Site-directed RNA repair of endogenous Mecp2 RNA in neurons

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Contributed by Gail Mandel, September 22, 2017 (sent for review August 30, 2017; reviewed by Gordon G. Carmichael and Stuart Cobb)

Rett syndrome (RTT) is a debilitating neurological disorder caused by mutations in the gene encoding the transcription factor Methyl CpG Binding Protein 2 (MECP2). A distinct disorder results from MECP2 gene duplication, suggesting that therapeutic approaches must restore close to normal levels of MECP2. Here, we apply the approach of site-directed RNA editing to repair, at the mRNA level, a disease-causing guanosine to adenosine (G > A) mutation in the mouse MeCP2 DNA binding domain. To mediate repair, we exploit the catalytic domain of Adenosine Deaminase Acting on RNA (ADAR2) that deamidates A to inosine (I) residues that are subsequently translocated as G. We fuse the ADAR2 domain, tagged with a nuclear localization signal, to an RNA binding peptide from bacteriophage lambda. In cultured neurons from mice that harbor an RTT patient G > A mutation and express engineered ADAR2, along with an appropriate RNA guide, a human mutation in MECP2 that causes RTT in humans result in MECP2 overexpression. This successful use of site-directed RNA editing to repair an endogenous mRNA and restore protein function opens the door to future in vivo applications to treat RTT and other diseases.

RNA editing | MECP2 | Rett syndrome | ADAR2

Rett syndrome (RTT) is a neurodevelopmental disorder due to sporadic mutations in the transcription factor, Methyl CpG Binding Protein 2 (MECP2) (1). MECP2 is located on the X chromosome. Because of dosage compensation mechanisms in mammals, females affected with RTT are mosaic, with an ~50:50 split between wild-type and mutant cells. Females with MECP2 mutations undergo regression of early developmental milestones, such as speech and purposeful hand motions, and then acquire severe motor abnormalities, including respiration, and die on average by age 40 (2, 3). Males with mutations in MECP2, with a single X chromosome, have an even more profound disease, usually succumbing before 2 y of age (4). There is no cure for RTT.

Mice engineered with mutations in Mecp2 that cause RTT in humans, either germ line or confined to neural cells, exhibit growth abnormalities, anxiety, and motor deficits, which are similar to RTT patients (5–7). Studies in mice indicate that the most robust RTT phenotypes are neurological, affecting both neurons and glia (6, 8), although many other tissues are also likely affected (9). As in humans, male Rett mice have a more severe disease than female mice. For example, female Rett mice live a normal lifespan, while male mice die between 3 and 4 mo of age (5, 7). At the cellular level, neural cells in Rett male and female mice have smaller somas, nuclei, and reduced process complexities (10–16), reminiscent of affected human cells (17–20). Importantly, restoration of MeCP2 in Mecp2-null mice, via conditional Cre recombinase (21) or gene therapy approaches (22–25), reverses many of the Rett-like symptoms and cellular deficits, even in late stages of the disease. The phenotype reversals suggest that in humans, RTT may be amenable to gene replacement strategies (14, 22–25). However, duplications spanning the MECP2 gene in humans result in MECP2 overexpression and a severe neurological disorder (26). Further, MeCP2 in mice is expressed to different levels in different neural cell types, and perhaps as a consequence, loss of MeCP2 function in mice results in cell-specific alterations in gene expression (27–31). These findings underscore the challenges for MECP2 gene replacement that must be finely tuned to restore normal MECP2 levels and cellular physiology across diverse cell types in the nervous system. We hypothesized that repairing MECP2 mutations at the level of mRNA could circumvent the problems of both MECP2 overexpression and cell type-specific regulation.

To test this hypothesis, we targeted guanosine to adenosine (G > A) mutations that underlie RTT (32). We selected this group of mutations as a starting point because there is a family of naturally occurring enzymes, Adenosine Deaminase Acting on RNA (ADAR), which hydrolytically deaminates A to inosine (I) (33–37) in endogenous mRNAs. Base pairs with cytosine (C) and is translated by the ribosome as G (38). One ADAR family member, ADAR2, is expressed to high levels in brain where it posttranscriptionally alters protein functions, such as ion channel permeability, through deamination of the primary transcript (39–41). In addition to its catalytic activity, natural editing by ADAR2 requires recognition of a double-stranded RNA structure, mediated by an intron in the pre-mRNA, which appropriately positions the target A in an exon for editing (39, 42–46). Similar to strategies

Significance

Rett syndrome (RTT) is a neurological disease caused by mutations in the gene encoding the global transcriptional regulator, Methyl CpG Binding Protein 2 (MECP2). We exploit a strategy to repair mutant Mecp2 mRNA that if successful should reverse symptoms. The strategy utilizes the catalytic activity of a naturally occurring enzyme, Adenosine Deaminase Acting on RNA (ADAR2), which in brain alters the mRNA sequence and function of proteins. In cultured Rett neurons co-expressing a modified ADAR2 protein and an appropriate RNA guide, a human mutation in Mecp2 mRNA is repaired efficiently. RNA repair restores MeCP2 function, consistent with reversal of the pathological consequences of the RTT mutation. Our strategy holds promise for new therapeutic approaches to RTT and other neurological diseases.


Reviewers: G.G.C., University of Connecticut Health Center; and S.C., University of Glasgow.

The authors declare no conflict of interest.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1715320114/-/DCSupplemental.

www.pnas.org/cgi/doi/10.1073/pnas.1715320114

PNAS | Published online October 16, 2017 | E9395-E9402
used to exploit functional domains in other modular enzymes, a cloned catalytic domain in human ADAR2 (hADAR2) has been harnessed, in various configurations, to target G > A repair in heterologously expressed mRNAs, usually at stop codons (47–52). In one approach, replicated in our present study, the native RNA binding domains in ADAR2 are replaced with an RNA binding peptide from bacteriophage lambda (λN; ref. 52) that binds to a specific short RNA hairpin with nanomolar affinity (53). Targeted editing of heterologous mRNAs is then achieved by expression of the hybrid ADAR2 protein along with an RNA guide that contains the λN-recognized stem loops and a region complementary to the target mRNA (51, 52).

No endogenous mRNAs have been repaired by site-directed RNA editing. Nonetheless, we were encouraged to test this approach for G > A mutations in endogenous Mecp2. First, the mutations in Mecp2 are in domains that encode well-established functions. Second, the fidelity of repair can be monitored by sequence analysis, Western blotting, and at the single cell level, immunochromatography. Here, we exploit the recombinant hADAR2-λN protein (hereafter, Editase) to test for effective repair of G > A mutations within endogenous Mecp2 transcripts. After determining parameters for Editase editing in transfected mouse neuroblastoma (N2A) cells, we used adenovirus-associated virus (AAV) to transduce primary neuronal cultures from a P0 RTT mouse model that contains a severe form of RTT (54) (Fig. 1A). The number and position of the stem loops residues in the NCoR interaction domain (NID) (32, 54) (Fig. 1B). The number and position of the stem loops relative to the target A were based on previous studies (51, 52) and determined empirically by us for Mecp2 in transfection analyses before this study. Editing is optimal with a C mismatch at that site in the complementary guide (50, 56, 57), and all mRNAs contain this mismatch.

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The N2A cells were cotransfected with separate plasmids encoding Editase, MeCP2-GFP, and a third plasmid either containing or lacking the guide sequences. After 3 d, we used Sanger sequencing to analyze cDNAs synthesized from the targeted region of Mecp2-gfp mRNA (Fig. 1 C and D). Editing efficiency was measured by determining relative peak heights at the targeted A position. All three Mecp2 mutations were edited in a guide-dependent manner, consistent with ADAR2-mediated editing requiring double-stranded RNA (Fig. 1 C and D). The percent editing for a targeted A varied with the 5′ nucleotide context, similar to the sequence preference of the ADAR2 catalytic domain determined in previous studies (58, 59). Specifically, based on in vitro screens, the optimal 5′ nucleotide hierarchy for A deamination by ADAR2 catalytic domain is U > A > G > C and the most optimal 3′ nucleotides are C ∼ G ∼ A > U. W104X (UAG) was edited most efficiently (76 ± 10%), followed by R306H (CAC, 34 ± 3%) and R106Q (CAA, 25 ± 2%), which for Mecp2 were not statistically different (Fig. 1D). To further optimize the Editase system for repairing Mecp2 G > A mutations, we focused on R106Q because in human patients it is more common than the W104X mutation and leads to a more severe form of RTT than R306H (32, 60).

A Mutation in the Deaminase Domain, E488Q, Increases Editing Efficiency of the Hybrid Editase. Previous studies have reported that hADAR2 catalytic domain containing an E488Q mutation increase A > G editing efficiency by increasing both the catalytic rate (51, 61) and the affinity of the catalytic domain for substrate RNA (59). This feature allows the E488Q mutation to achieve higher editing levels of unfavorable 5′ and 3′ contexts (51, 61). To test whether EditaseE488Q would increase the editing efficiency of the target A in Mecp2 (GenBank AF006285), which has a suboptimal 5′ C, we cotransfected N2A cells with Mecp2gfp and EditaseE488Q cDNAs. Sequence analysis indicated that guide expression was required for editing and that the percent editing of Mecp2 mRNA was increased twofold with EditaseE488Q compared with wild-type Editase (51 ± 11% vs. 22 ± 5%, n = 3, P < 0.01) (Fig. 2A). Using either wild-type hADAR2 or hADAR2E488Q catalytic domains in the hybrid Editase, we detected one off-target editing site within the guide region of transfected Mecp2gfp cDNA (Fig. 2B). Editing at this site results in a silent codon change, T105T (ACA > ACG). Previous studies indicated that a G mismatch at the off-target site reduced off-target editing in transfected substrates (49). To determine whether a G mismatch would also reduce off-target editing in Mecp2 mRNA, we analyzed editing efficiency in N2A cells transfected with plasmids coding for Mecp2gfp (Fig. 2C). The amount of off-target editing was reduced significantly when the Editase was targeted with a guide containing the A–G mismatch (4.9 ± 0% with mismatch, 33 ± 5% without mismatch, n = 3, P < 0.0001; Fig. 2 D and E), with no significant effect on editing at the target A (Fig. 2 D and F). All of the editing events required the presence of the guide RNA (Fig. 2 E and F).

Site-Directed RNA Editing Repairs an Endogenous Rett-Causing Mutation, Restoring Protein Levels and MeCP2 Function. Next, we tested whether EditaseE488Q could (i) repair the R106Q missense mutation in the endogenous Mecp2 mRNA, (ii) recover protein levels, and (iii) restore the ability of MeCP2 to bind to heterochromatin, a hallmark functional feature required to reverse Rett-like symptoms in mice (24). For these tests, we isolated neurons from mice engineered to contain the R106Q mutation in the endogenous Mecp2 gene (genotyping data in Fig. S1). The cultured neurons were transduced with either of two AAVs (AAV1/2). Both viruses expressed EditaseE488Q under control of the human Synapsin 1 promoter (62), and one virus additionally contained six copies of the guide (off-target mismatch guide; Fig. 2C) each under control of the human U6 promoter. The other virus served as a control and lacked all guide sequences.

Hippocampal neurons were generated from P0 Mecp2R106Q/Δ mice and transduced with either guide-containing or control AAV vectors carrying the AAV1/2 hybrid capsids at 7 d in vitro.
(DIV 7). After allowing expression of the virus for an additional 7 d, MeCP2 cDNA was prepared from experimental and control cultures and analyzed by Sanger sequencing. We found that 72 ± 5% of the MeCP2 mRNA was repaired in the cultures expressing both Editase and guide (Fig. 3A), while there was no detectable editing in neurons transduced with the control virus that lacked guide. In addition to editing at R106Q, sequence analysis also identified several off-target editing sites within the MeCP2 cDNA (Fig. 3B). The off-target sites occurred primarily within the region complementary to the guide RNA, although one event occurred outside the guide (N126S).

We first tested the functional consequences of the RNA editing by measuring the amount of MeCP2 protein in the AAV1/2 transduced cultures by Western blotting. Similar to other mutations in the MBD (63, 64), MeCP2<sup>R106Q</sup> protein levels are decreased compared with wild-type levels (Fig. 4). The reduced levels of mutant MeCP2 protein are likely due to destabilization (63). Expression of the Editase and guide in the mutant primary neurons increased MeCP2 protein levels by ~threefold compared with expression of Editase alone (Fig. 4; 35.3 ± 2% with guide compared with 12.9 ± 1% without guide, n = 3, P < 0.001).

MeCP2 binds with high affinity to methyl-CpGs, both in vitro and in vivo (27, 65), a property critical to normal function. In mouse cells, mutations in the MBD of MeCP2 reduce binding to heterochromatin that contains amplified satellite sequences rich in mCG (64, 66). MeCP2<sup>R106Q</sup>, an MBD mutation, also shows reduced binding to methyl-CpGs in vitro (67). To determine whether MeCP2<sup>R106Q</sup> has similarly reduced binding in cells and whether editing of G > A mutant MeCP2 RNA restores enrichment in heterochromatin, we immunolabeled nuclei in MeCP2<sup>R106Q</sup> neuronal cultures transduced with AAV1/2 encoding HA-tagged Editase, with or without guide as a control (Fig. 5). We used DAPI (4′, 6-diamidino-2-phenylindole), a fluorescent indicator that binds strongly to A–T-rich regions in DNA, to identify nuclei and heterochromatin. In cultures from wild-type neurons (MeCP2<sup>+</sup>), nuclei showed classical MeCP2 enrichment in the DAPI-stained heterochromatin (foci), reflecting a functional MBD (Fig. 5A). In contrast, in cultures prepared from MeCP2<sup>R106Q</sup>y siblings transduced with Editase virus that lacked guide sequences, MeCP2 immunofluorescence was distributed diffusely throughout the nucleus, as expected for a mutation in the MBD that prevents binding to DNA (63, 66) (Fig. 5B). The intensity of staining was also less than in wild-type nuclei, presumably reflecting the destabilized MeCP2 protein. In contrast, MeCP2<sup>R106Q</sup> neurons expressing both Editase and guide RNA showed a clear increase in MeCP2 immunofluorescence, to a level similar to wild-type nuclei, and enrichment of MeCP2 protein at heterochromatic foci, indicating functional restoration of the MBD (Fig. 5C and D). To quantify the immunofluorescence results, we first determined in three experiments that Editase was expressed in the same percentage of cells irrespective of the presence of guide (Editase alone 67 ± 7%, Editase and guide 67 ± 10%; n = 134 and 137 cells, respectively; Fig. 5E). We then determined that in the cultures transduced with Editase and guide, 74 ± 11% of the cells expressing Editase (Fig. 5F) and 49 ± 8% of the total cells showed MeCP2 enrichment in heterochromatic foci (Fig. 5G). We never detected enrichment of MeCP2 within heterochromatic foci in MeCP2<sup>R106Q</sup> nuclei transduced with virus lacking guide, consistent with our sequencing results showing that editing depended upon the presence of guide.

Discussion

The idea to use ADAR to repair G > A mutations in exogenous mRNAs was first proposed 20 y ago in experiments using Xenopus oocytes (68). Many iterations of this approach have been tested recently, but our study demonstrates that site-directed RNA editing, using an engineered hADAR2 catalytic domain,
can repair an endogenous mutant mRNA and reverse a cellular defect caused by the mutation.

Three genes encode ADAR proteins in mouse and human, but only ADAR1 and ADAR2 exhibit A-to-I catalytic activity (69). Native ADAR-mediated editing is critically important for posttranscriptionally modulating protein function in the brain, first shown for ion channels and receptors (39–41) but now known to extend to many other proteins and noncoding RNAs (reviewed in refs. 70 and 71). We zeroed in on the ADAR2 catalytic domain because of precedence showing its ability to edit heterologous mRNAs (49–52) and because of its well-characterized editing mechanism (61, 72). Indeed, we found increased editing efficiency of Mecp2 mRNA when the Editase contained an E488Q mutation within the catalytic domain shown previously to increase efficiency (51, 61, 73). The elucidation of the structure of the hADAR2 catalytic domain complexed to double-stranded RNA (72) now provides a valuable resource for generating other versions of Editase were modified in pcDNA3.1 by overlapping PCR of wild-type Editase and cloned into pcDNA3.1+.

In future studies, any off-target editing and potential interference with endogenous ADAR activity need to be quantitatively assessed. In transfected cells, the higher editing efficiency with EditaseE488Q at the targeted A also resulted in higher off-target editing at one site within the guide region. We were able to attenuate the single off-target editing site by using a G–A mismatch as published by others (50). In a previous study using transfected cells, sequencing of five cDNAs representing highly expressed mRNAs, other than the target mRNA, did not indicate off-target editing (51). However, and surprisingly, in our study with neurons, off-target editing sites were different between transfected and endogenous Mecp2 mRNA. Specifically, in endogenous repaired Mecp2 mRNA, we noted several additional off-target editing sites within, and one outside, the guide region that were absent from our Mecp2 mRNA expressed from cDNA (Fig. 3B). The difference in off-target editing sites between transfected and endogenous Mecp2 mRNAs likely reflects sequence differences that can affect RNA folding and other downstream processing events. Importantly, none of the off-target sites in the endogenous Mecp2 mRNA are reported to cause RTT (32). However, it is essential to determine the extent of off-target editing by global transcriptomic analysis, and it will be best to perform these studies in mice where functional and physiological correlates may be accomplished at the same time. RTT mouse models are an ideal test system for assessing this important issue in the future because cellular and behavioral symptoms can be reversed by restoration of wild-type MeCP2 in symptomatic mice (21–25).

Materials and Methods

Plasmid Constructions. A pcDNA 3.1+ plasmid Thermo Fisher Scientific coding for the N-terminally tagged ADAR2 catalytic domain (Editase) has been described previously (51, 52). The EditaseE488Q cDNA was generated by overlapping PCR of wild-type Editase and cloned into pcDNA3.1+. Both versions of Editase were modified in pcDNA3.1+ by inserting two copies of
Fig. 3. Sequence analysis of endogenous MeCP2 mRNA following AAV1/2 transduction of primary neurons. (A) Quantification of editing (mean ± SD; n = 3) by sequence analysis of cDNA isolated from MeCP2R106Q hippocampal neurons (DIV14), 7 d following transduction with AAV1/2 virus. +guide refers to AAV1/2 that contains Editase under control of the neuronal Synapsin I promoter (62) and six copies of the guide, each expressed under control of a U6 promoter. The guide contains a C mismatch at the targeted A for R106Q and a G mismatch at the off-target A T105T. The control virus contains Editase under control of the Synapsin I promoter but lacks any guide sequences (−guide). ***P < 0.0001 by unpaired two-tailed t test. (B, Top) MeCP2 mRNA and primary amino acid sequences relative to the guide RNA region (green). The target A is bolded, and asterisks indicate off-target edited A residues. The hairpins in the guide represent the positions of the Box8 sequences recognized by 2N peptide. (B, Bottom) Quantitation of editing at the off-target sites within MeCP2 mRNA (mean ± SD; n = 3). Residue N126S lies outside the guide region.

Fig. 4. Site-directed RNA editing increases MeCP2 protein levels. (Top) Representative Western blot of whole-cell lysates from MeCP2R106Q or wild-type (WT, MeCP2++) sibling hippocampal neurons (DIV14) transduced 7 d earlier with AAV1/2 expressing either Editase alone or Editase and guide. The guide contains a C mismatch at the R106Q site and a G mismatch at the off-target A T105T. (Bottom) Quantification of Western blots (mean ± SD, n = 3), each condition normalized to β-actin. Light-gray bar, cells transduced with Editase alone; dark-gray bar, cells transduced with Editase and guide. ***P < 0.001 by unpaired two-tailed t test.
Fig. 5. Site-directed RNA editing restores the ability of MeCP2 to bind to heterochromatin. Shown are representative confocal images of hippocampal neurons (DIV14) immunolabeled for Editase (HA) and MeCP2. DAPI staining outlines the nuclei and shows heterochromatic foci. Insets demarcate the cells imaged at higher magnification and higher gain in the adjacent panels. (A) Wild-type (Mecp2野生型) neuronal cultures. (B) Mecp2R106Q/y neuronal cultures transduced with AAV1/2 virus expressing Editase alone (no guide). These neurons never exhibited MeCP2 enrichment in heterochromatin. (C and D) Mecp2R106Q/y neuronal cultures transduced with AAV1/2 virus expressing Editase and guide containing the C mismatch at the target A. In D, + and − indicate nuclei with the presence and absence, respectively, of MeCP2 enrichment in heterochromatin. While there appears to be a relationship between Editase/guide expression and MeCP2 heterochromatic enrichment in this image, overall we were not able to confidently support this relationship. (Scale bar, 10 μm.) (E and G) Each histogram represents quantification of cells (Editase alone, n = 134; Editase and guide, n = 137) from three fields in each of three slides (mean ± SD). (E) Percentage of Editase+ cells identified by HA nuclear staining after thresholding signals from uninfected cells. Percentages are relative to the total number of DAPI+ cells. (F) Percentage of Editase+ cells with MeCP2 enrichment in heterochromatin (foci) and (G) percentage of all cells with MeCP2 enrichment in heterochromatin (foci). ns, not significant.
medium change to remove cellular debris. Half medium changes were done every 2–3 d. Cells were maintained at 37 °C in 5% CO2.

RNA Editing. For analysis of N2A cells, cells were seeded at a density of 1.3 × 10^5 cells per well in a 12-well plate. After 24 h, cells were transfected with plasmids containing wild-type or E488Q Edtase (pGM1090 and 1091), one copy of guide (pGM1099, pGM1181 or pGM1108), and Mecp2-edg DNA (pGM1174, pGM1172, or pGM1173) using a 2:1 ratio of Lipofectamine 2000 (Thermo Fisher Scientific) and DNA in Opti-MEM reduced serum media (Thermo Fisher Scientific). The amount of plasmid DNA added per well was 125 ng target, 250 ng Edtase, and 2.5 μg guide. After 72 h, cells were harvested and total RNA was isolated using the Purelink RNA Mini kit (Ambion) according to the manufacturer’s instructions. Residual plasmid DNA was removed using the TURBO DNA-free kit (Ambion). Total RNA was reverse transcribed using the SuperScript III First-Strand Synthesis System (Life Technologies) and primed using oligo dT. The transfected Mecp2-edg DNA were amplified for sequence analysis by PCR using a 5’ primer in the CMV promoter in pEGFP-N3 and a reverse primer in the egfp gene. For editing analysis of primary neurons, at DIV7, 5 × 10^5 hippocampal primary neurons were transfected with AAV1/2 at a multiplicity of infection of 3–6 × 10^6 viral genomes per cell. Viral volume did not exceed 5% of total medium volume. Cells were harvested 1 wk posttransduction and analyzed for editing activity by measuring the A/G peak heights for the transfected cells.

The efficiency of A to I editing was determined by reverse transcription PCR (RT-PCR) and direct sequencing of PCR products. Quantification of the sequencing peak heights from the antisense strand was determined by processing the four-dye-trace sequences using the Bioedit Software package (www.mbio.ncsu.edu/BioEdit/bioedit.html; File → Batch Export of Raw Sequence Trace Data). The amount of editing at each site was then determined using the percentage of the unedited and C (edited) peaks at a given site and calculating the percentage of CDNA edited (100 % × [C height/(T height + C height)]). A detection limit of 5% editing was determined by measuring G–A peak heights in mixtures containing decreasing ratios of 10:90 mutant to wild-type Mecp2 plasmids. The G/C peak heights of the antisense strand was quantified because it is more accurate than using the A/G peak heights of the sense strand (58), however for clarity all chromatograms are shown in the reverse complement.

Western Blotting. Primary hippocampal neurons, transfected with AAV1/2, were lysed in 100 μL of whole-cell lysis buffer (25 mM Tris, pH 7.6, 150 mM NaCl, 1% Igepal CA-630; Sigma), 1% deoxycholate, 0.1% SDS, protease inhibitor (Complete EDTA-free; Roche), 1 mM beta-mercaptoethanol, and 1% Igepal CA-630; Sigma), 1% deoxycholate, 0.1% SDS, protease inhibitor (Complete EDTA-free; Roche), 1 mM beta-mercaptoethanol, and 1% Igepal CA-630; Sigma). Lysates were centrifuged at 9,300 × g for 10 min at 4 °C and the soluble fraction isolated. Protein concentrations were measured using the BCA protein assay kit (Pierce Biotechnology). Equal amounts of protein lysates were separated on NuPage 4–12% Bis-Tris gels (Thermo Fisher Scientific) in Mops-SDS running buffer (Thermo Fisher Scientific), and proteins were blotted onto nitrocellulose membrane (GE Healthcare Life Sciences). Membranes were blocked with 3% BSA in 1× TBST-BS for 0.5% Tween-20 for 1 h, then incubated with either rabbit anti-Mecp2 (Covance) or rabbit anti-j–β-actin (Barnes & Noble) overnight at 4 °C. After washing three times with 1× TBST, blots were incubated with anti-rabbit IgG DyLight 680 (1:10,000 dilution; Thermo Scientific) for 1 h. Blots were quantified using the Odyssey Imaging System (LI-COR Biosciences).

Immunostaining. Hippocampal primary neurons were fixed in 4% para- formaldehyde in PBS for 20 min at room temperature.Fixed cells were washed twice with 1× PBSTG (0.1 M glycine in 1× PBS) at room temperature for 10 min. Then, cells were blocked and permeabilized (0.5% Igepal CA-630, Sigma; 3% BSA (source) in 1× PBST) for 1 h at 4 °C and incubated with primary antibodies raised against MeCP2 (rabbit mAb D4F3; Cell Signaling) and HA (rat mAb 3F10; Roche) in a humidified chamber overnight at 4 °C. Cells were washed three times in 1× PBS containing 0.5% Igepal and incubated with secondary antibodies Alexa 488 and Alexa 568 (Thermo Fisher Scientific) for 1 h. After another wash with 1× PBS containing 0.5% Igepal, cells were incubated with 300 nM DAPI for 5 min, then washed again with 1× PBS. The cells were mounted using ProLong Gold antifade reagent (Thermo Fisher Scientific) overnight. All images were acquired as z-stacks of 0.5-μm optical sections on a Zeiss 710 confocal microscope using a 40x water immersion objective. HA and MeCP2 fluorescent images were taken using the same settings across all samples. Total cell number or numbers of antibody-positive cells were determined by ImageJ cell counter plugin [National Institutes of Health, https://imagej.nih.gov/ij/, version 1.60 (22 bit)].

Statistical Analysis. All statistics were performed using GraphPad version 6.0 software (Prism). The percentage of A to I editing in N2A cells was analyzed using one-way ANOVA followed by Bonferroni post hoc tests. The level of A to I editing in Mecp2170609 transduced neurons, Western blots comparing MeCP2 protein levels, and the number of neurons showing MeCP2 enrichment at heterochromatic foci were each analyzed using unpaired t tests. All experimental results are expressed as mean ± SD.

ACKNOWLEDGMENTS. We thank Drs. Maria Montiel-Gonzalez and Joshua Rosenthal (Marine Biological Laboratories) for kindly providing the initial Editase construct and for useful discussions. We also thank Dr. Paul Brehm (Vollum Institute, OHSU) and members of the G.M. laboratory for advice during the course of the work and Ms. Christine Schmidt (Vollum Institute, OHSU) for excellent technical support. We acknowledge the laboratories of Drs. Michael E. Greenberg (Harvard Medical School) and Adrian Bird (Wellcome Trust Centre for Cell Biology, University of Edinburgh) for advice and encouragement through the Rett Syndrome Research Trust (RSRT) Consortium. This work was supported by NIH Grants 5NS087726 (to G.M.) and 5NS088399 (to H.N.) and the Rett Syndrome Research Trust (G.M.).


