[88]. For example, a speculative hypothesis would be that MeCP2 functions as a repressor when it binds to DNA containing 5mC but as an activator when it interacts with 5hmC[16].

Alternative models of MeCP2 function

In addition to being implicated in transcriptional repression and activation, various alternative models for MeCP2 function have been proposed. MeCP2 might regulate chromatin structure — for example, ectopic expression of MeCP2 by transient transfection of mouse myoblasts resulted in clustering of chromocentres[89], and loss of a chromatin loop was reported in *Mecp2*-knockout mice[90]. An analogous effect was observed *in vitro*, where recombinant MeCP2 binds to and compacts nucleosomal arrays[65,91]. Experiments using *in vitro* assembled chromatin indicated that MeCP2 was able to compete with histone H1 for binding to these substrates[72]. Furthermore, these proteins seem to compete for the same binding sites *in vivo* in mouse fibroblasts, as microinjection of each protein led to accelerated FRAP kinetics of the other[92]. One line of evidence indicating that these observations could be relevant in the brain is that neurons from *Mecp2*-null mice display increased levels of histone H1 (REF. 24). Together, these results suggest that displacement of histone H1 might represent a mechanism whereby MeCP2 modulates higher-order chromatin architecture, perhaps in order to control gene expression. Moreover, as chromatin is the substrate for many different nuclear processes such as DNA replication, repair and translocation, there are various other possibilities. However, the precise role that altered chromatin structure might have in RTT pathology has not been defined.

Consistent with the finding that MeCP2 can bind to RNA *in vitro*[93], it has also been proposed that MeCP2 regulates processes such as alternative splicing[94,95] and miRNA processing[96]. MeCP2 might therefore also serve to regulate gene expression at a post-transcriptional level. MeCP2 is reported to interact with YB1, a Y-box transcription factor that can regulate alternative splicing, and ectopic expression of MeCP2 by transient transfection promotes the inclusion of variable exons in a reporter minigene[94] (FIG. 2). It has also been reported that depletion of MeCP2 in human cell lines leads to aberrant alternative splicing events[95]. However, the degree to which MeCP2 is involved in these functions in the brain, and therefore the role of defective splicing in RTT pathology, has yet to be established. Furthermore, various miRNAs are upregulated in the hippocampus of *Mecp2*-null mice compared with wild-type mice, and it has been proposed that MeCP2 negatively regulates miRNA processing by interacting with DGCR8 and interfering with the assembly of the DGCR8–Drosha complex[96] (FIG. 2). MeCP2 also reportedly interacts with a plethora of different proteins, including many chromatin-modifying factors (TABLE 2). One interpretation of this aggregated information is that MeCP2 might function through a diverse set of binding partners. However, the physiological roles of many of these interactions and their relevance to RTT pathology remain to be established.

Do changes in gene expression have an impact on mouse mutant phenotypes? An important question concerning the relevance of models that invoke MeCP2 as a transcriptional regulator is as follows: do the observed changes in gene expression cause the phenotypes associated with MeCP2 mutants? This issue has been addressed in mice, in which it is possible to experimentally manipulate the expression levels of putative MeCP2 target genes. For example, corticotropin-releasing hormone (*Crh*) and opioid receptor mu-1 (*Oprm1*), two genes linked to anxiety, are upregulated in mice overexpressing MeCP2, and genetically reducing the expression levels of these two genes modulates the anxiety phenotype observed in those animals[97]. Furthermore, expression of brain-derived neurotrophic factor (BDNF), an autocrine factor that promotes neuronal growth and survival, is reduced in *Mecp2*-null mice. Transgene-mediated overexpression of BDNF in *Mecp2*-null mice ameliorates the phenotype, including improved survival[98]. Similarly, administration of exogenous BDNF has been reported to improve synaptic function in *Mecp2*-null mice[99]. Cholesterol biosynthesis genes are initially upregulated in the brains of *Mecp2*-null mice before being downregulated as the animals become symptomatic[100]. Consistent with these changes being causally relevant to disease pathology, a mutation in the cholesterol biosynthesis pathway was identified in a screen for suppressor mutations that reduce the severity of the *Mecp2*-null phenotype[100].

This is strong evidence that the changes in gene expression seen in mouse models of RTT, although modest, have phenotypic consequences, and the cumulative effect of many such changes might provide a complete explanation of the pathology of RTT. However, such a conclusion should only be drawn cautiously. One study found that gene expression patterns vary more between wild-type mice from different litters than between wild-type and *Mecp2*-mutant mice that are littermates[101]. This highlights the fact that RTT-associated changes in gene expression, although clearly important in some cases, are generally rather subtle.

Human mutations underlying disease pathology

Although the prevalence of RTT is relatively low, the availability of large patient databases such as RettBASE[102] makes it possible to examine a broad spectrum of disease-causing *MECP2* mutations. RTT-causing mutations, as well as polymorphisms not associated with the disease, are curated from the literature and also directly submitted by clinicians.

Chromocentres
Aggregations of heterochromatin in mouse nuclei that stain readily with DAPI (4′,6-diamidino-2-phenylindole). These regions are enriched in major satellite repeat elements.

Nucleosomal arrays
Arrays that can be assembled *in vitro* by reconstituting recombinant histones with DNA. They represent a useful tool to study the effect of other proteins on chromatin structure.

Table 2 | **MeCP2 binding proteins***

Proteins	Unbiased	Biochemical	Two-hybrid	Endogenous	Refs
ATRX	+	+	+	+	111,137
SKI		+		+	75
Brahma		+		+	138
NCOR–SMRT	+	+		+	138
Huntingtin		+		+	139
EHMT2		+		+	140
PRMT6	+	+			138
HP1α, HP1β and HP1γ		+		+	138
PRPF3	+	+		+	141
CDKL5		+			138
HMGB1	+	+			138
Lamin B		+		+	138
IKKα		+			142
SOX2		+		+	138
DNMT1		+			138
MBD2		+			143
TDP43	+	+		+	144
TET1		+		+	145
DGCR8		+		+	96
YY1		+		+	138
TFIIB		+			138
xp20	+	+			138
HIPK2	+	+	+	+	138
LEDGF	+	+		+	146
SIRT1		+			138
p300		+			138
SIN3		+		+	73
HLCS		+			147
YB1	+	+		+	94
PU.1	+	+			138
MYCN		+		+	148
SMC1 and SMC3		+		+	138
FBP11	+	+			138
HYPC		+			138
CBF1		+			138
CREB1	+	+		+	82
EWSR1	+		+		138
SP1 and SP3		+		+	138
SUV39H1		+			138
Polyubiquitin C	+	+		+	138

ATRX, α-thalassemia/mental retardation syndrome X-linked; CBF1 is also known as Jκ-recombination signal-binding protein (RBPJ); CDKL5, cyclin-dependent kinase-like 5; CREB1, cyclic AMP-responsive element-binding protein 1; DNMT1, DNA (cytosine-5)-methyltransferase 1; EHMT2, euchromatic histone-lysine *N*-methyltransferase 2 (also known as G9a); FBP11, formin-binding protein 11 (also known as PRPF40A); HIPK2, homeodomain-interacting protein kinase 2; HLCS, holocarboxylase synthetase; HMGB1, high-mobility group protein B1; HP1, heterochromatin protein 1; HYPC, Huntingtin yeast partner C; IKKα, inhibitor of NF-κB kinase-α; LEDGF, lens epithelium-derived growth factor (also known as PSIP1); MBD2, methyl-CpG binding domain protein 2; NCOR, nuclear receptor co-repressor; p300, histone acetyltransferase p300; PRMT6, protein arginine *N*-methyltransferase 6; PRPF3, pre-mRNA-splicing factor 3; SIRT1, sirtuin 1; SMC, structural maintenance of chromosomes protein; SMRT, silencing mediator of retinoic acid and thyroid hormone receptor; SUV39H1, suppressor of variegation 3–9 homologue 1; TDP43, TAR DNA-binding protein 43; TET1, TET methylcytosine dioxygenase 1; TFIIB, transcription initiation factor IIB; xp20, oviduct protein p20; YB1, Y-box transcription factor; YY1, transcriptional repressor protein YY1. *The evidence for each interaction (biochemical or two-hybrid) is indicated, together with details on whether an association was demonstrated with endogenous proteins. Also indicated are references and whether the interaction was uncovered using an unbiased (rather than candidate) approach.

The collaborative efforts involved in creating such databases have enabled patient information to directly inform our understanding of the protein implicated in this disorder.

Whereas alterations resulting in the loss of large parts of the protein yield little information on structure–function relationships, RTT-causing missense mutations, in which only single amino acids are changed, point to crucial features of the MeCP2 protein. Plotting missense mutations onto the primary structure of MeCP2 illustrates that the MBD and the NID are both vitally important for proper MeCP2 function[18,19] (FIG. 1). Under-representation of these regions among neutral polymorphisms from the Exome Aggregation Consortium (ExAC) data set (a collaboration that provides a data set of exome sequencing from 60,706 individuals who are not affected by severe paediatric disease) further highlights their likely importance (FIG. 1). The four RTT-associated missense mutations in the NID all abolish its interaction with the NCOR–SMRT co-repressor complex, as well as the ability of MeCP2 to silence reporter genes[18]. The fact that these single-amino-acid changes are sufficient to cause RTT strongly suggests that NID-mediated interaction with NCOR–SMRT is vital for MeCP2 function. Furthermore, the relatively weak binding to SIN3A was not disrupted by any of these mutations, which brings into question the relevance of this co-repressor interaction to transcriptional repression and RTT[18]. However, it is also possible that this domain has essential functions other than NCOR–SMRT binding (see below).

Three confirmed RTT-associated missense mutations lie outside the two crucial functional domains of MeCP2 described above[18]. Two (P225R and P322L) involve proline residues and therefore potentially disrupt the overall fold of the protein. A third (A2V) affects only one of the two known MeCP2 isoforms[11,12], and data regarding the importance of the residues specific to this isoform are conflicting[21,103,104]. Some studies have detected isoform-specific functions[104], whereas others have argued that the functional significance of the few differing amino acids are subtle or absent[21,103]. Regardless of this uncertainty, A2V affects the residue next to the start codon of the most abundant MeCP2 isoform[11,12], and this mutation may therefore impair the translation of MeCP2. Resolution of these unknowns awaits the creation and characterization of the relevant mouse models. Importantly, a major class of RTT mutations (~10%) involves frameshifts in the carboxyl terminus of MeCP2 that also leave both the MBD and the NID intact[102]. One interpretation is that a crucial domain exists in the C terminus of MeCP2. Alternatively, the addition of missense residues after a frameshift might result in destabilization or inappropriate folding of MeCP2. Compatible with this scenario is the observation that mutations in the stop codon that add extra amino acids to the complete MeCP2 sequence nevertheless cause RTT[102]. Also consistent with the idea that the C terminus is not intrinsically essential is the finding that mice with MeCP2 truncated upstream of the site of these frameshifts[105] have neurological impairments that are much milder than those of animals with RTT mutations, such as *Mecp2*-null mutations[13,14] or mutations of the MBD[19,70] and the NID[18,19]. The distribution of RTT-causing nonsense mutations in MeCP2 is also consistent with the notion that there are two critical regions in the protein, as all of the frequently occurring mutations in this category lie upstream of the NID[18,19,102].

Studies with large cohorts of patients have revealed that the nature of the *MECP2* mutation affects RTT severity[106–110]. In one study, a discrete boundary between different truncation mutations found in patients identified a transition in severity depending on the presence or absence of only three amino acids of the MeCP2 protein. The existence of this boundary was confirmed in mice expressing forms of MeCP2 with the corresponding R270X and G273X truncation mutations[20]; mice with the latter mutation survived longer, as is the case in human males. The region affecting RTT severity included an AT-hook motif (FIG. 1), which was found to contribute to MeCP2 binding to heterochromatin in the mouse brain as assessed by chromatin immunoprecipitation[20]. However, unlike the MBD, no confirmed RTT missense mutations affecting this AT-hook have been reported in patients (FIG. 1), suggesting that its absence alone might not be sufficient to cause the disease.

How do MeCP2 mutations cause RTT?

For a model of MeCP2 function to properly describe the pathology of RTT, it must explain how the same syndrome can arise from both null mutations and missense mutations predominantly in two discrete regions of the protein. One such model proposes that the principal function of MeCP2 is to serve as a bridge between DNA and the NCOR–SMRT co-repressor complex, interactions with which are affected by RTT-associated missense mutations[18]. Any one of these three types of mutation (null, MBD or NID) would prevent the recruitment of the NCOR–SMRT co-repressor to chromatin by MeCP2 (FIG. 3a).

An alternative model proposes that the primary function of MeCP2 is to modulate the 3D architecture of chromatin via multifaceted interactions with DNA (FIG. 3b). The NID includes a basic cluster, the primary function of which may be as an auxiliary DNA-binding domain[19], as the R306C mutation is reported to impair the interaction of this region of MeCP2 with DNA. Together with work demonstrating that AT-hook 2 of MeCP2 contributes to the severity of RTT, these observations correspond to the view that the critical interactions of MeCP2 are with DNA. If this model accounts for RTT pathology, then a prediction is that all of the disease-causing mutations in the NID would abolish its interaction with DNA. Although there is evidence that all NID missense mutations prevent the NCOR–SMRT interaction[18], the effect of all of these changes on DNA binding has yet to be assessed[19].

Although both the co-repressor recruitment and the chromatin compaction models are consistent with the mutation spectrum of RTT, other models remain possible. For example, the regions of MeCP2 that are mutated in most patients with RTT — the MBD and the NID — could perform essential functions other than binding to DNA and to NCOR–SMRT. In this regard, the MBD has been found to interact with the ATP-dependent helicase ATRX[20,111]. The relationship between MeCP2 and ATRX is not fully understood, as some RTT mutations — such as R270X and G273X, which have no effect on the interaction between MeCP2 and ATRX — nevertheless lead to loss of association of ATRX with heterochromatin[20]. However, RTT mutations in the MBD do not affect ATRX binding, and so loss of this particular interaction seems unlikely to be central to RTT pathology[111].

The RTT mutation spectrum is a valuable tool for evaluating alternative models that link MeCP2 function with RTT pathology. For example, a model in which MeCP2 functions to cluster chromocentres[89,112] is not supported by these data because although mutations in the MBD abolish chromocentre clustering[112], the MBD alone is sufficient to condense heterochromatin in this manner[89]. Therefore, this model does not provide an explanation for RTT pathology caused by mutations that affect the NID. The idea that MeCP2 acts mainly as a transcriptional activator also lacks supporting evidence from mutational studies, as RTT-associated missense mutations have not been reported to cause defective interaction with activators such as CREB1. Similar arguments can be made about the models in which MeCP2 functions to modulate alternative splicing and miRNA processing

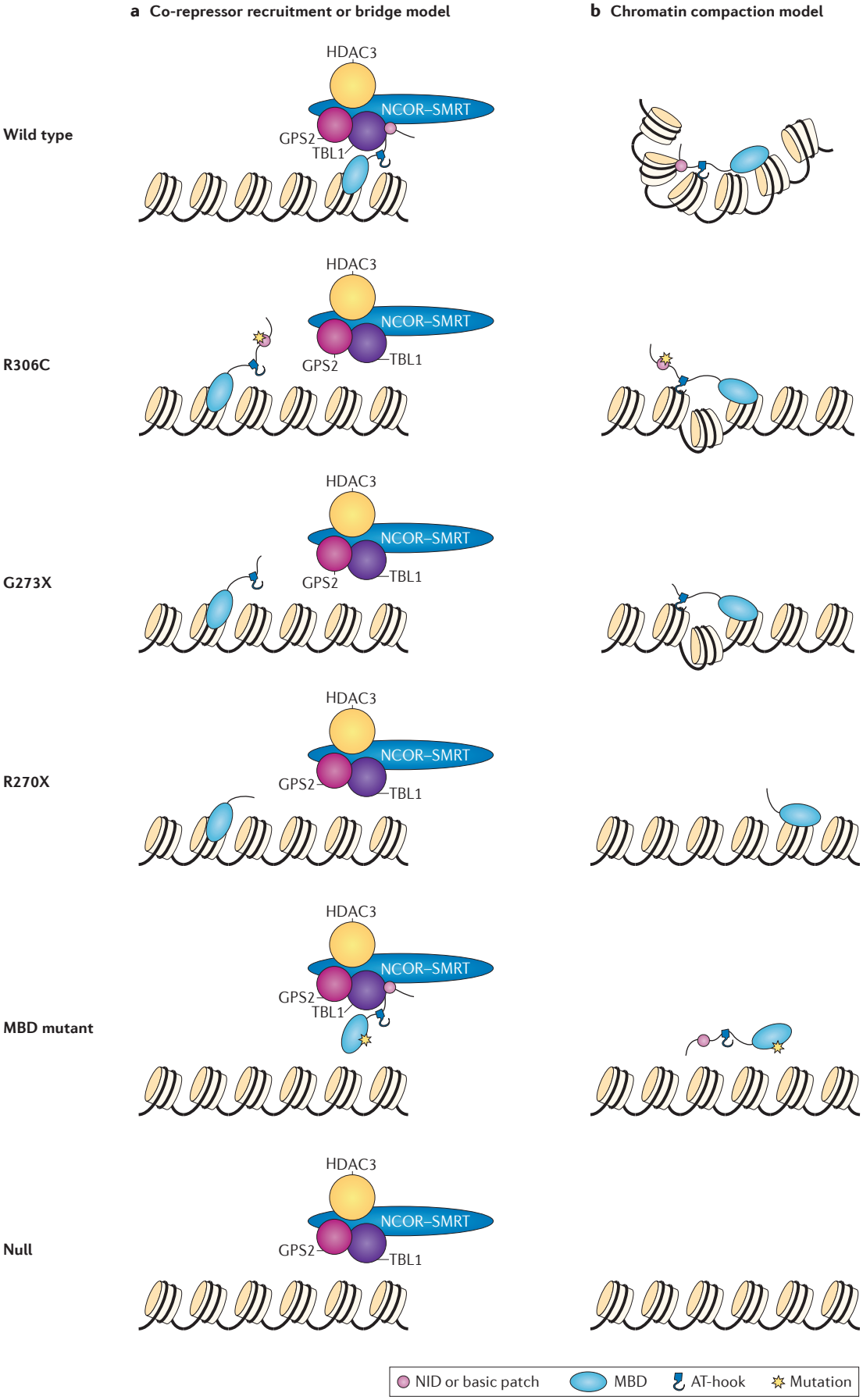

Figure 3 | **Two models of MeCP2 function are consistent with the mutation spectrum of Rett syndrome.** **a** | In the co-repressor recruitment or bridge model, methyl-CpG-binding protein 2 (MeCP2) serves as a bridge between chromatin and the nuclear receptor co-repressor (NCOR)–SMRT (silencing mediator of retinoic acid and thyroid hormone receptor) complex. Mutations can disrupt the bridge through defective binding to DNA by mutations that disrupt methyl-CpG-binding domain (MBD) function, by truncating MeCP2 upstream of the NCOR–SMRT interaction domain (NID; also referred to as a 'basic cluster') (for example, R270X and G273X nonsense mutations), or by abolishing binding of the NID to NCOR–SMRT (for example, the R306C missense mutation). **b** | In the chromatin compaction model, MeCP2 regulates chromatin architecture by interacting with the genome through its different DNA-binding domains. Loss of the NID (for example, the R306C or G273X mutations) means that MeCP2 can still bind to DNA but cannot fully affect chromatin structure. In the presence of an earlier truncation mutation that additionally abolishes an AT-hook motif (R270X), MeCP2 still binds to DNA but cannot mediate changes to chromatin structure. R270X therefore gives rise to a more severe disease phenotype in patients and in mice than G273X or R306C. Mutations in the MBD, which prevent MeCP2 from binding to DNA and amount to complete loss of MeCP2 function, also result in severe phenotypes. GPS2, G protein pathway suppressor 2; HDAC3, histone deacetylase 3; TBL1, transducin β-like protein 1.

by its interactions with YB1 and DGCR8, respectively. A caveat to these negative assessments is that some essential functions of MeCP2 might not be destroyed by a single amino acid substitution but might require the alteration of multiple residues. Such interactions would not be highlighted by the mutation spectrum. Testing this idea would require engineering mutations to abolish the putative essential interaction. Assessing the phenotypic consequences of such mutations would represent an interesting avenue of future research.

Conclusions and perspectives

Twenty-three years of research have painted a complex picture of MeCP2 function. Fortunately, the mutation spectrum of RTT can be used to critically assess various functional models, thus aiding our understanding of the pathology of this disorder. In our view, two models of MeCP2 function are consistent with the RTT mutation spectrum. First, MeCP2 might serve as a bridge between chromatin and the NCOR–SMRT complex[18] (FIG. 3a). Second, MeCP2 might influence the architecture of chromatin by binding to DNA via the MBD, an AT-hook[20] and a basic cluster[19] (FIG. 3b). These two models, together with the possibility that as-yet unidentified functions for the MBD and NID are key, provide a platform for future investigations into the molecular basis of RTT. Based on the first model, mice engineered to express mutant forms of the NCOR–SMRT complex that fail to bind to MeCP2 should have an RTT-like phenotype. Whether other crucial regions outside the MBD and the NID exist could be tested by deletion of presumed non-essential domains in mice.

Another outstanding question is as follows: what are the downstream pathways that depend on the essential molecular interactions of MeCP2? Despite the implication that MeCP2 is involved in transcriptional regulation, there is no compelling case to suggest that changes in gene expression underlie RTT pathology. Indeed, the NCOR–SMRT complex that is thought to be involved in MeCP2 repressor function is a large, multiprotein assembly that might mediate an array of functions that are not limited to gene regulation. However, a model has recently been put forward whereby MeCP2 modulates transcription in a gene-length-dependent manner[38,149], with the long genes that are most enriched with methylated CpA dicnuleotides[149] being preferentially upregulated in isolated brain regions from *Mecp2*-null mice. Given the abundance of MeCP2 and the fact that it seems to bind throughout the genome, it is plausible that MeCP2 might not serve to regulate specific target genes but may affect broad categories of transcription in a global manner. Approaches that aim to identify common features of genes dysregulated in MeCP2 mutants, such as methylation status[149], length[38,149] and expression level[16], might be promising avenues for attempting to understand the logic behind the effects of MeCP2 on transcription.

Several downstream consequences of MeCP2 deficiency in the nucleus have been reported, particularly affecting the cell biology of RTT neurons from patients, as well as in animal and cellular models of the disorder. For example, mitochondria function abnormally[113], protein synthesis is impaired[86] and synaptogenesis is reduced[114]. Although all of these phenomena are strong candidates for involvement in the pathology of RTT, distinguishing them from secondary consequences of pathology in the brain or in cultured cells remains a challenge. Future priorities are to test and refine models of the molecular function of MeCP2, bearing in mind the mutation spectrum underlying RTT, and to elucidate the cellular defects that are the most relevant to RTT pathology. Armed with new genetic, cell biological and biochemical technologies, the research field is well placed to make advances in understanding this presently enigmatic protein and, ultimately, to facilitate the development of therapeutic approaches.

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Competing interests statement
The authors declare no competing interests.

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