

ARTICLE

Transgenic complementation of MeCP2 deficiency: phenotypic rescue of *Mecp2*-null mice by isoform-specific transgenes

Bredford Kerr¹, Jessica Soto C^{1,2}, Mauricio Saez^{1,2}, Alexander Abrams³, Katherina Walz^{1,3} and Juan I Young^{*,1,3}

Rett syndrome (RTT) is a disorder that affects patients' ability to communicate, move and behave. RTT patients are characterized by impaired language, stereotypic behaviors, frequent seizures, ataxia and sleep disturbances, with the onset of symptoms occurring after a period of seemingly normal development. RTT is caused by mutations in methyl-CpG binding protein 2 (*MECP2*), an X-chromosome gene encoding for MeCP2, a protein that regulates gene expression. *MECP2* generates two alternative splice variants encoding two protein isoforms that differ only in the N-terminus. Although no functional differences have been identified for these splice variants, it has been suggested that the RTT phenotype may occur in the presence of a functional MeCP2-e2 protein. This suggests that the two isoforms might be functionally distinct. Supporting this notion, the two variants show regional and age-related differences in transcript abundance. Here, we show that transgenic expression of either the MeCP2-e1 or MeCP2-e2 splice variant results in prevention of development of RTT-like phenotypic manifestations in a mouse model lacking *Mecp2*. Our results indicate that the two MeCP2 splice variants can substitute for each other and fulfill the basic functions of MeCP2 in the mouse brain.

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INTRODUCTION

Rett syndrome (RTT, MIM 312750) is a progressive neurodevelopmental disorder that affects predominantly females. It is one of the leading causes of intellectual disability and autistic features in females.¹ Clinical features of RTT include psychomotor regression, intellectual disability, communication dysfunction, seizures, postural hypotonia, stereotypic hand movements, tremors, autonomic dysfunction and growth failure.²

The vast majority of RTT cases are caused by mutations in the X-linked gene *MECP2*,³ encoding for methyl-CpG binding protein 2 (MeCP2). MeCP2 is a nuclear protein that binds to methylated CpGs and regulates gene expression.^{4,5} In addition, mutations in *CDKL5* and *FOXG1* have been also associated with RTT.^{1,6}

In mammals, *MECP2* generates two alternative splice variants, encoding protein isoforms that differ only in the N-terminus.^{7,8} The MeCP2-e1 mRNA splice variant, in which exon 2 is spliced out, produces a 496-amino acid polypeptide with an acidic N-terminus translated from an ATG initiation codon in exon 1. The second variant, MeCP2-e2, encodes a slightly shorter protein (486-aminoacids) translated from an ATG in exon 2.

Splicing variants often encode functionally diverse protein isoforms.⁹ Evidence that this could also be the case for MeCP2 splice variants comes from the findings that MeCP2 variants show regional and age-related differences in transcript abundance in the mouse brain – *MECP2*-e1 is the predominant form in most adult brain structures¹⁰ – and that *Mecp2-e1* and *Mecp2-e2* transcripts

appear to show different preferences for alternative polyadenylation sites within the long 3'-UTR.¹⁰ In addition, research into the relationship between genotype and phenotype in RTT provides further support to the notion that MeCP2-e1 and MeCP2-e2 could be functionally distinct; several mutations in *MECP2* exon 1 have been identified in classic RTT patients,^{8,11–17} including point mutations that allegedly do not affect transcription or translation of MeCP2-e2,^{16,17} suggesting that endogenously expressed MeCP2-e2 is unable to compensate for the lack of MeCP2-e1.

On the other hand, it was reported that the sole expression of MeCP2-e2 was able to rescue the phenotype of *Mecp2*^{-/-} mice, leading to the conclusion that expression of MeCP2-e2 – in the absence of MeCP2-e1 – is sufficient for attaining normal neuronal function.^{18–20}

These seemingly contradictory results could stem from mouse–human differences in MeCP2 requirements, but could also be reconciled by the hypothesis that MeCP2-e1 and MeCP2-e2 could perform similar functions in brain cells, if they were expressed at comparative levels. Thus, MeCP2-e2 cannot compensate for the lack of MeCP2-e1 in the reported patients carrying mutations that specifically affect MeCP2-e1, because there is not enough MeCP2-e2 in these cells. However, in mice engineered to express MeCP2-e2 from a heterologous promoter, the expression of MeCP2-e2 could suffice to compensate for the lack of MeCP2-e1. Supporting this hypothesis, both MeCP2 isoforms show similar intranuclear localization and chromatin binding kinetics in transfected cells.²¹

¹Department of Biology, Centro de Estudios Científicos, Valdivia, Chile; ²Department of Biochemistry, Universidad Austral de Chile, Valdivia, Chile; ³Department of Human Genetics, John P Hussman Institute for Human Genomics, Miller School of Medicine, University of Miami, Miami, FL, USA

*Correspondence: Dr JI Young, Department of Human genetics, John P Hussman Institute for Human Genomics, Miller School of Medicine, University of Miami, 1501 NW 10th Avenue, BRB-514 (M-860), Miami, FL 33136, USA. Tel: +1 305 243 1027; Fax: +1 305 243 2703; E-mail: Jyoung3@med.miami.edu

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Mutations that affect specifically the expression of MeCP2-e2 have not been described, precluding a definitive answer to the functional significance of the two differing MeCP2 splice variants.

We reasoned that if the two MeCP2 splice variants subserved different functions, then the extent of phenotypic rescue displayed by mice lacking endogenous *MeCP2*, but expressing either MeCP2-e1 or MeCP2-e2 cDNAs, should be distinct.

Thus, we generated transgenic mice expressing either MeCP2-e1 or MeCP2-e2 cDNAs in brain cells, crossed them with mice lacking *MeCP2*, and compared the ability of each of the variants to compensate for the lack of the endogenous gene. We observed that expression of either of the isoforms mitigated the phenotypic consequences from the lack of *MeCP2* and allowed *MeCP2*^{-/-} mice to survive to adulthood, indicating a significant degree of functional overlap.

MATERIALS AND METHODS

Animals

MECP2 transgenes were generated by linearizing pcDNA3.1A-MECP2B-myc and pEGFP-MECP2 (kindly provided by B Minassian and S Kudo, respectively) to release vector sequences and microinjected into the pronuclei of B6CBF2 zygotes. MeCP2-e1-myc and EGFP-MeCP2-e2 transgenic mice were crossed with 129/SvJ *MeCP2*^{-/-} mice.²² Animals were kept in an animal room under SPF conditions, in a 12/12 h light/dark cycle with free access to food and water. All the experiments were approved by the Centro de Estudios Científicos Animal Care and Use Committee.

Fluorescent immunohistochemistry

Floating cryostat sections (40 μ m) were incubated overnight with primary antibodies anti-MeCP2 1:100 (Upstate/Millipore, Billerica, MA, USA), anti-Myc 1:1000 (Sigma, St Louis, MO, USA) and anti-GFP 1:500 (Molecular Probes, Carlsbad, CA, USA). Secondary antibodies conjugated to Alexa Fluor 488 (Molecular Probes) or Cy3 (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) were used at a 1:500 dilution. Images were captured using a Zeiss Axiovert 100M confocal microscope equipped with a QImaging 3.3 RTV cooled CCD camera (Zeiss, Göttingen, Germany) and Adobe Photoshop 7.0 (Adobe Systems, San Jose, CA, USA).

Western blot analysis

Brain regions were dissected from 1 mm coronal sections and homogenized in lysis buffer containing 125 mM Tris (pH 6.8) and 2% SDS supplemented with 1 \times protease inhibitor cocktail (Sigma, P8340). Thirty microgram of protein were electrophoresed and transferred on PVDF membranes (Bio-Rad, Hercules, CA, USA). Membranes were incubated with anti-MeCP2 1:2500 (Upstate) or anti- β -Tubulin 1:1000 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and anti-rabbit HRP-conjugated IgG 1:40 000 (Pierce, Biotechnology, Rockford, IL, USA). Densitometry of immunoreactive bands was quantitated with Quantity One software (Bio-Rad).

Phenotypic characterization

Starting from 3 weeks old, mice were tested on a weekly basis for survival, body weight determination, and presence and severity of stereotypic hand movements and clasping. Severity of clasping was determined according to an arbitrary scaling (from 0 to 3), based on how fast mice clasp their feet together when picked by the tail and also on their capability of releasing the posture: 0 (no clasping), 1 (reversible clasping in which only hind-limbs press into the stomach), 2 (delayed, but irreversible) and 3 (immediate and irreversible). At 7 weeks of age, each mouse was subjected to a battery of behavioral tests performed always in the same order: elevated plus maze, open field, elevated beam test and hanging wire test. For all experiments, the data are presented as mean \pm SEM. The data were analyzed using the Student's *t*-test. Statistical significance was set at a minimum of $P < 0.05$. Survival analysis was conducted by means of a Kaplan–Meier survival analysis.

Elevated plus maze

Mice were placed in the center of a cross-shaped maze elevated 45 cm from the floor with two open and two closed arms. The behavior of the mice was

observed and the time spent in either the closed, open or at the center of the maze was recorded.

Elevated beam test

The elevated beam test consists of two elevated platforms connected through a 70 cm long dowel of 0.7 cm radius. Before testing, mice were placed on the dowel 10 cm away from one of the platforms. Only those mice that reached the platform in the first 60 s were further assessed. Next, mice were placed in the middle of the dowel and the time of first arrival, the total number of arrivals and the number of falls were recorded for 2 min.

Hanging wire test

The hanging wire test was performed by hanging the mice by its forepaws from a suspended wire and recording the number of falls in 2 min.

RESULTS

Generation of transgenic mice for either MeCP2-e1 or MeCP2-e2

We generated transgenic mice carrying either the cDNA for MeCP2-e1 or MeCP2-e2 under the control of the human cytomegalovirus immediate-early promoter/enhancer region (CMV promoter), which drives consistently strong expression of genes in both cultured cells and in differentiated neurons. In order to easily differentiate between the two isoforms, MeCP2-e1 was fused C terminally to a myc tag, whereas EGFP was added to the N-terminus of MeCP2-e2 (Figure 1a). The functionality of these constructs was confirmed by testing the ability to repress an *in vitro* methylated reported construct, to modulate alternative splicing of reporter constructs and to correctly localize to heterochromatic foci (Supplementary Figure 1). As previously reported, both constructs completely colocalize in N2A-transfected cells in interphase, as well as in cells undergoing active replication (Figure 1b). These data suggest that the addition of the tags does not alter functionality of the isoforms.

Several founder mice were obtained for both constructs. Two independent transgenic lines expressing different amounts of MeCP2-e1-myc in the brain, e1-TgH and e1-TgL, were selected for this study. As it was already shown that *MeCP2-e2* expression rescued lethality, normalized body weight regulation and restored motor activity of *MeCP2*^{-/-} mice to wild-type levels,^{18–20} we decided to study only one MeCP2-e2 transgenic line that exhibited widespread neuronal expression of the transgene to confirm these previous observations (e2-Tg).

Expression of isoform-specific cDNAs in transgenic mice

The expression pattern of the transgenes on the selected transgenic lines is shown in Figure 2, determined by indirect immunofluorescence for the Myc tag and EGFP in the MeCP2-e1 and MeCP2-e2 transgenics, respectively. Transgene expression was widely distributed in the brain for all three lines. There are qualitative and quantitative differences in the expression of MeCP2-e1 in e1-TgH and e1-TgL lines. In line e1-TgH, the intensity of the fluorescent signal was similar across all brain regions, with the exception of the cerebellum, in which the intensity was higher, and the lateral nucleus of the thalamus and the CA1 area of the hippocampus, which showed decreased labeling (Figure 2a). Myc labeling in e1-TgL was also widespread, but exhibited lower levels of expression than e1-TgH in general and more cell to cell heterogeneity, with highest expression in cortical layer 2 and lowest expression in the cerebellum (Figure 2a). Line e2-Tg showed brain-wide EGFP–MeCP2-e2 expression at comparable levels throughout the brain, excluding the dentate gyrus of the hippocampus where the expression was undetectable (Figure 2a). Co-staining of transgenic MeCP2 and the intermediate filament marker glial fibrillary acidic protein (GFAP) indicated that none of the transgenic lines express

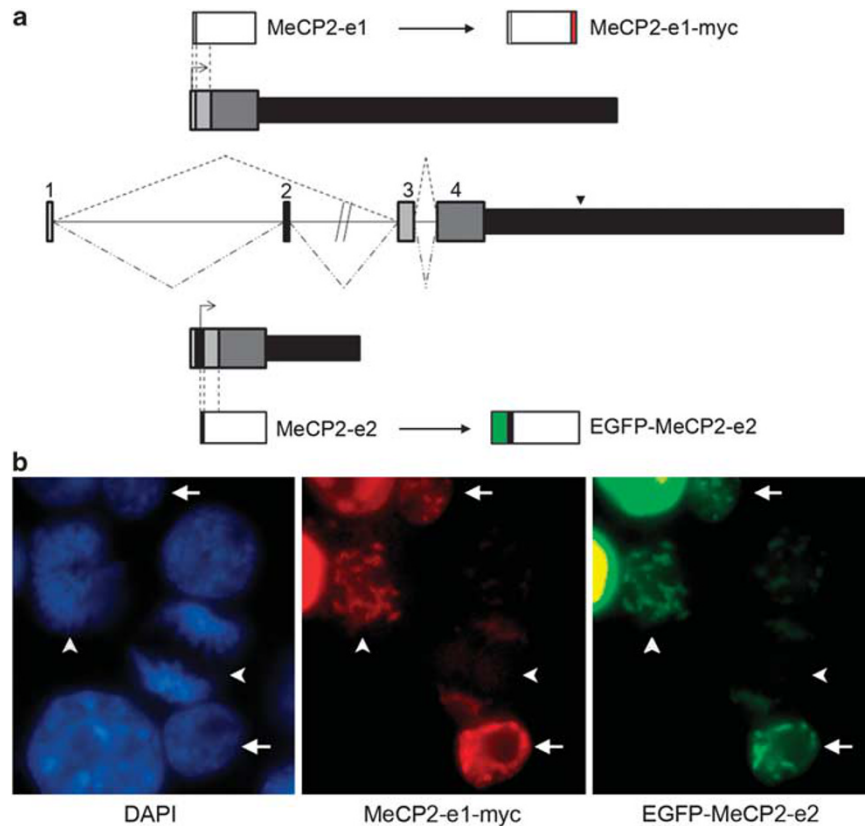


Figure 1 Isoform-specific MeCP2 transgenes. (a) Scheme of the MeCP2 isoforms generated by alternative splicing and splice variant-specific transgenic proteins. Exclusion of exon 2 generates MeCP2-e1 (top), whereas its inclusion produces MeCP2-e2 (bottom). The polyadenylation site predominantly used by MeCP2 is marked by a black arrowhead. The tagged versions of the isoforms are depicted. (b) Immunofluorescence for the detection of the myc epitope (in red) in cells co-transfected with MeCP2-e1-myc and EGFP-MeCP2-e2 shows colocalization with EGFP (in green) in cells in interphase (arrows), as well as cells in different phases of cell division (arrowheads). DNA was stained with DAPI (in blue).

MeCP2 in astrocytes at detectable levels (Supplementary Figure 2a). However, as it has been shown that endogenous MeCP2 expression in glial cells is not easily revealed due to its low levels,²³ we analyzed the expression of the transgenes in primary cultures of mixed cortical cells and found expression of MeCP2 transgenes in some GFAP-positive glial cells in the three transgenic lines studied (Supplementary Figure 2b).

To determine the percentage of neurons expressing transgenic MeCP2-e1 or MeCP2-e2 in the different brain regions, we co-stained for MeCP2, which labels neurons expressing both endogenous MeCP2, as well as the transgenic MeCP2-e1 or MeCP2-e2, depending on the transgenic mice analyzed (Figures 2b–d). Co-immunofluorescence analysis using anti-MeCP2 and anti-myc indicated that transgenic line e1-TgH expressed MeCP2-e1 in 16 to 98% of neurons, depending on the brain region (Table 1). Line e1-TgL expressed transgenic MeCP2-e1 in 7–96% of neurons, and MeCP2-e2 was expressed in 9–88% in the e2-Tg transgenic line (Table 1 and Supplementary Figure 3).

It has been demonstrated that neurons are sensitive to inappropriate MeCP2 expression levels.^{24,25} Thus, we determined the level of expression of transgenic MeCP2 by comparing the immunoreactivity for MeCP2 in protein extracts obtained from a variety of brain regions of transgenic and wild-type mice. Analysis of the western blot results indicated that expression of MeCP2-e1 in e1-TgH and e1-TgL varied between brain regions, averaging at 40 and 15% of MeCP2 wild-type expression, respectively (data not shown). A higher level of expression was observed in cerebellum of line e1-TgH, in which transgenic

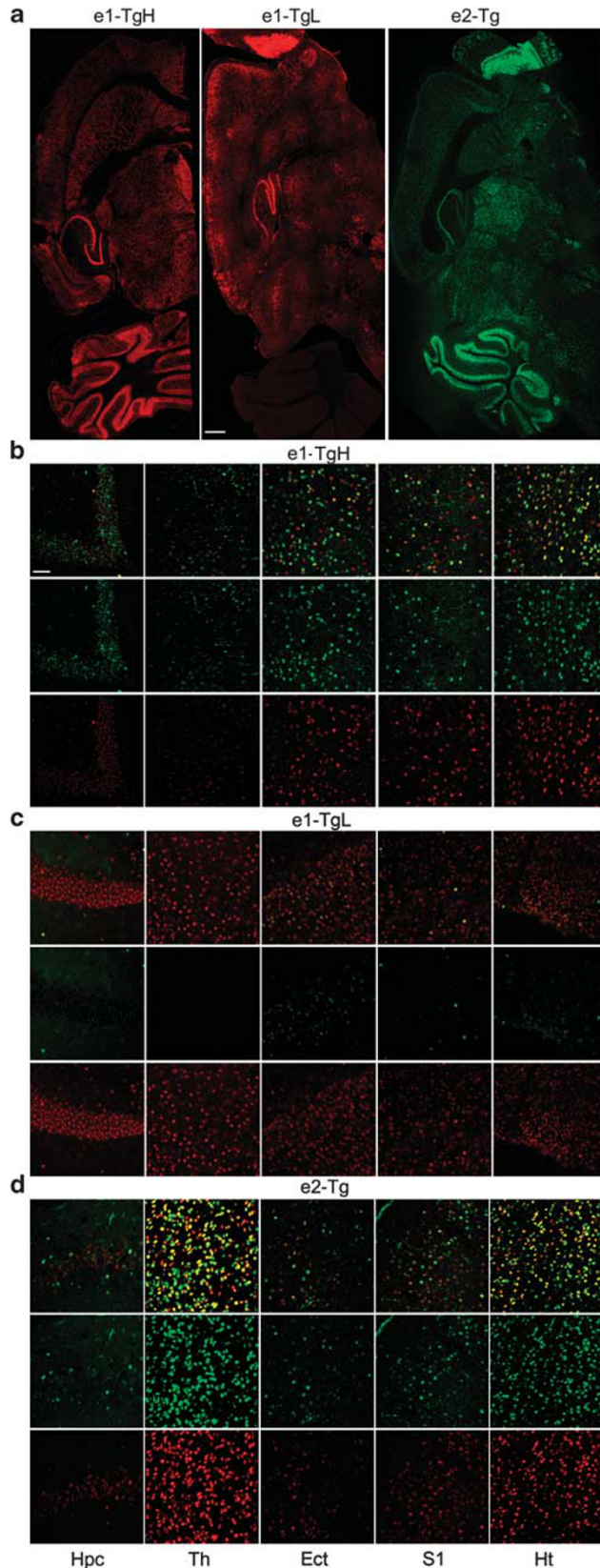
MeCP2-e1 was approximately 75% of the wild-type MeCP2 expression (data not shown). Line e2-Tg expressed 30–40% of wild-type MeCP2 in regions such as the brain stem, cerebral cortex and hippocampus, but expressed close to 100% in the caudate/putamen areas, hypothalamus and thalamus (Supplementary Figure 4). These results were confirmed by quantitating the amount of MeCP2 expressed by transgenic mice crossed into a *Mecp2* null background (*Mecp2*^{-/-};e1-TgH, *Mecp2*^{-/-};e1-TgL and *Mecp2*^{-/-};e2-Tg (Figures 3a and b). Thus, considering the percentage of cells expressing the transgene in the different brain regions (Table 1) and the total relative amount expressed per region (Figure 3b), the estimated expression of transgenic proteins per cell amounts to approximately 30–80% in e1-TgH, 35–95% in e1-TgL (higher than 40% only in cerebellum, with approximate expression of MeCP2-e1 of 95%) and 40–140% in e2-Tg, compared with the expression of endogenous MeCP2.

Interestingly, we did not observe phenotypic abnormalities in e1-TgL mice. However, e1-TgH mice, modestly overexpressing MeCP2-e1 developed a recognizable late onset phenotype that includes hypoactivity and premature death at 12–16 months of age (Abrams *et al*, unpublished).

Transgenic MeCP2-e1 is enough to prevent Rett-like phenotypes in MeCP2-deficient mice

The ability of MeCP2-e1 to compensate for loss of endogenous MeCP2 was tested in male mice derived from matings of *Mecp2*^{-/+} females crossed with e1-TgH, e1-TgL or e2-Tg (as positive controls).

Two independent blind observers were able to easily and accurately sort out *Mecp2*^{-f/y} mice from mice of all other genotypes after 7 weeks of age, by simply looking at the mice in their home cage. The main



differentiating parameters were activity and body posture. *Mecp2*^{-f/y} mice appeared less active, hesitant and usually adopted a crouched posture, as compared with the rest of the mice in the cage, suggesting that both transgenes had a modifier effect on the neurobehavioral phenotype of the *Mecp2*^{-f/y} mice. We then performed a more systematic phenotypic evaluation by determining life span, measuring body weight, testing motor skills, evaluating anxiety-related behaviors and documenting seizures.

Life span. Fifty percent of *Mecp2*^{-f/y} mice did not survive past 10 weeks and all mice with this genotype died before 30 weeks (Figure 3c). Notably, more than 80% of *Mecp2*^{-f/y};e1-TgH were still alive at 43 weeks, with some mice surviving past 16 months. Thus, MeCP2-e1 was able to fully prevent the early death seen in *Mecp2* null mice.

Body weight. *Mecp2*^{-f/y} mice show a characteristic weight gain pattern: weight escalates around week 8 and then starts declining significantly at 14 weeks (Figure 3d and data not shown). The weight gain curve of the *Mecp2*^{-f/y};e1-TgH mice was indistinguishable from wild-type littermates, demonstrating that the presence of this isoform by itself may prevent the development of this weight gain phenotype.

Hindpaw clasping. To determine whether the neurobehavioral decline shown by *Mecp2*^{-f/y} mice was modified by expression of the MeCP2-e1 variant, we quantitatively assessed paw clasping. At 3 weeks of age, *Mecp2*^{-f/y} mice displayed hindpaw clasping of a severity level of 1, and by 8 weeks, it progressed to 2. By 12 weeks of age, all *Mecp2*^{-f/y} mice studied presented grade 2–3 clasping. Expression of MeCP2-e1 significantly improved ($P < 0.01$) the average clasping score of the mutant mice (Figure 4a). No clasping was observed in *Mecp2*^{-f/y};e1-TgH mice until 9 weeks of age, when some began to show a subtle clasping that by 18 weeks was classified as of level 1.

Anxiety. As it has been previously reported, *Mecp2*^{-f/y} mice exhibited a behavior that correlates with decreased anxiety when tested in the plus maze, they did not show a preference for the closed (commonly interpreted as safe) arm of the maze, as wild-type mice did (Figure 4b). *Mecp2*^{-f/y} mice spent a similar amount of time in both, open and closed arms of the maze, a behavior that was significantly different than that exhibited by wild-type and *Mecp2*^{-f/y};e1-TgH mice.

Motor control. We subjected the mice to the elevated beam test, in which a mouse is induced to walk on a thin wooden dowel to reach the safety of a platform. *Mecp2*^{-f/y} mice performed significantly worse than their wild-type littermates (Figure 4). The performance of *Mecp2*^{-f/y};e1-TgH mice was similar to the wild-type littermates

Figure 2 Transgene expression in brains of e1-TgH, e1-TgL and e2-Tg mice. (a) Myc immunoreactivity (in red) was detected throughout the brain in 40 μ m sections from e1-TgH (left panel) and e1-TgL (middle panel) mice. EGFP fluorescence (in green) is also widely observed in the brain of e2-Tg mice (right panel). (b–d) Representative images of double immunofluorescence for MeCP2 (in red) and MeCP2 transgenic proteins (in green) from the indicated areas in brain cryosections from e1-TgH (b), e1-TgL (c) and e2-Tg (d). Top panels show the merged figures. Note that the anti-MeCP2 antibody labels endogenous and transgenic MeCP2. Thus, every cell labeled by the anti-tag antibody (in green) should also be stained by the anti-MeCP2 antibody (in red), and red-only cells demarcate the minority of cells in which transgenic MeCP2 is not expressed. All images were captured at $\times 25$ magnification. Scale bars represent 500 μ m (a) and 50 μ m (b–d). Hpc, hippocampus; Th, thalamus; Ect, Ectorhinal cortex; S1, somatosensory cortex; Ht, hypothalamus.

Table 1 Percentage of neurons expressing MeCP2 transgenes in selected areas of the brain

	<i>e1-TgH</i>	<i>e1-TgL</i>	<i>e2-Tg</i>
<i>Cortex</i>			
Somatosensory (S1; %)	67 ± 18	32 ± 11 (except layer 2: 96 ± 16)	63 ± 12
Ectorhinal (Ect; %)	81 ± 19	40 ± 14	79 ± 13
Entorhinal (MEnt; %)	84 ± 16	76 ± 26	82 ± 16
Striatum (CPu; %)	72 ± 12	27 ± 11	77 ± 18
<i>Hippocampus</i>			
CA1 (%)	16 ± 8	23 ± 11	86 ± 14
CA2 (%)	86 ± 15	19 ± 14	88 ± 9
CA3 (%)	85 ± 19	16 ± 9	76 ± 19
DG (%)	92 ± 22	22 ± 13	9 ± 5
Inferior colliculus (%)	96 ± 17	21 ± 8	88 ± 7
Cerebellum (Crus1; %)	98 ± 3	21 ± 8 (2% in the molecular layer)	77 ± 12
Thalamus (LPMR+AVDM; %)	64 ± 10	7 ± 7	82 ± 16
<i>Brain stem</i>			
Oral pontine reticular nucleus (%)	66 ± 15	51 ± 12	60 ± 16
Intermediate reticular nucleus (%)	42 ± 11	46 ± 9	32 ± 14
Inferior olive (%)	68 ± 19	31 ± 16	79 ± 22

Abbreviations: MeCP2, methyl-CpG binding protein 2; S1, primary somatosensory cortex; Ect, ectorhinal cortex; MEnt, entorhinal cortex, medial part; CPu, caudate/putamen; Crus1, superior semilunar lobule; LPMR, lateral posterior nucleus; AVDM, anteroventral nucleus, according to MBL (Mouse Brain Atlas Nomenclatures). Brain sections of 40 μm obtained from *Mecp2^{+/+};e1-TgH*, *Mecp2^{+/+};e1-TgL* and *Mecp2^{+/+};e2-Tg* mice of 4 weeks of age were stained for anti-MeCP2 and anti-myc or anti-EGFP. The percentage of cells expressing tagged versions of MeCP2 was calculated as cells positive for anti-myc or anti-GFP/cell expressing MeCP2 × 100. At least 50 cells were counted per region per animal (*n*=3). The specific area from which cell counting was performed is indicated between brackets.

(Figures 4c–f). To further characterize the motor phenotype of the transgenic mice, we measured their ability to remain suspended from a horizontal wire. Wild-type and *Mecp2^{-/-};e1-TgH* mice outperformed the *Mecp2^{-/-}* mice in this test (Figure 4g). Collectively, these results indicate that MeCP2-e1 was able to normalize the motor phenotype caused by lack of endogenous MeCP2.

The phenotypic rescue exerted by transgenic MeCP2-e1 is dosage-dependent

Transgenic expression of MeCP2-e2 is sufficient to prevent the RTT-like phenotype in MeCP2 null mice;^{18–20} however, the fact that MeCP2-e2 seems to be unable to compensate for the absence of the MeCP2-e1 variant in human RTT patients carrying mutations that exclusively affect MeCP2-e1^{16,17} raises the possibility of a MeCP2 dose-dependency in the extent of rescue. We explored this possibility by comparing the extent of rescue elicited by the *e1-TgH* and *e1-TgL* MeCP2-e1 transgenic lines, which showed different levels of transgene expression. Although initial home cage observation suggested phenotypic rescue in *Mecp2^{-/-};e1-TgL* mice (see above), a more detailed analysis showed that this rescue was only partial. The presence of the *e1-TgL* transgene extended the median life span (0.5 probability survival rate) of the *Mecp2^{-/-}* mice from 10 weeks in *Mecp2^{-/-}* to 25 weeks in *Mecp2^{-/-};e1-TgL* mice, but this effect is noticeably less significant than the one observed in the *Mecp2^{-/-};e1-TgH* mice. In addition, *Mecp2^{-/-};e1-TgL* mice showed partial amelioration in clasping severity (Figure 4a) and only a trend for rescue in anxiety and

motor performance (Figure 4), suggesting a gene dose-dependent effect on severity of these phenotypes.

Transgenic MeCP2-e2 also prevents Rett-like phenotypes

To compare the rescuing capacity of MeCP2-e1 and MeCP2-e2, we characterized the phenotype of the *Mecp2^{-/-};e2-Tg* mice. *Mecp2^{-/-};e2-Tg* mice have a longer life expectancy than *Mecp2^{-/-}*, with a median life span of 42 weeks and some animals surviving for more than 14 months (Figure 3a). Body weight and behavior on the plus maze were also normalized (Figure 3b). Thus, both MeCP2 isoforms were able to prevent the early death and the phenotypes seen in *Mecp2* null mice to comparable extents. However, hindlimb clasping and motor abnormalities were only partially rescued in *Mecp2^{-/-};e2-Tg*, raising the possibility of minor differences in brain function of both isoforms (Figure 4).

In vivo colocalization of transgenic MeCP2 isoforms

Transfected MeCP2-e1 and MeCP2-e2 show an apparent similar intracellular localization when overexpressed in cultured cells. However, it is possible that *in vivo*, both isoforms have distinct intracellular localization and the observed complete colocalization could be an artifact of *in vitro* transient overexpression. Our mice expressing transgenic MeCP2 isoforms at levels comparable to the endogenous proteins allows for intracellular colocalization studies of the transgenic MeCP2 isoforms in intact brain cells. We crossbred our transgenic mice into a *Mecp2* null background to generate mice expressing both *e1-TgH* and *e2-Tg* in the absence of endogenous MeCP2. Confocal analysis of tissue sections obtained from *Mecp2^{-/-};e1-TgH;e2-Tg* mice indicate that transgenic MeCP2-e1 and MeCP2-e2 appear to colocalize completely in cells in the brain, skeletal muscle, heart, eye and kidney (Supplementary Figure 5).

DISCUSSION

This study was designed to determine whether the two MeCP2 isoforms derived from alternative splicing have diverse or completely redundant functions. MeCP2-e1 and MeCP2-e2 differ only in the N-terminus; MeCP2-e1-specific N-terminus contains the sequence MAAAAAAPS GGGGGGEEERL, which in MeCP2-e2 is changed to MVAGMLGLR. The presence of polyA and polyG repeats in the MeCP2-e1 N-terminus is suggestive of a differential function of the isoforms, as there is accumulating evidence that tandem repeats may serve functional roles within coding sequences, in addition to be regulatory elements or mutational hotspots.²⁶ However, most *in vitro* studies failed to detect any functional difference between the two isoforms.²¹ In spite of the limited information about MeCP2-e2 expression in human brain, the existence of Rett-causing mutations in *MECP2* that apparently do not interfere with MeCP2-e2 expression, but affect MeCP2-e1, suggests that the sole presence of MeCP2-e2 is not enough to provide the full functional role. Although, as with most cases of neurological disorders, integrating and interpreting human and mouse data is not completely straightforward, the available mouse data suggest that expression of MeCP2-e2 is sufficient to prevent the development of disease signs in mice lacking endogenous MeCP2 expression.^{18–20} Our data show for the first time that the sole expression of MeCP2-e1 was able to compensate for the lack of MeCP2 in mice. The rescuing effect of transgene expression seemed to be dose dependent, as transgenics expressing lower levels of MeCP2-e1 were only partially rescued, suggesting that the hypothetical inability of MeCP2-e2 to compensate for the absence of MeCP2-e1 in patients carrying mutations affecting only MeCP2-e1 is most probably due to insufficient endogenous expression levels.

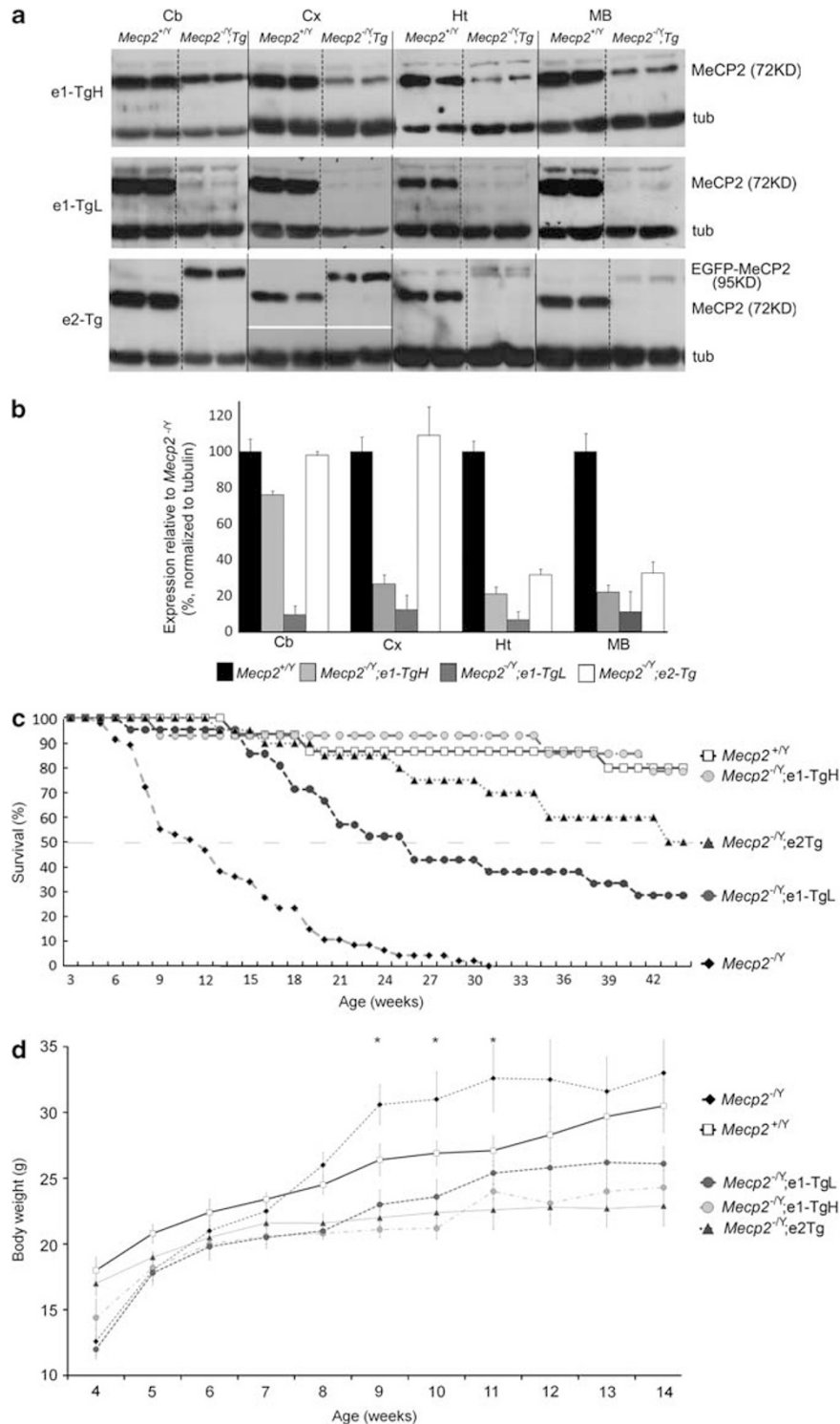


Figure 3 Determination of transgene expression levels and their effects on life span and body weight. **(a)** Western blots for the immunodetection of MeCP2 using whole protein extracts obtained from cerebellum (Cb), cerebral cortex (Cx), hypothalamus (Ht) and midbrain (MB) of wild-type mice (*Mecp2*^{+/Y}) and transgenic mice carrying isoform-specific MeCP2 transgenes in a *Mecp2*-null background (*Mecp2*^{-Y};Tg). Anti-tubulin antibody was used as a normalization control (tub). Representative images of western blots from *Mecp2*^{-Y};e1-TgH (top), *Mecp2*^{-Y};e1-TgL (middle) and *Mecp2*^{-Y};e2-Tg (bottom) are shown. Note that the band recognized by the anti-MeCP2 antibody in the *Mecp2*^{-Y};e2-Tg lanes has a higher molecular weight due to the fusion to EGFP. **(b)** Densitographic quantification of the western data. Values (means \pm SEM) of MeCP2/tubulin from three independent mice were normalized to wild-type (*Mecp2*^{+/Y}) controls. **(c)** Kaplan–Meier plot showing the survival of *Mecp2*^{+/Y} ($n=11$), *Mecp2*^{-Y} ($n=45$), *Mecp2*^{-Y};e1-TgH ($n=9$), *Mecp2*^{-Y};e1-TgL ($n=19$) and *Mecp2*^{-Y};e2-Tg ($n=17$) mice up to 45 weeks of age. Log-rank test was used to evaluate significance of differences between groups. Median life span represents the age at which 50% of the population within groups remained alive. Note that *Mecp2*^{-Y} mice in this genetic background exhibited longer survival than usually reported²². **(d)** Growth curves of *Mecp2*^{+/Y} ($n=11$), *Mecp2*^{-Y} ($n=32$), *Mecp2*^{-Y};e1-TgH ($n=9$), *Mecp2*^{-Y};e1-TgL ($n=21$) and *Mecp2*^{-Y};e2-Tg ($n=17$) mice. All curves were significantly different from the *Mecp2*^{-Y} curve. * $P<0.05$.

We have previously reported that transgenic mice expressing MeCP2-e2 under the NSE or CamKIIa promoters, when crossed to *Mecp2*^{-/-} elicited no significant behavioral or lifespan improvements.²⁷ Contrasting this previous study with the current

data, in which we observe significant phenotypic prevention with widespread brain expression of MeCP2-e2, suggests that not only level, but also location of expression is important for MeCP2's overall activity in brain function. In addition, the expression of transgenic MeCP2 in glial cells in the transgenic mice described in this report could contribute to the behavioral amelioration and might explain the different results observed.^{23,28}

Do our results mean that MeCP2-e1 and MeCP2-e2 are practically interchangeable? Life span, body weight control and anxiety were equally rescued by both isoforms, suggesting equal function at similar levels of expression. However, the rescue exhibited by both isoforms was not 100% concordant; the degree of rescue of the clasping and motor phenotypes was significantly higher for MeCP2-e1. This might suggest differential relevance of the two isoforms for the development of motor and clasping phenotypes, but the possibility of differences due to variable expression of the transgenes in different brain tissues cannot be ruled out. Further, the study of phenotypes not analyzed in this study could potentially reveal unidentified functional differences between the two isoforms.

Notably, the approximate expression of MeCP2-e1 in the transgenic line that showed a significant normalization of the Rett-like phenotypes presented by *Mecp2*^{-/-} mice was only 40–60% of endogenous, suggesting that equaling endogenous level of expression is not strictly necessary for a significant phenotypic rescue. Even more remarkable is the observation that *Mecp2*^{-/-};e1-TgL mice showed rescue effects that, although partial, were in most cases equivalent or more significant than what was seen in the *Mecp2*^{-/-};e2-Tg mice. This implies that only minor amounts of MeCP2-e1 are sufficient to elicit dramatic behavioral recovery, and that the influence of the two forms on phenotypic function might not be completely equivalent.

We and others have shown that mice carrying a *Mecp2* floxed allele and expressing approximately 40% less MeCP2 than wild-type mice exhibited discernible phenotypes, suggesting that even mild reductions in MeCP2 protein might cause neuronal dysfunction.^{24,29} This apparent inconsistency could be due to small differences in patterns of transgene expression, or to the different time point in which the mice were tested (7 weeks of age (this study) versus 12 weeks). Also, subtle phenotypic manifestations not analyzed in this study, such as social behavior, could still be present in *Mecp2*^{-/-} mice expressing transgenic MeCP2-e1.

The finding that expression of MeCP2-e1 by itself was sufficient to significantly extend the life span of *Mecp2*^{-/-} mice, to prevent the development of common manifestations of neurological dysfunction

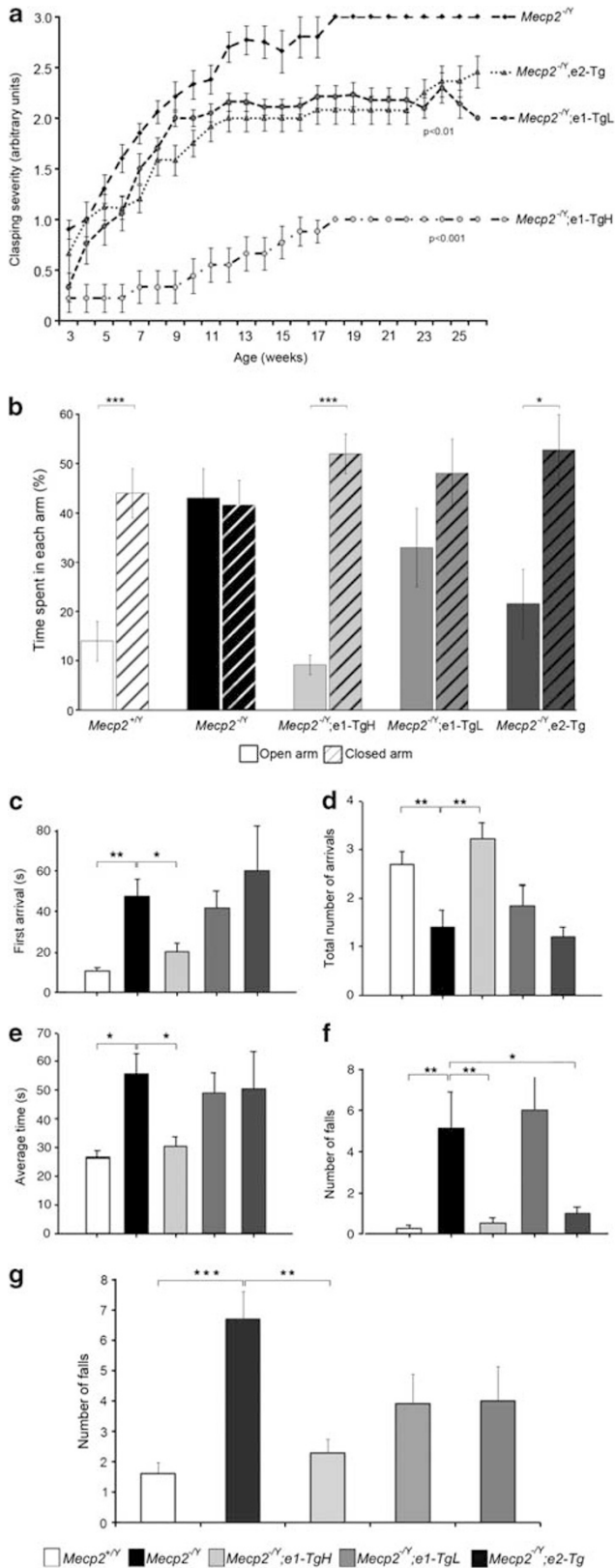


Figure 4 Isoform-specific transgene expression rescues neurophenotypes exhibited by *Mecp2*-null mice. (a) Severity of clasping. *Mecp2*^{-/-};e1-TgH (*n*=9) and *Mecp2*^{-/-};e1-TgL (*n*=15) mice had significantly less severe clasping than *Mecp2*^{-/-} (*n*=32) and *Mecp2*^{-/-};e2-Tg (*n*=17) mice. *Mecp2*^{+/+} mice had no noticeable clasping and were therefore not included in the analysis. All curves were significantly different from the *Mecp2*^{-/-} curve. (b) The behavior of mice of the indicated genotypes in the elevated plus maze is shown as percentage of time spent in the open (filled bars) and closed (hatched bars) arms of the maze. All results are presented as means ± SEM (*n*=10 for *Mecp2*^{+/+}, *n*=21 for *Mecp2*^{-/-}, *n*=14 for *Mecp2*^{-/-};e1-TgH, *n*=13 for *Mecp2*^{-/-};e1-TgL and *n*=3 for *Mecp2*^{-/-};e2-Tg). (c–f) Elevated beam test. Time of the first arrival to the platform displayed by *Mecp2*^{-/-};e1-TgH mice was significantly reduced (c), the total number of arrivals to the platforms was significantly higher (d), the average time to traverse the dowel was decreased (e), and the total number of falls was reduced (f) as compared with *Mecp2*^{-/-} mice. Note that this motor phenotype is not rescued in *Mecp2*^{-/-};e1-TgH mice or *Mecp2*^{-/-};e2-Tg. (g) Hanging wire test. Data represent the mean ± SEM. ****P*<0.001, ***P*<0.01 and **P*<0.05 by Student's *t*-test.

in mice, such as clasping, and to normalize anxiety-related and motor phenotypes, is noteworthy and relevant to the design of efficient strategies aiming to restore MeCP2 activity, including gene therapy³⁰ and protein administration approaches.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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