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## INVITED REVIEW

## State-of-the-art therapies for Rett syndrome

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Rett syndrome (RTT) is an X-linked neurogenetic disorder caused by mutations of the *MECP2* (methyl-CpG-binding protein 2) gene. Over two decades of work established MeCP2 as a protein with pivotal roles in the regulation of the epigenome, neuronal physiology, synaptic maintenance, and behaviour. Given the genetic aetiology of RTT and the proof of concept of its reversal in a mouse model, considerable efforts have been made to design therapeutic approaches to re-express MeCP2. By being at the forefront of the development of innovative gene therapies, research on RTT is of paramount importance for the treatment of monogenic neurological diseases. Here we discuss the recent advances and challenges of promising genetic strategies for the treatment of RTT including gene replacement therapies, gene/RNA editing strategies, and reactivation of the silenced X chromosome.

## RETT SYNDROME: FROM ANDREAS RETT TO BENGT HAGBERG

Rett syndrome (RTT, Mendelian Inheritance in Man [MIM] 312750) is a rare genetic disorder leading to severe and progressive intellectual disability, almost exclusively affecting female children with an incidence of approximately 1 in 15 000 live births. It was first characterized in 1966 by Andreas Rett, a psychiatrist from Austria who specialized in neurodevelopmental disorders.<sup>1</sup> However, it was not until two decades later that RTT became officially recognized after a Swedish neurologist, Bengt Hagberg, observed patients with similar symptoms and decided, with French and Portuguese colleagues, to publish an

article describing the syndrome.<sup>2</sup> RTT alone accounts for 10% of cases of profound intellectual disability of genetic origin in females.<sup>3</sup> RTT is a severe developmental disorder.<sup>4,5</sup> Females with RTT begin life apparently 'healthy'. However, from 6 to 18 months of age they undergo regression of early milestones, with deterioration of motor skills, eye contact, speech, and motor control; they then develop a range of neurological symptoms, including anxiety, respiratory dysrhythmias, and seizures.<sup>5</sup> Since most cases are sporadic, identifying a causative locus has been fraught with difficulties. Initially RTT was considered a purely nervous system pathology including neurons and astrocytes but in recent years it has emerged that RTT is also a neurometabolic pathology involving cholesterol abnormalities.<sup>6,7</sup>

**Abbreviations:** AAV, adeno-associated virus; ADAR, adenosine deaminase acting on RNA; gRNA, guide RNA; KO, knockout; *MECP2*, methyl CpG-binding protein 2 gene; miRARE, MiRNA-responsive autoregulatory element; RTT, Rett syndrome.

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## MECP2 mutations are the cause of Rett syndrome

In 1999, Huda Zoghbi's team identified heterozygous pathogenic variants of the *MECP2* gene, located in position Xq28 on the long arm of the X chromosome, in patients with RTT.<sup>4,8</sup> This gene encodes an eponymous protein that is ubiquitous but expressed mainly in the nervous system. MeCP2 has two main isoforms: MeCP2-E1 and MeCP2-E2, differing in a few amino (N)-terminal amino-acid residues. MECP2-E2 was isolated first and used to define the general roles of MECP2 in, targeting of transcriptional regulatory complexes, and its disease-causing impact in RTT.<sup>9</sup> It was later found that MECP2-E1 is the most abundant isoform in the brain and that exon 1 mutations cause RTT.<sup>9</sup> However, differential functions of MeCP2-E1 and -E2 remain largely unexplored and, until now, gene replacement strategies have aimed at the re-expression of MeCP2-E1.<sup>10</sup> It is estimated that more than 95% of typical RTT cases are caused by an *MECP2* variant.<sup>11</sup> Since the link between *MECP2* and RTT was identified, thousands of *MECP2* variants have been reported in patients worldwide.<sup>5</sup> These variants are of different types: missense and nonsense mutations, deletions, and insertions with or without reading frameshifts. Apart from RTT-associated variants, it is also well established that some polymorphisms are not associated with the disease.<sup>5</sup> Many researchers have tried to make genotype–phenotype associations to predict the evolution of patients' symptoms. Some variants such as A140V, R133C, or carboxy (C)-terminal domain deletions are known to generate a moderate phenotype.<sup>12–14</sup> On the contrary, other variants are associated with severe forms of RTT such as those affecting the MeCP2 protein nuclear localization signal or leading to truncated proteins by insertion of a premature stop codon.<sup>15</sup> The phenotypic variability is also due to the X-chromosome inactivation profile. Indeed, in females, one of the two X chromosomes is randomly inactivated in each cell. This phenomenon (X-chromosome inactivation), discovered in 1961, explains the greater severity of X-linked dominant pathologies in males. In a heterozygous female, in general, about half of the cells express the mutant protein; in contrast, in hemizygous males, all the cells express the mutant protein. However, sometimes the inactivation profile of the X chromosome is skewed, with one allele being more expressed than the other. This inactivation bias can be in favour of the healthy allele or the mutated allele, hence the difficulty in performing a strong correlative study between genotype and phenotype where both the type of mutation and the degree of X-chromosome inactivation should be considered. In addition to loss-of-function mutations leading to RTT, duplication or triplication of the *MECP2* locus led to severe intellectual disability, autistic features, and motor dysfunction, as observed in males with *MECP2* duplication syndrome.<sup>16</sup> The regulation of *MECP2* gene dosage must be finely tuned as both its absence (severe encephalopathy in male children and RTT in females), and its overexpression (*MECP2* duplication syndrome) leads to serious neurological consequences.<sup>17</sup>

### What this paper adds

- Recent advances shed light on the promises of gene replacement therapy with new vectors designed to control the levels of MeCP2 expression.
- New developments in DNA/RNA editing approaches or reactivation of the silenced X chromosome open the possibility to re-express the native MeCP2 locus at endogenous levels.
- Current strategies still face limitations in transduction efficiency and future work is needed to improve brain delivery.

## MeCP2 cellular functions and mouse models

MeCP2 is one of the most abundantly expressed proteins and its main locus of action is the nucleus.<sup>18</sup> Its expression is low during early developmental stages but progressively increases in line with a key role in neuronal differentiation, maturation, circuit refinement, and synaptic maintenance.<sup>19</sup> Its distribution seems already high and uniform during early postnatal periods in the brain. In the adult, the levels peak in cortical regions, the hippocampus, cerebellum, and olfactory bulb. On the other hand, expression of MeCP2 is also high in the lungs and spleen, but seems significantly lower in the kidneys, heart, liver, stomach, and intestine.<sup>20</sup> MeCP2 has been conditionally knocked out in different tissues, brain areas, and cell types. Several studies showed that the removal of MeCP2 was particularly detrimental when achieved in neurons and astrocytes.<sup>21</sup>

Although MeCP2 is understood to be an epigenetic regulator, its precise role and biological functions remain debated. Historically, MeCP2 is described as a transcriptional repressor. The mechanism behind this canonical role involves MeCP2 binding to methylated DNA through its methyl-binding domain and compaction of the chromatin through interaction with corepressing factors and complexes within the transcription repression domain such as Sin3A, or NCoR and SMRT (though the NCoR1/SMRT interaction domain). These factors further serve as a scaffold for other DNA binding proteins and recruit histone deacetylases, thus making the DNA less accessible to transcription factors.<sup>22–24</sup> Other proteins interacting with MeCP2, including chromatin remodellers, DNA/histone methyltransferases, or helicase/ATPases, contribute to MeCP2-mediated gene silencing.<sup>25</sup> This model emphasizes the importance of MeCP2 in coupling DNA binding to gene silencing. In favour of this role, RTT-causing mutations in the methyl-binding domain or the NCoR1/SMRT interaction domain are particularly detrimental both in humans and in mice.<sup>5</sup> Strikingly, mice expressing a minimal *Mecp2* protein consisting of the methyl-binding domain, NCoR1/SMRT interaction domain, and linker regions develop only a mild RTT-like

phenotype. In the same study, genetic reactivation or virally driven mini-Mecp2 gene delivery ameliorated symptoms in Mecp2-deficient mice, thus highlighting the necessity of the DNA binding and repressive functions of MeCP2.<sup>26</sup> Besides this consensual repressive role, some studies have suggested an activating role in the expression of certain genes.<sup>27</sup> Interpretations about the repressive or activating role of MeCP2 have also been challenged because of the ability of MeCP2 to recognize methylated base-pair couples guanine-cytosine and cytosine-adenine, hydroxymethylated cytosines, and because these modifications are associated with either repression (methylated cytosine-adenine/methylated guanine-cytosine) or activation (hydroxymethylated cytosines) of transcription.<sup>28</sup> For years, many efforts were deployed to unambiguously determine the target sequences of MeCP2. Recent work has shown that MeCP2-induced repressive effects on transcription are directed by the pattern of methylation established by DNMT3A at cytosine-adenine sites during early life.<sup>29</sup> Intriguingly, amid studies claiming the binding of MeCP2 on hydroxymethylated cytosines and methylated cytosine at cytosine-adenine sites, questions about the enrichment of such modifications in the brain, the enrichment of MeCP2 binding on these cytosine-adenine repeats in the brain, and the correlation between cytosine-adenine repeats and MeCP2-dependent gene expression are still highly debated.<sup>30</sup> Several laboratories are also trying to decipher whether the activity of MeCP2 is genome-wide or restricted to certain genes, such as long genes (100 kilobases on average).<sup>18,31</sup> Apart from these repression/activation roles, the function of MeCP2 seems to extend to post-transcriptional levels. MeCP2 was shown to regulate alternative splicing through its interaction with the Y-box transcription factor and suppress microRNAs processing through alteration of the assembly of Drosha-DGCR8 complex.<sup>32,33</sup> Finally, several studies also pointed towards the involvement of MeCP2 in gene expression through its impact on the chromatin higher-order structure.<sup>25</sup>

After the discovery of the causal link between MeCP2 mutation and RTT, many cellular and animal models have been generated. A variety of mouse models exist, including either constitutive or conditional knockouts, knockin, hypomorph, and duplicated mice.<sup>34</sup> In many aspects these mice strongly recapitulate the respective pathologies. RTT mouse models seem healthy at birth but then show gradual pathological manifestations as seen in female patients. The major morphological abnormalities of the central nervous system (CNS) related to RTT are an overall decrease in brain weight and volume as well as reduced neuronal cell size and an alteration in neurons' electrical properties.<sup>19</sup> A role for MeCP2 in corticogenesis was recently shown.<sup>35</sup> However, so far, there is no evidence of neuronal degeneration or apoptosis in line with the possibility of rescuing the pathology in adult mice.<sup>25</sup> The loss of MeCP2 also affects the number of axonal boutons and axonal arborization.<sup>37</sup> These observations show that RTT is a neurodevelopmental rather than a neurodegenerative disorder. A research team showed that MeCP2 floxed mice are hypomorph, thus expressing 50% of the

MeCP2 levels of a wild-type mouse. The work helped to understand the quantity of MeCP2 that is necessary for the proper functioning of the organism.<sup>38</sup> Despite their normal survival curve and body weight, and lack of hindlimb clasping, hypomorph mice exhibit motor disturbances, deficits in social behaviours, nest-building, learning, and an altered respiratory pattern. These results indicate that expression of only half of wild-type MeCP2 levels is not enough to avoid significant phenotypic manifestations.

## Innovative treatment in RTT

RTT is, so far, an incurable pathology whose only available treatments aim to alleviate evident symptoms and improve patients' quality of life. Several pharmacological interventions have been proposed, thanks to the discovery of targets of interest in RTT mouse models. Despite the rarity of the pathology, RTT represents one of the rare diseases that is the most advanced in the development of pharmacological treatments with more than 60 clinical trials finished or in progress. However, these treatments are not sufficient to cure the disease. In this review we have voluntarily decided not to address the pharmacological approaches but to focus on the most innovative strategies which only aim at allowing each cell to express a functional Mecp2 and potentially to cure the pathology. Because of the importance of *MECP2* during neuronal development and the time window of its expression, it was long thought that the damage caused by its absence was irreversible, despite the absence of neuronal death. A key study led by Bird answered this question by demonstrating that restoration of the expression of Mecp2 in adult symptomatic mice can reverse the phenotype.<sup>36</sup> In turn, specific elimination of MeCP2 in adult mice demonstrated the dependence of the mature brain on MeCP2-related functions and that potential treatments must be provided throughout life.<sup>39</sup> These results fostered the development of different strategies aimed at the re-expression of MeCP2 in RTT mice and the design of translational approaches to treat patients with RTT. Nonetheless, as stated above, issues related to the fine-tuning of MeCP2 gene dosage remain to be addressed to restore physiological levels without risking its overexpression.<sup>16,17</sup>

## Gene therapy

The most common type of preclinical innovative studies concern gene therapy, which can take several forms. Either new genetic material can be introduced to compensate for the lack of the mutated protein (gene replacement technique) or the genome/transcriptome of the organism affected by the disease can be modified (genome or RNA editing). Among available viral vectors that can be used for gene therapy in humans, adeno-associated viruses (AAVs) rapidly became the preferred option given its ability to achieve long-term expression profiles in both dividing or non-dividing cells,

and to offer cell-type-specific tropisms depending on the used serotype. Moreover, AAVs elicit a very mild immune response *in vivo*, making them thus far vectors with high biosafety profiles. So far, a limited number of *in vitro*/cellular studies have suggested the use of MeCP2-expressing lentiviral vectors.<sup>40</sup> The transfer of these strategies to humans seems to be more complex as they are not superior to AAVs, and thus warrants further investigation.

## Gene replacement strategies

The first published gene therapy paper in a mouse model of RTT, in 2013, showed that intracerebral administration of a single-strand AAV9-Mecp2 vector in a neonatal *Mecp2*-KO (knockout) mouse (P1–P3) induced MeCP2 expression in brain structures (up to 41.5% of transduced cells) and ameliorated the mouse phenotype.<sup>41</sup> The same authors administered a scAAV9-pME (229 base pair)-*Mecp2* vector intravenously in 30-day-old *Mecp2*-KO mice. This injection improved the survival of treated mice (although the mice showed signs of hepatotoxicity). The biodistribution of this AAV (injected into the tail vein) was assessed, showing that the liver is the most transduced organ, followed by the heart, spleen, kidney, and CNS (the main target, although <4% of the cells were transduced).<sup>41</sup> These very low transduction rates in the brain could explain the limited phenotype improvement in treated mice. Also in 2013, another study showed significant improvements in *Mecp2*-KO males and heterozygous female mice, both treated at a symptomatic stage with a scAAV9-pME (700 base pair)-*Mecp2* vector.<sup>42</sup> Despite using a higher dose than in the work by Gadalla et al.,<sup>41</sup> no increase in mortality was observed (although the authors did not assess potential side effects). However, they did observe enhanced AAV transduction with up to 25% of the cells expressing MeCP2 in some brain areas of the *Mecp2*-KO-treated mice. These differences may be related both to the dose administered and to the design of the vector, which features a promoter three times longer than the one used before, thus enabling a better control of transgene expression.<sup>29</sup> In 2017, several other studies were published.<sup>26,43–45</sup> A recent study by our laboratory confirmed that intravenous injection of an AAV9 containing an optimized codon version of *Mecp2* in heterozygous females resulted in functional improvements but also led to hepatic pathology.<sup>46</sup>

Indeed, RTT affects exclusively females, which are genetic mosaics (~50% of cells express wild-type MeCP2, 50% mutant). Importantly, MECP2 overexpression is also pathogenic, as patients with *MECP2* duplication syndrome suffer from a neurodevelopmental disorder and intellectual disability.<sup>14</sup> It is therefore necessary to avoid treating both healthy and mutated cells simultaneously, which would result in MeCP2 overexpression in cells with basal MeCP2 levels. Studies using hypomorphic or MeCP2-overexpressing mice aim to identify optimal MeCP2 levels that might be tolerated by the cells.

Following their 2013 study, Gadalla et al. modified their vector to decrease the hepatic side effects.<sup>44</sup> They used an scAAV9.47-pME (426 base pair)-*Mecp2* with a 3' untranslated region to feature key regulatory elements and limit MeCP2 overexpression.<sup>32</sup> This injection improved both the survival and body weight of treated mice without inducing hepatic pathology, despite a high transduction in the liver.

To improve the low transduction rate of AAV in the brain after systemic injection, an AAV9-PhP.B was generated. This novel AAV has the ability, following an intravenous injection, to selectively transduce CNS neurons.<sup>47</sup> This new tool was used in 2020 to deliver an 'unstable' *Mecp2* transgene with a reduced half-life (to reduce the risk of *Mecp2* overexpression) both in male and in female mouse models of RTT.<sup>48</sup> The results of this study showed an interesting phenotypic improvement thanks to a high vector transduction rate in the CNS. An immune response was still observed in injected males with the production of anti-Mecp2 antibodies, but not in heterozygous females. Transduction in the liver was verified and no side effects related to potential hepatotoxicity were observed.<sup>48</sup> While the results of this study are very encouraging, work showing efficacy of PhP.B limited to C57Bl/6J mice limits the translational potential of this study.<sup>49</sup> Tillotson et al. proposed that the full *Mecp2* sequence is not necessary and that administration of an scAAV9-pME (426 base pair)-*Mecp2*-truncated intracerebrally in P1–P2 *Mecp2*-KO males allows expression of *Mecp2* in up to 50% of brain cells and a significant improvement of the phenotype.<sup>26</sup> These results are particularly interesting because different studies have shown that the lack of regulatory elements due to the limited space in AAV9 induced an overexpression of toxic *Mecp2*, mainly in the liver. The possibility of using a mini *Mecp2* transgene would therefore free up space to provide different additional regulatory elements. To limit the problems associated with transgene overexpression, a team developed an AAV with a 3' untranslated region carrying targets of microRNAs that regulate *Mecp2* expression. For instance, targets of miRNA-122 decrease transgene expression in the liver.<sup>50</sup> Their microRNA target panel is called miRARE (MiRNA-responsive autoregulatory element). Their pME (426 base pair)-*Mecp2* truncated-miRARE construct was then placed into a scAAV9 but also AAV.PhP.B.<sup>50</sup> The intrathecal administration in P30 *Mecp2*-KO males mainly improved survival. However, the addition of miRARE-like regulatory sequences seems to be a real advantage to control transgene expression better.

A very different strategy to limit effects of MeCP2 overexpression could be to target the nuclear transport of MeCP2 to fine-tune its nuclear levels. Previous studies claimed the involvement of importin  $\alpha 3$  and  $\alpha 4$  in MeCP2 nuclear shuttling, while *in vivo* analyses showed a specific deficit of MeCP2 nuclear transport in the hippocampus of importin  $\alpha 5$ -deleted mice.<sup>51–53</sup> Importin  $\alpha$  exhibit overlapping cargo specificities *in vitro* and in heterologous systems, while showing exquisite specificity *in vivo*.<sup>53–56</sup> Future studies in this direction could help to design genetic and pharmacological strategies to specifically modulate MeCP2 nuclear

transport that could accompany *MeCP2* gene replacement therapies.

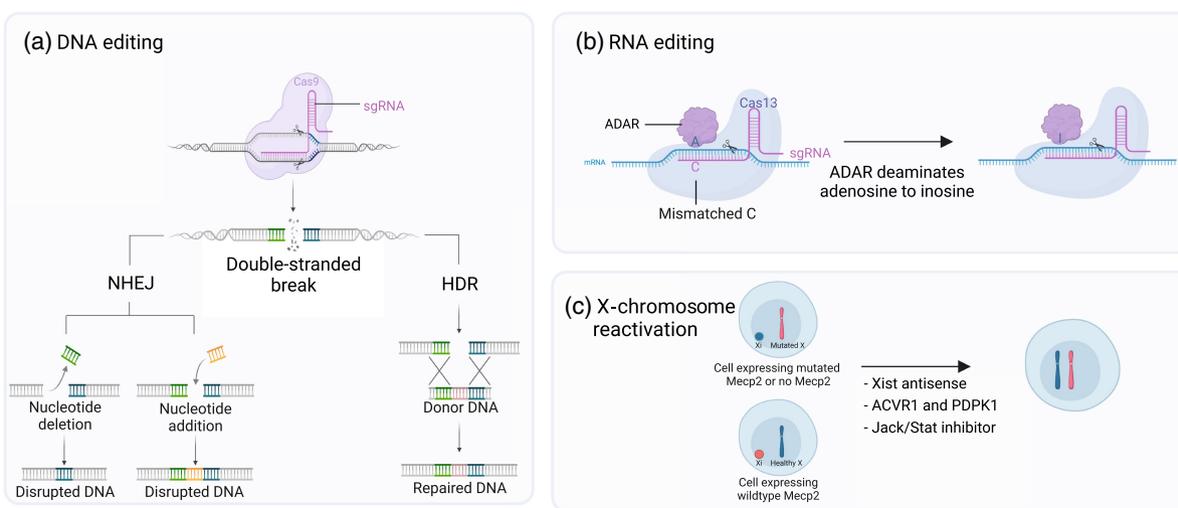
## Genome editing

By directly repairing the faulty gene in the cell in a targeted, stable, and permanent way, genome editing of *MECP2* offers the promise of the expression of a healthy protein at endogenous levels, therefore avoiding overexpression side effects observed with the replacement strategy. To perform genome editing, a nuclease capable of cutting the genome where necessary and a segment of DNA or RNA used for repair must be imported into the cell. The CRISPR–Cas9 system, which is composed of the Cas9 enzyme and a synthetically engineered guide RNA (gRNA) rapidly became the most popular gene editing method.<sup>57</sup> Cas9 and gRNA form a ribonucleic complex capable of recognizing and cleaving a target sequence. Following double-stranded DNA cleavage, the cut is repaired either by non-homologous end joining (NHEJ) or by homology-directed repair that allows precise genome repair against a donor DNA template (Figure 1). The system is efficient, fast, and easy to use, thus making it a leading tool for the treatment of genetic diseases. However, two caveats limit its use in gene therapy. The first is off-target modifications on sequences that differ by only a few nucleotides from the target sequence, which can cause alteration of essential genes (the longer Cas9 is expressed, the greater the risk of off-target modifications).<sup>58</sup> Second, to co-express Cas9 and gRNA one must transduce the same cell with two different AAVs, the encapsidation size of one being insufficient. Two approaches to genome editing are being developed for RTT. Beam therapeutics is aiming at personalized medicine by targeting single point mutations. A study in patients' fibroblasts and neurons derived from induced pluripotent

stem cells showed the success of this approach to correct the T158M mutation.<sup>59</sup> There, the Cas9 expression was limited by adding a sequence recognized by Cas9 between its own sequence and its promoter to allow for its self-cleavage. This two-vector approach allowed editing of about 45% of the cells on average. Alternatively, Jonathan Watts' team at the University of Massachusetts is performing exon editing by replacing exons 3 and 4. This approach would allow, with the same technology, to correct 97% of RTT-causing mutations. A first in vitro study conducted by another team showed 20% to 30% efficacy in cell lines derived from patients with RTT for mutations located in exon 4.<sup>60</sup> In this work, they used two vectors containing two gRNAs targeting only the mutated allele, a wild-type DNA template to do homology-directed repair over a large portion of exon 4 (2.5 kilobases) and Cas9. These interesting proofs of concept still face limited CNS tissue accessibility as with gene replacement therapy, an issue compounded by the necessity for cotransduction of two vectors into the same cell.

## RNA editing

There is an innate cellular machinery featuring enzymes (editases or adenosine deaminases acting on RNA [ADARs]) that can edit messenger RNAs if an error has been made during transcription.<sup>61</sup> In contrast to genomic editing, RNA is rapidly degraded and the modifications are not permanent. To target a specific RNA, some studies use fusions of ADAR domains with a protein capable of binding to RNA such as Cas13 as well as a specific gRNA of the target sequence.<sup>62</sup> Historically, the only editing available was for G>A variants. The deamination of adenosine (A) to inosine (I) by the editase allows the cell to consider the base as a guanine (G) upon translation of the messenger RNA into protein. Many



**FIGURE 1** Innovative therapeutic developments in Rett syndrome. (a) DNA editing. Double-strand breaks guided by Cas9 and its guide RNA can be repaired by non-homologous end joining (NHEJ) or by homology-directed repair (HDR) in the presence of donor DNA. (b) RNA editing. A modified Cas13 fused to adenosine deaminase acting on RNA (ADAR) which deaminates an adenosine to an inosine at a specific target site. The inosine is then read as a guanine by the translation machinery. (c) X-chromosome reactivation. In female cells, one of the two X chromosomes is randomly inactivated to balance the expression of genes. Pharmacological strategies allow the reactivation of the inactive X (Xi) carrying the healthy *MeCP2* gene.

laboratories modify editases to extend their capabilities, and it is now possible to correct 6 out of 12 point mutations: C>T, A>G, C>G, G>A, T>C, G>C. Of the pathogenic variants causing RTT, 36% are G>A or C>T variants that introduce stop codons.<sup>63</sup> A knockin mouse was generated with a 317G>A (R106Q) pathogenic variant in the *Mecp2* gene.<sup>63</sup> In vitro, RNA repair and functionality of the subsequently translated protein were demonstrated in cultures of primary neurons from the R106Q mouse.<sup>64</sup> These neurons were transduced with an AAV containing a hyperactive ADAR2 enzyme fused to a bacteriophage peptide, and a guide sequence. The same team then successfully tested the technology in vivo by injecting the AAV vector into the hippocampus of P30 mice.<sup>65</sup> By analysing the off-target modifications, they counted no less than 2984 RNA editing sites, very few in the sequence covered by the gRNA but rather distributed throughout the transcriptome. The extent of off-target editing correlates with the level of ADAR2 present in the cells. It is likely that peripheral administration of AAV (vs intracerebral administration) results in lower levels of ADAR in the cells and thus limits off-target modifications. The effects of this RNA editing on mouse model phenotype have not been studied yet. As with other technologies, accessibility of AAVs to the brain will be a constraint. It is also important to generate more efficient enzymes that induce fewer off-target modifications to limit side effects.

## REACTIVATION OF THE INACTIVE X CHROMOSOME

X-chromosome inactivation is a random phenomenon occurring in female cells to balance the expression of genes carried by one of the two X chromosomes. The inactivation is initiated by the non-coding RNA Xist, and achieved through methylation and heterochromatinization of the inactivated chromosome.<sup>66</sup> Xist is required for the establishment of X-chromosome inactivation during embryogenesis and its maintenance throughout life.<sup>67</sup> Thanks to this mechanism, only one X chromosome is active and the genes it carries can be expressed in the female. Given the randomness of inactivation, females are therefore mosaics. RTT being caused by heterozygous variants, cells randomly express either a healthy or a mutated MeCP2 protein. The discovery of healthy carriers of an *MECP2* mutation due to X-chromosome inactivation bias<sup>68</sup> suggests that reactivation of the unmutated X chromosome in patients with RTT is a promising therapeutic strategy. Pharmacological inhibition of the factors responsible for X inactivation allowed reactivation of the X chromosome carrying the healthy *MECP2* allele in patients' fibroblasts and mouse neurons, resulting in activation of both X chromosomes.<sup>69</sup> To determine the side effects related to the upregulation of the different genes carried by the X chromosome following the activation of both X chromosomes in the cell, the authors studied a female *Stc1*<sup>-/-</sup> mouse with a lack of random inactivation of the X chromosome. The results showed that these mice had a

normal life expectancy and, surprisingly, did not overexpress most (98%) of the genes carried by the X chromosome. This suggests that compensatory mechanisms may prevent a general gene overexpression. In 2018, it was shown in vitro that the combined use of a small-molecule inhibitor of DNA methylation and an antisense oligonucleotide against Xist enables the reactivation of *MECP2*.<sup>70</sup> However, with this approach, the reactivation was not specific to *MECP2* but affected the entire X chromosome. Another team described the pharmacological reactivation of *Mecp2* carried by the inactive X chromosome in vivo in mouse cortical neurons by intracerebral injection of two different molecules, ACVR1 (activin A receptor type I) and PDPK1 (pyruvate dehydrogenase kinase 1).<sup>71</sup> Laboratories have been trying since 2017 to identify other regulators of Xist that could be targeted pharmacologically.<sup>72</sup> Inhibitors of the JAK/STAT pathway have been shown in vitro to reactivate the inactive X chromosome.<sup>73</sup> However, the efficiency of reactivation is tissue- and cell-type-dependent.

A grey area remains with the reactivation of the X chromosome in the case of hypomorphic *MECP2* mutant alleles such as R133C. So far, increased expression of MeCP2 in female R133C mice could correct some deficits but caused *MECP2* duplication syndrome-like motor, anxiety, and fear memory phenotypes, but similar strategies in female T158M or R255X mice did not 'overcorrect' these phenotypes.<sup>74-76</sup> Thus it will be important to test the therapeutic impact of the X-chromosome reactivation in mouse models expressing different *Mecp2* variants.

Some questions also remain about transcriptional and post-transcriptional regulation of the *MECP2* gene in the context of the silenced X reactivation. As discussed above, MeCP2 exists as two isoforms presenting tissue specificity but also non-redundant functions.<sup>77</sup> In addition, both MeCP2 proteins are encoded by transcripts with different 3' untranslated region lengths, which are prone to microRNA-based regulations.<sup>78</sup> As of now, gene therapy has mostly focused on expressing MeCP2-E1, although transgenic rescue of MeCP2 deficiency could be achieved with either isoform in mice.<sup>79</sup> In this context, the reactivation of the silenced X chromosome should allow both re-expression and optimal regulations of MeCP2.

## CONCLUSION

Our review has focused on promising advances in the field of gene therapy. Most approaches developed by academic laboratories and industry aim at replacing MeCP2. Many of the strategies are facing issues inherent with the necessity to regulate MeCP2 levels precisely to avoid detrimental effects associated with its overexpression. Gene editing and X reactivation promise exciting strategies by editing or re-expressing the native locus and allowing endogenous expression of *MECP2*; by doing so, they avoid the risk of developing *MECP2* duplication syndrome overexpression phenotypes. In addition, gene replacement and gene editing

strategies still face a limitation in transduction efficiency and more work is required to improve brain delivery. In this sense, combining viral vector delivery with focus ultrasound technologies has shown promising results in the field of CNS delivery.<sup>80</sup> Although these different strategies have shown encouraging results in preclinical studies, further refinement is needed to devise the best genetic approach. It is also tempting to look towards new developments of bioinformatic pipelines that could shed light on new candidate drugs ameliorating RTT phenotypes.<sup>81</sup> We believe that such an integrative approach and the fast development of these fields will be key to finding a cure for RTT.

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## DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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