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## L1 retrotransposition in neurons is modulated by MeCP2

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Long interspersed nuclear elements-1 (LINE-1 or L1s) are abundant retrotransposons that comprise approximately 20% of mammalian genomes<sup>1–3</sup>. Active L1 retrotransposons can impact the genome in a variety of ways, creating insertions, deletions, new splice sites or gene expression fine-tuning<sup>4–6</sup>. We have previously shown that L1 retrotransposons are capable of mobilization in neuronal progenitor cells from rodents and humans and evidence of massive L1 insertions was observed in adult brain tissues but not in other somatic tissues<sup>7,8</sup>. In addition, L1 mobility in the adult hippocampus can also be influenced by the environment<sup>9</sup>. The neuronal specificity of somatic L1 retrotransposition in neural progenitors is partially due to the transition of a Sox2/HDAC1 repressor complex to a Wnt-mediated TCF/LEF transcriptional activation<sup>7,10</sup>. The transcriptional switch accompanies chromatin remodeling during neuronal differentiation, allowing a transient stimulation of L1 transcription<sup>7</sup>. The activity of L1 retrotransposons during brain development can impact gene expression and neuronal function, thereby increasing brain-specific genetic

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### Supplementary Information

Full Methods and respective references

Supplementary Figures 1-6

Supplementary Movie

### Authors Contributions

A.R.M. and M.C.N.M. are the leading authors. They contributed to the concept, designed, performed the experiments, analyzed the data, and wrote the manuscript. N.G.C. performed and analyzed qPCR experiments. R.O. performed tissue culture experiments and quantification. G.Y. helped with statistical analysis and data interpretation. K.N. contributed reagents, and performed data analyses and manuscript revision. F.H.G. contributed to the concept, analyzed the data, and revised the manuscript.

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mosaicism<sup>11,12</sup>. Further understanding of the molecular mechanisms that regulate L1 expression should provide new insights into the role of L1 retrotransposition during brain development. Here we show that L1 neuronal transcription and retrotransposition in rodents is increased in the absence of methyl-CpG-binding protein 2 (MeCP2), a protein involved in global DNA methylation and human neurodevelopmental diseases. Using neuronal progenitor cells derived from human induced pluripotent stem cells and human tissues, we revealed that patients with Rett syndrome (RTT), carrying MeCP2 mutations, have increased susceptibility for L1 retrotransposition. Our data demonstrate that L1 retrotransposition can be controlled in a tissue-specific manner and that disease-related genetic mutations can influence the frequency of neuronal L1 retrotransposition. Our findings add a new level of complexity to the molecular events that can lead to neurological disorders.

In neural stem cells, the repressor complex on the L1 promoter region (L1 5'UTR) includes the transcriptional factor Sox2 and the histone deacetylase 1 protein (HDAC1)<sup>7</sup>, a MeCP2 partner<sup>13,14</sup>. MeCP2 has been shown to interfere with the L1 5'UTR promoter activity in transformed cell lines<sup>15</sup>. To investigate the role of MeCP2 in the activity of L1 promoter in neural stem cells, we cloned the L1 promoter region upstream to the *luciferase* gene, generating the L1 5'UTR-*Luc* plasmid<sup>7</sup>. Methylation of the L1 5'UTR-*Luc* reduced the promoter activity in neural stem cells (Fig. 1a and Supplementary Fig. 1a). Reduction of MeCP2 levels using siRNAs, led to an increase in luciferase activity (Fig. 1b and Supplementary Fig. 1b). Transfection of the L1 5'UTR-*Luc* methylated plasmid in mouse neuroepithelial cells revealed that the L1 promoter activity was approximately 4 times more active in the MeCP2 knockout (KO) background than in wild-type (WT) (Fig. 1c and Supplementary Fig. 1c). Ectopic MeCP2 expression reduced the luciferase activity in MeCP2 KO cells close to WT levels (Fig. 1c).

We repeated the luciferase assay using neuroepithelial cells from a sibling MBD1 KO animal<sup>16</sup>. MBD1 (methyl-binding protein 1) is part of the methyl-binding protein family and has differential DNA specificity when compared to MeCP2<sup>17</sup>. The L1 promoter was not activated in MBD1 KO background, a finding that is consistent with the idea that L1 transcriptional repression is specific to MeCP2 (Fig. 1c). Moreover, the promoter activity correlated well with the level of L1 RNA, as measured by qPCR (Fig. 1d). Ectopic MeCP2 expression reduced L1 RNA levels in the MeCP2 KO background (Fig. 1d). We co-transfected neural stem cells with the methylated L1 5'UTR-*Luc* and a plasmid containing either the MeCP2 cDNA or the MeCP2 fused with the transactivator domain VP16. The overexpression of MeCP2 alone did not change the luciferase levels but the MeCP2-VP16 fusion increased luciferase levels 2-fold (Fig. 1e).

Using chromatin immunoprecipitation (ChIP) followed by quantitative PCR (qPCR), we detected high levels of MeCP2 in association with endogenous L1 promoter regions in neural stem cells compared to neurons (Fig. 1f). MeCP2 was also associated with other L1 regions (ORF2) but this association did not change during differentiation (Fig. 1g and Supplementary Fig. 1d, e). After treatment with 5-Azacytidine, the MeCP2 ChIP signal was reduced and L1 expression increased (Fig. 1h and Supplementary Fig. 1f). A set of the CpG sites within the L1 promoter had a tendency to de-methylate during neuronal differentiation,

indicating that DNA methylation may silence L1 expression in neural stem cells by attracting MeCP2 (Supplementary Fig. 1g, h).

To study L1 regulation *in vivo*, we compared the brains of the L1-EGFP transgenic mice in WT and MeCP2 KO backgrounds. L1-EGFP transgenic mice have a L1 indicator cassette that will only activate the EGFP reporter after retrotransposition<sup>7</sup> (Supplementary Fig. 2a). The numbers of EGFP-positive cells in the brains of MeCP2 KO mice were significantly higher than in WT (Fig. 2a, b). EGFP-positive cells were also observed in the germ line of MeCP2 KO at similar frequency as in WT animals, but not in other somatic tissues (Supplementary Fig. 2b). To visualize the distribution of EGFP-positive cells, we generated high-resolution, 3-dimensional maps of both MeCP2 KO and WT brains. Although MeCP2 KO brain sections had an average of 3.5-fold more EGFP-positive cells than WT, certain brain structures were more prone to L1 retrotransposition (Fig. 2b, c). Specifically, the cerebellum, striatum, cortex, hippocampus and olfactory bulb contained 4.2-, 5.3-, 2.8-, 6.3- and 3.8-fold more EGFP-positive neurons, respectively, in the MeCP2 KO genetic background than in WT (Supplementary Fig. 3 and Supplementary movie). More EGFP-positive cells may suggest an increased rate of L1 retrotransposition and/or higher rate of MeCP2 KO cell proliferation with the newly retrotransposed EGFP reporter. We found no evidence that neuroepithelial cells from the MeCP2 KO genetic background had a higher rate of division than WT (Supplementary Fig. 4a).

We next asked whether endogenous L1 retrotransposition was also increased in the MeCP2 KO brain. New retroelements insertions can be quantified using a qPCR approach<sup>8,18</sup>. To determine the activity of endogenous L1 elements, we developed a technique based on single-cell genomic qPCR that measures the frequency of mouse L1 sequences within the genome (Fig. 3a). We hypothesized that MeCP2 KO-derived neuroepithelial cells would have increased genomic content of L1 sequences compared to WT cells. Neuroepithelial cells from WT and MeCP2 KO sibling mouse embryos were synchronized in G1 phase and karyotyped, to avoid interference during genomic L1 detection (Supplementary Fig. 4b, c). Finally, single-cell amplification using primers for ORF2 from active L1 families confirmed the presence of the expected amplicons (Supplementary Fig. 4d). MeCP2 KO-derived neuroepithelial cells displayed significantly more ORF2 genomic copies than WT cells (Fig. 3b). Specific primers for the L1 5'UTR were also tested in neuroepithelial cells and did not reveal an increase in copy number in MeCP2 KO background (Fig. 3c). This lack of difference can be explained by the fact that, upon retrotransposition, the 5' region of the L1 sequence is frequently truncated<sup>19,20</sup>. Also, no difference between genetic backgrounds was observed when using primers for non-mobile 5S ribosomal RNA repetitive sequences (Fig. 3d). Another control experiment was performed using fibroblasts isolated from the two backgrounds (Fig. 3e). We did not observe a highly significant increase in L1 copy number in MeCP2 KO compared to WT.

Mutations on the MeCP2 gene cause RTT, characterized by arrested development in early childhood and autistic behavior at different levels of intensity<sup>21</sup>. To determine if L1 retrotransposition could occur in neuronal progenitor cells (NPC) derived from RTT patients, we generated induced pluripotent stem cells (iPSC) from a RTT patient's fibroblasts carrying a frameshift MeCP2 mutation and from a control, non-affected

individual. All clones were pluripotent and able to produce NPC and neurons (Supplementary Fig. 5). Thus, we decided to test if the iPSC-derived NPC supported L1 retrotransposition (Fig. 4a).

NPC from both WT and RTT iPSC expressed the neural markers Sox1, Musashi1, Nestin and Sox2 at similar rates at the time of the experiment (Supplementary Fig. 6a, b). RTT and WT cells were electroporated with the L1<sub>RE3</sub>-EGFP reporter construct<sup>22,23</sup>. EGFP expression was detected in both WT and RTT cells (Fig. 4b). The frequency of EGFP-positive cells was approximately 2-fold higher in RTT than in control cells. Moreover, MeCP2 complementation reduced the levels of EGFP-positive cells in RTT NPC (Fig. 4b,c and Supplementary Fig. 6c). PCR confirmed the presence of the retrotransposed EGFP and sequencing confirmed the precise splicing of the intron (Supplementary Fig. 6d). We concluded that L1 activity could be facilitated by loss of MeCP2 function in human cells. We extended the iPSC findings *in vivo* using postmortem human tissues. To analyze the amounts of L1 retrotransposition in RTT patients and controls, brain and heart tissue were obtained from the same individuals. After genomic DNA extraction, a qPCR was used to compare the number of L1 ORF2 sequences normalized by four distinct non-mobile repetitive sequences. The number of L1 ORF2 sequences in the brains of RTT patients was significantly higher than in age/gender-matched controls (Fig. 4d). Moreover, the number of ORF2 sequences was higher in brain tissues in both controls and RTT patients when compared to heart tissue from the same individuals.

Our findings support previous data demonstrating that L1 5'UTR sequences are MeCP2 targets that may be subject to methylation-dependent repression<sup>15,17</sup>. However, we cannot exclude an indirect effect of MeCP2 in regulating genes involved in L1 expression and/or in changing the chromatin epigenetic landscape to facilitate *de novo* L1 insertions. An additive effect of multiple mechanisms is likely. Using different strategies, we have shown that L1 retrotransposition can be modulated by MeCP2. First, we demonstrated that MeCP2 can downregulate L1 promoter activity. Second, L1 retrotransposition from the L1-EGFP transgenic mice was significantly higher in the brains of a MeCP2 KO background than in a WT sibling animal. The L1-EGFP indicator system underestimates the actual capacity of retrotransposition and does not take into account insertions that truncate or silence the reporter cassette, *in trans* retrotransposition of *Alus* or other RNAs<sup>24–26</sup>. Third, we developed a new technique based on single-cell genomic qPCR to measure the relative abundance of L1 sequences, revealing that MeCP2 KO neuroepithelial cells have more L1 sequences in the genome than WT cells. Lastly, RTT-NPC showed a higher L1 retrotransposition frequency than control cells. A qPCR experiment extended these observations to human brain samples from RTT patients compared to controls.

Our data provide evidence of a role for DNA methylation-dependent MeCP2 activity in controlling L1 mobility in the nervous system. Re-activation of MeCP2 expression was shown to reverse some of the neurological symptoms in MeCP2 KO mice<sup>27</sup>. The high rates of neuronal retrotransposition in the MeCP2 KO mice and RTT patients may be a consequence, rather than a cause, of the disease process. Nonetheless, new somatic insertions, especially at early developmental stages, may contribute to the genetic and epigenetic status of mature neurons at later stages of life. Early developmental structural and

functional modulations could have potential consequences for RTT, where the detrimental effects of MeCP2 mutation occur at later postnatal stages. It is plausible to conclude that the RTT process leads to an increased rate of somatic mutations in the brain. Increased L1 neuronal retrotransposition is a novel and unexpected characteristic of RTT pathology. Our findings add a new layer of complexity to the understanding of genomic plasticity and may have direct implications for individual variation and for neurological diseases.

## Methods Summary

For the *luciferase* activity experiments, rat neural stem cells were isolated, characterized and cultured as described<sup>28</sup>. Freshly isolated neuroepithelial cells from time-pregnant midgestation (E11.5) telencephalons from male WT, MBD1 KO, and MeCP2 KO sibling mouse embryos, from the same genetic background (C57BL/6J), were cultured for 2–3 passages in DMEMF12 media with N2 supplement and FGF2 as described elsewhere<sup>29</sup>. Plasmid and siRNA transfections were performed by electroporation (Lonza/Amara Biosystem). *Luciferase* activity was measured with the Dual-*Luciferase* reporter assay system (Promega) according to the manufacturer's protocol. Chromatin immunoprecipitation (ChIP) assays were performed following the manufacturer's protocol using a kit from Millipore/Upstate. Antibodies used were anti-MeCP2 and IgG (Upstate). After IP, recovered chromatin fragments were subjected to PCR using primers for the rat L1 sequence. QPCR values were normalized to the IgG precipitation and shown as fold enrichment. For human iPSC derivation, RTT and control fibroblasts were infected with retroviral vectors containing the *Oct4*, *c-Myc*, *Klf4* and *Sox2* human cDNAs as described previously by Yamanaka's group<sup>30</sup>. iPSC-derived neural progenitors were electroporated (Lonza/Amara Biosystem) with L1-EGFP plasmid and FACS sorted for EGFP to quantify L1 *de novo* insertion. Single-cell genomic quantitative PCR (qPCR) was performed in cell cycle arrested neuroepithelial cells and fibroblasts from wt and MeCP2 KO mice. The plates containing 1 cell/well were then snap frozen at  $-80^{\circ}\text{C}$  until the day of the qPCR. The qPCR was performed using the protocol available on the manufacturer's website (Applied Biosystems). Briefly, a solution containing forward/reverse primers and SYBR Green PCR Master Mix was added to the previously sorted cells and the detection of DNA products was carried out in a ABI PRISM 7900HT Sequence Detection System. For multiplex genomic qPCR in human tissues the qPCR strategy and L1 copy estimation were done as previously described<sup>8</sup>.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

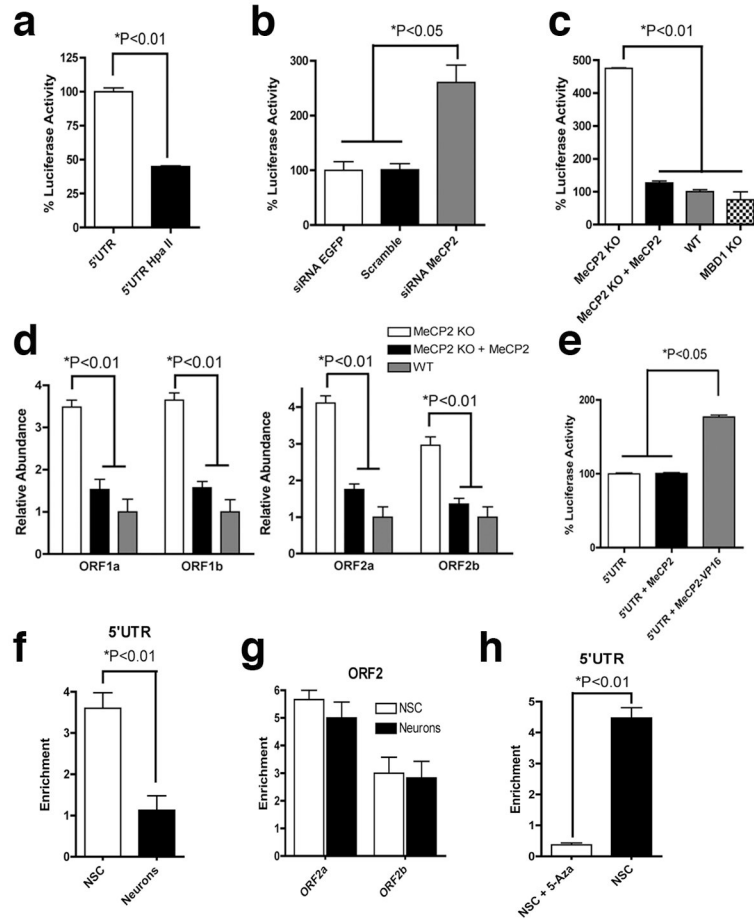
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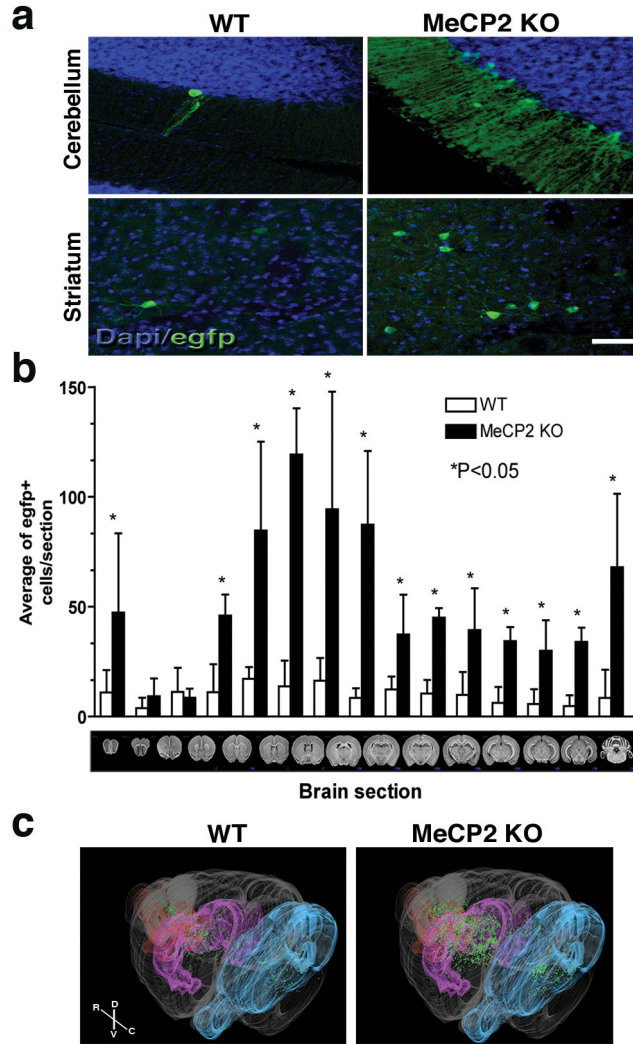
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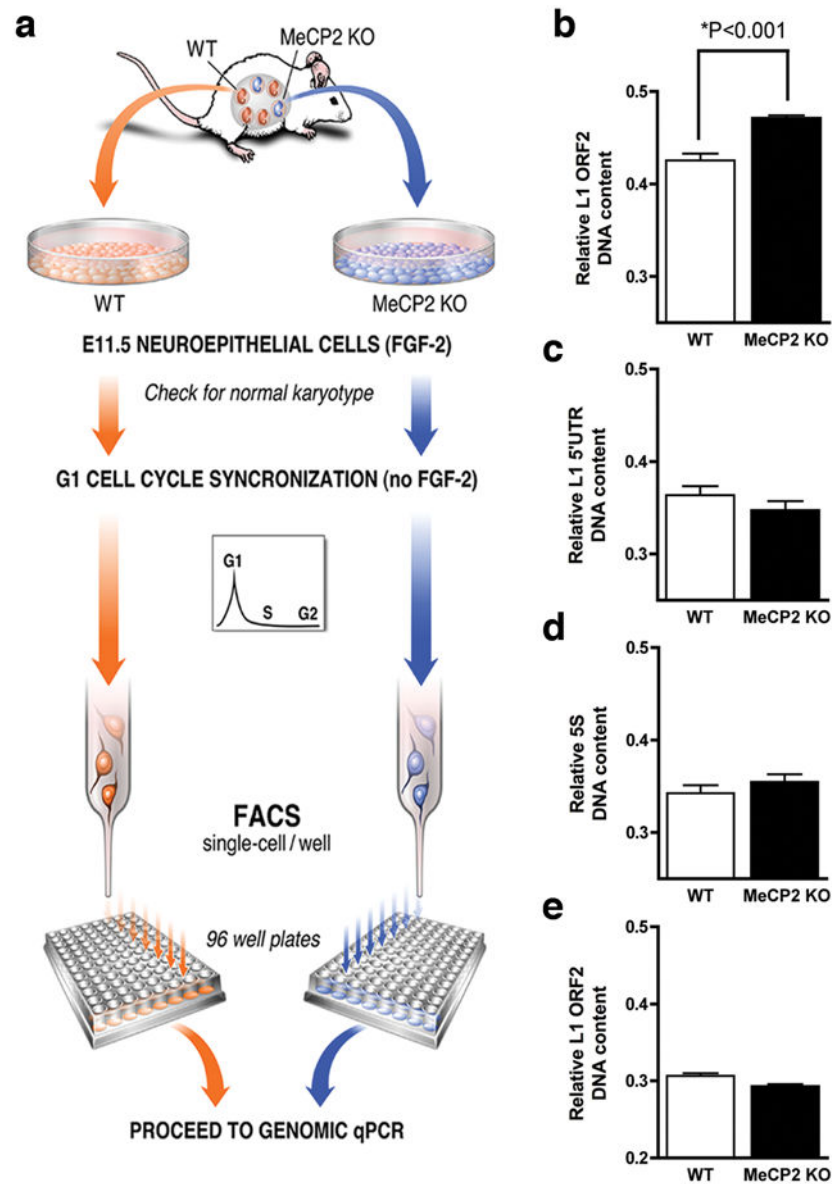


**Fig. 1.** MeCP2 silences L1 expression. **a**, Methylation of the L1 5'UTR-*Luc* reduced its transcriptional activity. **b**, Reduction of MeCP2 transcripts correlates with increased L1 promoter activity. **c**, Increased L1 promoter activity in the absence of MeCP2 but not MBD1. **d**, L1 RNA levels correlate with MeCP2 expression. **e**, Expression of the MeCP2-VP16 increased the activity of the L1 5'UTR promoter. **f** and **g**, Recruitment of MeCP2 on L1 sequences by ChIP in neural stem cells (NSC) or neurons, using 5'UTR primers (**f**) and two ORF2 regions (**g**). **h**, Occupancy of MeCP2 on the L1 promoter requires DNA methylation. Removal of DNA methylation with 5-Azacytidine (5-Aza) reduced MeCP2 association to L1 promoter. ChIP-qPCR shows enrichment over IgG control precipitation. All experiments show experimental triplicates. Error bars in all panels show s.e.m.

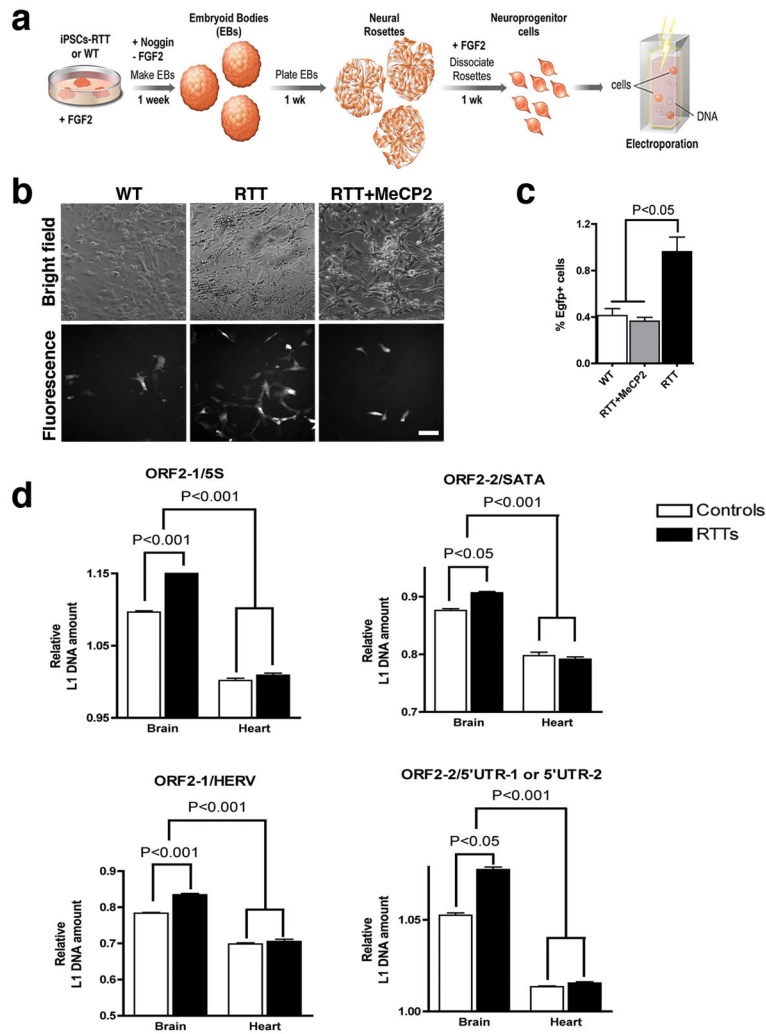




**Fig. 2.** MeCP2 modulates neuronal L1 retrotransposition *in vivo*. **a**, EGFP-positive cells, indicating *de novo* L1 retrotransposition, were found in several regions of the brain. The images were taken from sections that were highly affected by L1 retrotransposition. Bar=30  $\mu$ m. **b**, Quantification of brain sections in MeCP2 KO background revealed more EGFP-positive cells compared to WT (n=6 animals for each group). Error bars show s.d. **c**, Representative images from a 3-dimensional reconstruction of WT and MeCP2 KO brains carrying the L1-EGFP transgene. Single dots (green) represent neurons that supported L1-EGFP retrotransposition. Olfactory bulb is shown in red, striatum in magenta and cerebellum in cyan. R, rostral; C, caudal; D, dorsal and V, ventral.



**Fig. 3.** Endogenous L1 retrotransposition in mouse neuroepithelial cells. **a**, Neuroepithelial cells harvested from E11.5 sibling embryos were synchronized and sorted in individual wells followed by qPCR. **b**, Neuroepithelial cells in the MeCP2 KO background had higher L1 ORF2 DNA content than WT cells ( $P<0.001$ ). **c**, L1 5'UTR primers did not reveal a significant increase in copy number in MeCP2 KO background. **d**, Non-mobile 5S ribosomal genes were used as controls. **e**, The difference in the amount of L1 ORF2 DNA in fibroblasts from the different genetic backgrounds was smaller than in the neural lineage. All experiments show experimental triplicates ( $n=192$  cells for each primer pair). Error bars in all panels show s.e.m.



**Fig. 4.** L1 retrotransposition in RTT patients. **a**, Schematic view of the NPC differentiation from iPSC followed by L1<sub>RE3</sub>-EGFP electroporation. **b**, Representative images of iPSC-derived NPC expressing EGFP after L1 retrotransposition. Bar=30  $\mu$ m. **c**, Quantification of the EGFP-positive cells after transfection. **d**, Primers for ORF2 were used to multiplex with primers for control sequences, such as the 5S ribosomal gene (5S), the satellite alpha (SATA) region, the human endogenous retrovirus H (HERV) sequence and the 5'UTR. The inverse ratio of ORF2/5S represents the amount of L1 ORF2 sequence in each sample (n=5 individuals/group). Similar results were obtained when different primers/probe for ORF2 (ORF2-2) were multiplex/normalized to other control sequences, using two pair of primers (5'UTR-1 or 5'UTR-2). Error bars show s.e.m, and the experiments were performed in triplicate.