MeCP2²⁷⁰ Mutant Protein Is Expressed in Astrocytes as well as in Neurons and Localizes in the Nucleus

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Key Words

MeCP2 • R270X mutation • Rett syndrome • Transgenic mice

Abstract

The *MECP2* gene, located at Xq28, encodes methyl-CpG-binding protein 2 (MeCP2), which is frequently mutated (up to 90%) in Rett syndrome (RTT). RTT is a progressive neurodevelopmental disorder, which affects primarily girls during early childhood and it is one of the most common causes of mental retardation in females. R270X is one of the most frequent recurrent *MECP2* mutations among RTT cohorts. The R270X mutation resides within the TRD-NLS (Transcription Repression Domain-Nuclear Localization Signal) region of MeCP2 and causes a more severe clinical phenotype with increased mortality as compared to other mutations. To evaluate the functional role of the R270X mutation, we generated a transgenic mouse model expressing MeCP2^{270-EGFP} (human mutation equivalent) by BAC recombineering. The expression pattern of MeCP2^{270-EGFP} was similar to that of endogenous MeCP2. Strikingly, MeCP2^{270-EGFP} localizes in the nucleus, contrary to the conjecture that R270X could cause disruption of the NLS. In primary hippocampal cells,

we show that MeCP2^{270-EGFP} was expressed in astrocytes by colocalization with the astrocyte-specific marker glial fibrillary acidic protein. Our data showing expression of MeCP2^{270-EGFP} in transgenic mice astrocytes further reinforce the recent findings concerning the expression of MeCP2 in the glial cells.

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Rett syndrome (RTT) is a progressive neurodevelopmental disorder, which affects primarily girls during early childhood and it is one of the most common causes of mental retardation in females. RTT was first identified and described in 1966 by Andreas Rett in the Wiener Klinische Wochenschrift [German literature; Rett, 1966]. However, RTT was relatively unknown until the mid 1980s when Bengt Hagberg and his colleagues reported 35 cases of RTT in an English language journal [Hagberg et al., 1983]. The characteristic features of the disease include postnatal microcephaly, hand dyspraxia, stereotypic hand movements, ataxia, abnormal breathing, and growth retardation. In addition, tendency to develop social withdrawal and loss of the ability to speak were also

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observed; furthermore, many girls develop seizures and anxiety [Hagberg et al., 1983; Trevathan and Moser, 1988].

The gene *MECP2* (OMIM: *300005) encodes methyl-CpG-binding protein 2 (MeCP2), a nuclear protein that recognizes methylated CpG dinucleotides and binds symmetrically to target DNA sequence [Meehan et al., 1992; Nan et al., 1993]. Mutations in *MECP2* are the main cause of RTT [Amir et al., 1999]. *MECP2* located at Xq28 is comprised of 4 exons that code for 2 different isoforms of MeCP2 protein (e1 and e2) through alternative splicing of exon 2. The 2 isoforms differ only in their N-terminal sequence; the MeCP2-e1 isoform contains an additional 21 amino acids encoded by exon 1 but lacks 9 amino acids encoded by exon 2. In the MeCP2-e2 isoform, translation initiates from exon 2 [D'Esposito et al., 1996; Kriaucionis and Bird, 2004; Dragich et al., 2007]. The 3' untranslated region of *MECP2* contains multiple polyadenylation sites, which can further produce several transcript variants [Chahrour and Zoghbi, 2007].

The MeCP2 protein contains 2 conserved functional domains, a methyl-CpG-binding domain (MBD) and a transcriptional repression domain (TRD) with a 17-amino-acid nuclear localization signal (NLS) embedded within the TRD. The C-terminal residues of MeCP2 also comprise a domain named CTD [Adams et al., 2007]. Initial studies implicated MeCP2 as a transcriptional repressor, which silences target genes by recruiting co-repressors and histone deacetylases [Razin, 1998; Ng and Bird, 1999]. Recent advancements in the molecular function of MeCP2 suggest that it is a multifaceted nuclear protein. In terms of target gene regulation, MeCP2 acts both as a transcriptional activator as well as repressor [Chahrour et al., 2008; Ben-Shachar et al., 2009]. It also plays a prominent role in the modulation of global chromatin architecture by directly binding to chromatin fibers and facilitating its compaction by folding the chromatin [Georgel et al., 2003]. Moreover, MeCP2 also functions as a gene-specific splice regulator [Young et al., 2005].

To understand the underlying molecular mechanisms leading to symptoms of the RTT disease, several animal models have been generated. MeCP2-deficient mice recapitulate the pathological hallmarks of RTT symptoms. *Mecp2* null mice are phenotypically normal until 3–6 weeks of age; thereafter male mutant mice show stiffness, gait abnormalities, hypoactivity, tremor, hindlimb clasp-ing and irregular breathing. The symptoms are progressive in nature leading to severe weight loss and eventually death [Chen et al., 2001; Guy et al., 2001]. The female mice manifest a milder and delayed age of onset of the symptoms.

It is interesting to note that recent meta-analyses of *MECP2* mutations from several published and unpublished cases revealed that 8 recurrent missense and non-sense mutations (R106W, R133C, T158M, R168X, R255X, R270X, R294X, and R306C) accounted for up to 2 thirds of the pathogenic mutations identified in RTT [Dragich et al., 2000; Bebbington et al., 2008]. Among *MECP2* mutations, R270X (c.808 C > T) is one of the most frequent recurrent *MECP2* mutations among various cohorts irrespective of ethnic background [Dragich et al., 2000; Bebbington et al., 2008]. Further studies revealed that mutations in the NLS region within the TRD domain manifest a significantly more severe phenotype as compared to mutations located in other regions of the protein, particularly the region after the TRD-NLS including the C-terminal region [Huppke et al., 2002; Colvin et al., 2004]. R270X, located in the NLS of the TRD, manifests the most severe phenotype among the mutations, in contrast to R294X, which was also found in the TRD domain but after the NLS and had a much milder phenotype [Colvin et al., 2004]. In the epidemiological context, patients harboring R270X are prognosed to lose skills such as motor function, hand use and social interaction much earlier than their counterparts with the R294X. Patients with the latter type of mutation, on the other hand, are likely to be mobile and less likely to have growth or nutritional problems [Colvin et al., 2004].

The molecular mechanism leading to a severe phenotype by R270X mutation is still elusive. To dissect the effect of R270X, we generated a mouse model expressing the R270X mutant fused with green fluorescent protein (MeCP2^{270-EGFP}) under the control of the endogenous promoter.

Material and Methods

Generation of *Mecp2*^{270-EGFP} Transgenic Mouse Line

To generate the *Mecp2*^{270-EGFP} transgenic mouse line, a BAC (Bacterial Artificial Chromosome) clone B22804 (kindly gifted by Dr. M. Platzer, Jena, Germany) consisting of the murine *Mecp2* gene and its flanking regulatory regions was used to generate a transgenic construct by inserting an enhanced green fluorescent protein/kanamycin-resistant gene (EGFP/Kan) cassette into exon 4 of *Mecp2* by BAC recombineering (recombination-mediated genetic engineering) via the pGET recombination system [Orford et al., 2000]. The EGFP/Kan cassette was amplified by a primer pair consisting of 50-bp sequence flanking *Mecp2* exon 4 on either side of amino acid 270. Furthermore, 2 glycine residues were inserted between the truncated MeCP2 protein and the EGFP protein in-frame to facilitate the 2 proteins to fold and function independently. The correct insertion of EGFP/Kan cassette after the recombination event into the BAC DNA was confirmed by sequenc-

ing. The BAC clone B22804 also contained transcriptional units of *Opsin1* and part of *Irak1* genes upstream and downstream of the *Mecp2* gene. The sequences of both genes were deleted from the BAC clone by additional GET recombination steps using the zeocin selection cassette (containing the homology arms and Zeocin antibiotic marker gene driven by EM7 promoter from pSELECT vector) (InvivoGen).

The final BAC construct pBAC_Mecp2²⁷⁰_EGFP was linearized with *Mlu*I enzyme and micro-injected into the male pronuclei of the fertilized mouse oocytes derived from the FVB/N strain. Next, the injected oocytes were transplanted into the uteri of the foster mothers. The newborn pups were screened for the presence of the transgene by PCR analysis on genomic DNA isolated from tail biopsies. The transgenic product of 632 bp was amplified by primer pair Ctrl Mecp2_F1 (5'-TCCAGTTTGTTCCTTGTTCACTTA-3') and EGFP_Rev1 (5'-CCGTTTACGTCGCCGTC-CAGC-3') under the following conditions: 95°C for 30 s (denaturation), 55°C for 40 s (annealing), 72°C for 55 s (extension) for 35 cycles. To amplify the endogenous *Mecp2* allele (398 bp), we used the primer pair oIMR1436 (5'-GGTAAAGACCCATGTGACCC-3') and oIMR1438 (5'-GGCTTGCCACATGACAA-3').

Western Blot Analysis

Proteins were extracted from fresh or frozen mouse tissues by homogenization in the tissue lysis buffer (62 mM Tris/HCl pH 6.8, 2% SDS, 5 mM β -2-mercaptoethanol, 0.15 mM NaCl) containing protease inhibitors (Sigma). Lysates were sonicated on ice and centrifuged at 12,000 g for 10 min at 4°C. Supernatant consisting of total protein extract was used for further analysis. In brief, 50 μ g protein lysates were denatured by boiling in a buffer containing sodium dodecyl sulfate and dithiothreitol, which was resolved by SDS-PAGE using a NuPage 4–12% Bis-Tris precast gel in MES buffer (Invitrogen). The resolved proteins were electro-transferred to a polyvinylidene difluoride membrane (Macherey-Nagel). The membrane was blocked in 5% dried milk in PBS and then incubated with either anti-MeCP2 antibody (dilution 1:5,000) raised in rabbit using MeCP2-strep tag fusion protein as antigen (Eurogentec) or anti-GFP antibody (dilution 1:4,000) at 4°C overnight. Next, the immunoblot was incubated with secondary antibodies conjugated with alkaline phosphatase (dilution 1:30,000) (Sigma) for 1 h at room temperature (RT). The resolved proteins were visualized using nitroblue tetrazolium, 5-bromo-4-chloro-3-indolyl phosphate.

Immunohistochemistry

The mice were transcardially perfused with 4% paraformaldehyde in PBS. The whole brain was dissected and immersed in 30% sucrose solution for 3 days and then cryosectioned to 7–10 μ m thickness. The brain sections were washed 3 times with PBS for 5 min each and then permeabilized with 0.2% Triton-X for 30 min at 4°C. Then slides were again washed 3 times with PBS for 5 min and incubated in 5% BSA for 1 h at RT. The brain sections were then incubated with anti-MeCP2 antibody (1:200; Eurogentec), anti- β 3-tubulin (1:400, Abcam), anti-GFAP (1:500, kind gift from Dr. T. Manzke, Goettingen, Germany) or anti-GFP (1:200, Abcam) at RT for 2 h in 2% BSA solution, which was followed by incubation with secondary antibody conjugated with Cy3 or FITC (Sigma) for 1 h at RT in 2% BSA solution. After final washing, the slides were mounted with coverslips and were observed using a BX60 fluorescence microscope (Olympus).

Primary Cell Culture and Immunocytochemical Analysis

Mice were decapitated and the whole brain was isolated and put into dissection solution (0.72 g bovine albumin, 0.347 g MgSO₄ in 240 ml Hanks medium) on ice. Hippocampi were carefully dissected and cut into small pieces in dissection solution. The tissue samples were then digested at RT for 5 min with 6 mg trypsin and 60 μ g DNAase (2 K units/ μ l) in 2 ml of digestion solution (0.8 g NaCl, 35 mg NaHCO₃, 37 mg KCl, 99 mg Na₂HPO₄ in 100 ml H₂O, pH adjusted to 7.3–7.4). The reaction was stopped by addition of trypsin inhibitor (Invitrogen). The tissues were then incubated at RT in a solution of 0.5 ml horse serum and 2.5 ml dissection solution for 10 min, and afterwards washed 3 times with dissection solution. The hippocampal cells were triturated with 3 different pore sizes of pipettes coated with Sigmacote (Sigma). The homogenate was centrifuged at 1,400 g at 4°C for 5 min. The cells were resuspended in 1 ml dissection solution. The cell suspensions were seeded (2,000–3,000 cells per well) in pre-incubated culture plates with coverslips coated with poly-D-lysine and laminin in dissection medium and incubated at 37°C for 30 min in 5% CO₂, thereafter, culture medium A (0.5 g D-glucose, 0.2 ml (12.5 mg/ml) insulin, 1 ml (200 mM) glutamax, 50 μ l (10 mg/ml), gentamicin, 10 mg transferrin and 10 ml horse serum up to 100 ml DMEM) was added to the cultured neurons. After overnight incubation with culture medium A, the cells were washed with PBS to remove cell debris. After 4 days, medium A was replaced with culture medium B (50 μ l gentamicin, 125 μ l bFGF, 250 μ l L-glutamine, 2 ml B27 supplement, 125 μ l cytosine arabinoside (4 mM) up to 100 ml neurobasal medium) and cultured until day 7. Medium was changed every 2 days until the day of neuronal cell analysis, when cells were fixed and immunostained with anti- β 3-tubulin (dilution 1:500) (Abcam). The evaluation of neuronal parameters such as cell soma volume and neurite length was performed by Image J software (<http://rsb.info.nih.gov/ij/>).

Results

Establishment of the Mecp2²⁷⁰_EGFP Transgenic Mouse Line

The founder mice obtained by microinjection of linearized pBAC_Mecp2²⁷⁰_EGFP into the fertilized mouse oocytes derived from the FVB/N strain were tested for the integration of the transgene into the genomic sequence by PCR genotyping using a primer pair specific for the transgene (fig. 1B). As can be seen from the PCR genotyping, the transgenic mice in addition to endogenous *Mecp2* allele also carry the *Mecp2*²⁷⁰_EGFP transgene (fig. 1B). The mice which were positive for the transgene were bred with FVB/N mice to set up a stable *Mecp2*²⁷⁰_EGFP transgenic mouse line. To determine whether the *Mecp2*²⁷⁰_EGFP is expressed in the transgenic mice, we performed Western blot analysis with total protein extracted from brain biopsies from the transgenic mice. The immunoblot with antibody against MeCP2 revealed expression of 2 MeCP2 proteins, in addition

Fig. 1. Schematic diagram illustrating the generation of the *Mecp2*^{270_EGFP} transgenic construct using GET recombination in the BAC clone. An EGFP/Kan cassette was inserted in-frame at amino acid 270 of *Mecp2* in exon 4 and an arginine was replaced with 2 glycine residues in the BAC clone by homologous recombination (A). PCR genotyping of mice. The transgenic allele was amplified by the primer pair Ctrl *Mecp2*_F1 and EGFP_Rev1, yielding a PCR product of 632 bp. The endogenous *Mecp2* allele of 398 bp was amplified using primer pair oIMR1436 and oIMR1438. T²⁷⁰/-, transgenic; -/-, wild-type; N, negative control (H₂O) (B). Western blot analysis using MeCP2 antibody on the total protein extract isolated from brain samples revealed in addition to the endogenous MeCP2 (70 kDa) an additional MeCP2 protein corresponding to MeCP2^{270_EGFP} (65 kDa) in the transgenic mice. Further immunoblots with anti-GFP antibody confirmed that the 65-kDa size protein was indeed MeCP2^{270_EGFP} (C).

to the endogenous MeCP2 (70 kDa), a transgenic MeCP2^{270_EGFP} protein of 65 kDa was detected in the total protein extract isolated from the brain of transgenic mice (fig. 1C). In contrast, in the total protein extract isolated from the brain of wild-type mice, only endogenous MeCP2 was detectable (fig. 1C). Furthermore, immunoblot analysis with GFP antibody detected the same 65-kDa transgenic MeCP2^{270_EGFP} protein, which was identified by MeCP2 antibody (fig. 1C).

Transgenic MeCP2^{270_EGFP} Protein Shows a Nuclear Expression Pattern

The transgenic pBAC_*Mecp2*^{270_EGFP} construct consists of a mouse genomic fragment of ~150 kb, which contains all 4 exons of *Mecp2*, including the entire regulatory regions necessary for temporal and spatial expression of MeCP2. To determine the expression pattern of MeCP2^{270_EGFP} protein, we performed immunostaining on brain sections derived from transgenic mice (T²⁷⁰/-) (note: T²⁷⁰ refers to the transgenic allele and - corresponds to the wild-type allele) with GFP antibody. The expression of MeCP2^{270_EGFP} was widespread throughout different regions of the brain with predominant expression in the hippocampus, cerebellum and olfactory bulb (data not shown). Next, the hippocampal sections from the transgenic mice were stained with MeCP2 and GFP

antibodies to evaluate the expression of MeCP2 from the transgene as compared to the endogenous protein. The MeCP2^{270_EGFP} protein revealed a similar expression pattern as shown by the endogenous MeCP2 (fig. 2B–D; see online supplementary figure S1, www.karger.com/doi/10.1159/000315906). In the primary structure of MeCP2, the NLS spans 267–283 amino acids, therefore in the transgenic MeCP2^{270_EGFP} protein the NLS is likely to be abolished. To determine the intracellular localization of MeCP2^{270_EGFP} protein, we analyzed the hippocampal sections at a higher magnification (60×), immunostaining with GFP antibody, which revealed MeCP2^{270_EGFP} protein localized in the nucleus (fig. 2E) as confirmed by counterstaining the cells with DAPI (fig. 2F). Furthermore, staining the cells with MeCP2 antibody showed MeCP2^{270_EGFP} protein co-localized with the endogenous MeCP2 (fig. 2G) predominantly at the heterochromatin foci of the nuclei (fig. 2E–G).

Phenotypic Analysis of Mecp2^{270_EGFP} Transgenic Mice

Quantification of expression of MeCP2^{270_EGFP} in the transgenic mice revealed that the level of transgenic protein was comparable to that of endogenous MeCP2 suggesting that a single copy of the transgene was integrated into the genome of the transgenic mice (data not shown).

Fig. 2. Schematic diagram showing structural domains of the MeCP2 protein. N, NH₂-terminal; C, COOH-terminal; MBD, methyl binding domain; TRD, transcription repression domain; CTB, proline-rich C-terminal domain; NLS, nuclear localization signal (A). Expression analysis of MeCP2 in the hippocampus showed a similar expression pattern for the transgenic MeCP2²⁷⁰_{EGFP} protein as compared to endogenous MeCP2 (B–D). Analysis of the cryosection at the higher magnification revealed that both transgenic MeCP2²⁷⁰_{EGFP} as well as endogenous MeCP2 proteins localize in the nucleus (E–G). Scale bar, 50 μ m (B–D), 10 μ m (E–G).

To maintain the transgenic mouse line, the heterozygous T²⁷⁰/– mice were backcrossed with FVB/N mice. From such breeding, we obtained 50% T²⁷⁰/– mice and 50% wild-type littermates, thus excluding the possibility of prenatal lethality. Furthermore, we obtained equal numbers of male and female mice, which suggests that integration of the transgene was in an autosomal chromosome. Postnatally, the transgenic mice appeared normal with no obvious abnormalities. To assess neurological abnormalities, hind-limb clasp tests were performed on 5 adult (6-month-old) mice; however, the transgenic mice did not show clasping of their hind-limb when suspended by their tails (fig. 3A).

To determine any defect in the process of neuronal development and differentiation, hippocampal neurons from the transgenic mice (P7 stage) were cultured for 7 days and stained with neuronal marker β 3-tubulin (fig. 3B). For evaluation of neuronal parameters, the stained primary neurons were analyzed by Image J software. There was no significant change in neurite length as well as in cell soma size in the *Mecp2*²⁷⁰_{EGFP} transgenic neurons as compared to the wild-type neurons (fig. 3C, D).

Expression of MeCP2²⁷⁰_{EGFP} in Astrocytes

In the primary hippocampus culture of transgenic mice, when cells were stained with neuronal marker β 3-tubulin, we observed that several cells expressing MeCP2²⁷⁰_{EGFP} were negative for expression of β 3-tubulin (fig. 4A). When these hippocampal cells were stained with astrocyte marker GFAP, co-expression of MeCP2²⁷⁰_{EGFP} with GFAP was observed in the astrocytes (fig. 4B).

Discussion

Mutational spectrum analysis of MeCP2 in RTT shows that mutations are distributed throughout the structural architecture of the protein. However, certain point mutations occur recurrently and account for up to 2 thirds of the pathogenic mutations identified in RTT [Dragich et al., 2000]. R270X is one of the most frequent recurrent *MECP2* mutations amongst different cohorts irrespective of ethnic background and accounts for 6.4% of all reported mutations [Dragich et al., 2000; Laccone et al.,

Fig. 3. Image showing both *Mecp2*^{270-EGFP} transgenic as well as wild-type FVB/N mice were negative for hind-limb clasp test. Both mice, when they were held by the tail, showed normal spreading of hind-limbs. T^{270/-}, transgenic; -/-, wild-type (A). Immunostaining of the transgenic *Mecp2*^{270-EGFP} primary neuronal cells with the neuronal marker β3-tubulin. The stained neuron images were analyzed by Image J software. Scale bar, 50 μm (B). Image J analysis of the neuronal parameters; neurite length and cell soma size. Measurement of neurite lengths of the transgenic and wild-type neurons after 7 days of ex vivo culture showed no significant difference (C). Evaluation of cell soma size of the transgenic and wild-type neurons also failed to detect any difference, which was statistically significant. WT, wild type; T-270, *Mecp2*^{270-EGFP}, transgenic mice (D).

Fig. 4. Immunocytochemical analysis of *Mecp2*^{270-EGFP} hippocampal cell culture. Staining of cells with neuronal marker β3-tubulin revealed several *Mecp2*^{270-EGFP}-expressing cells were negative for the β3-tubulin (A). Immunostaining with astrocyte marker GFAP confirmed expression of *Mecp2*^{270-EGFP} in astrocytes (B). Scale bar, 50 μm (A), 10 μm (B).

2001; Colvin et al., 2004; Jian et al., 2005]. The R270X mutation resides within the TRD-NLS region of MeCP2. Individuals with mutations in the TRD-NLS region manifest a more severe clinical phenotype than those with mutations in other domains [Huppke et al., 2002; Colvin et al., 2004; Jian et al., 2005]. Moreover, it is suggested that the R270X mutation is associated with increased mortality [Jian et al., 2005]. Mutations in the TRD region appeared to have a less severe influence on the phenotype [Huppke et al., 2002; Colvin et al., 2004]. A representative mutation in this group is R294X [Colvin et al., 2004]. Mutations involving the TRD-NLS region might result in failure of the truncated MeCP2 protein (assuming a stable protein) to be imported into the nucleus of the cell, thus resulting in a dramatic reduction in the MeCP2-specific transcription activity, which could lead to a severe clinical phenotype [Colvin et al., 2004]. On the other hand, proteins with truncating mutations occurring distal to the NLS in the TRD could still be translocated into the nucleus, where the mutation could exert a partial effect on transcription activity thus rendering a milder effect [Colvin et al., 2004]. However, to our knowledge, no functional studies of mutations involving the TRD-NLS have been performed so far.

To evaluate the functional role of the R270X mutation, we generated a transgenic mouse model expressing MeCP2^{270-EGFP}, which is equivalent to the human mutation. The BAC construct, which was used for generation of the transgenic mice, consist of an ~150-kb genomic fragment with ~50 kb upstream and downstream of the *Mecp2* locus, which is supposed to contain along with the promoter also further regulatory elements necessary for the spatio-temporal expression pattern of MeCP2 [Collins et al., 2004]. The BAC clone also contained a transcriptional unit of *Opsin1* and *Irak1* genes upstream and downstream of the *Mecp2* gene, which we deleted, thus ensuring expression of the *Mecp2* locus only from the BAC construct. When expression of MeCP2^{270-EGFP} from the transgene was compared with endogenous MeCP2 in the wild-type as well as transgenic mice, we observed a similar expression pattern thus confirming that the BAC transgene mimics the endogenous MeCP2 expression. Previously, Collins and colleagues reported that expression of a transgenic MeCP2 from a human PAC containing a 90-kb fragment of *MECP2* was similar to that of endogenous MeCP2 [Collins et al., 2004], which is consistent with our finding.

Strikingly, MeCP2^{270-EGFP} localizes in the nucleus, although it was speculated that R270X could cause disruption of the NLS within the TRD-NLS region [Colvin

et al., 2004]. In silico analysis using the PSORT II Prediction program (<http://psort.ims.u-tokyo.ac.jp/form2.html>) [Horton et al., 1997] revealed several bipartite NLS preceding the R270 amino acid. It is likely that the NLS at region R270 is not critical for translocation of MeCP2 into the nucleus and other NLS in the N-terminal region are sufficient for the nuclear localization of MeCP2^{270_EGFP} protein.

The nuclear localization of transgenic MeCP2^{270_EGFP}, particularly at the heterochromatic foci, suggests that the methyl-cytosine-specific binding property of MeCP2^{270_EGFP} is still intact. It is likely that the human MeCP2^{R270X} also localizes in the nucleus. Therefore, manifestation of clinically severe phenotype by patients harboring the R270X mutation could be due to either a gain of function or a loss of functional domain. Although in principle both proteins can localize in the nucleus, interestingly, there is a striking difference in the phenotype between MeCP2^{R270X} and MeCP2^{R294X} [Colvin et al., 2004], which suggests perhaps an important functional domain/motif of MeCP2 located within the 270–294 residues region is disrupted in MeCP2^{R270X}.

The transgenic mice expressing MeCP2^{270_EGFP} were phenotypically normal. There were no symptoms of neurological abnormalities as these mice were negative for hind-limb clasping, which is indicative of neurological impairment [Dietrich et al., 2005]. Evaluation of neuronal parameters, such as neurite length and cell soma size of the MeCP2^{270_EGFP} neurons, revealed no significant difference as compared to wild-type neurons. Overall, our data from transgenic mice do not support a gain of function or a dominant negative effect of the MeCP2^{270_EGFP} on the endogenous protein. Further investigation of the transgenic mice including neuro-physiological tests and behavioral analyses to assess cognitive learning and motor performance of mice will assist us in dissecting the role of the MeCP2^{270_EGFP} protein. This issue is of particular relevance for the development of therapeutic strategies for the Rett syndrome aimed at reintroducing a wild-type MeCP2 protein in the diseased cells.

Previous studies reported that MeCP2 was highly expressed in mature neurons but was absent in the glial cells [Shahbazian et al., 2002; Kishi and Macklis, 2004]. In the primary hippocampal cell culture derived from *Mecp2*^{270_EGFP} transgenic mice, staining with neuronal marker β 3-tubulin revealed that several cells expressing MeCP2^{270_EGFP} were negative for β 3-tubulin. Furthermore, staining hippocampal cells with astrocyte marker GFAP showed MeCP2^{270_EGFP} was expressed in astrocytes. Recently, 2 studies reported that MeCP2 expres-

sion in the brain was not limited only to the neurons, but also was expressed in all types of glia, including astrocytes, oligodendrocyte progenitor cells and oligodendrocytes [Ballas et al., 2009; Maezawa et al., 2009]. Our data showing expression of MeCP2^{270_EGFP} in astrocytes of the transgenic mice further reinforces these recent findings concerning expression of MeCP2 in the glial cells.

These recent studies showed that loss of MeCP2 in the astrocytes causes abnormalities in neighboring neurons probably by a non-cell-autonomous mode of action [Ballas et al., 2009; Maezawa et al., 2009]. MeCP2-deficient astrocytes interfere with proper development and maturation of neurons; both wild-type and *Mecp2* null neurons develop fewer and shorter dendrites and only a few neurons can survive beyond 6 days when co-cultured with MeCP2-deficient astrocytes [Ballas et al., 2009]. However, MeCP2 target proteins in the astrocytes still remain unknown. An appropriate mouse model is needed to evaluate the role of MeCP2 in the astrocyte dysfunction contributing to RTT pathogenesis. Towards this end, our *Mecp2*^{270_EGFP} transgenic mice can serve as a valuable model-system.

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