Supplemental File 1

The young females with classical RTT and with “fruste” RTT were diagnosed according to the international criteria based on the Rett Syndrome Diagnostic Criteria Work Group (Trevathan E and Moser HW, Ann Neurol 23:425–428, 1988). The patient with classical RTT had a de novo mutation (R270X), the patient with “fruste” form had a de novo mutation (c.1164-1207del). Mutations were detected according the procedure described by Roa’s group (Buyse IM et al., Am. J. Hum. Genet. 67:1428–1436, 2000). We used a two-tiered molecular diagnostic approach for RTT, to increase test efficiency while maintaining the sensitivity provided by sequence analysis. Prior to targeted sequence analysis, the patients’ genomic DNA was analyzed for sequence variations/mutations with Denaturing high-performance liquid chromatography (DHPLC) method, which is a highly sensitive PCR-based technique for nucleotide variant detection. DHPLC relies on the principle of heteroduplex analysis by ion-pair reverse-phase liquid chromatography under partially denaturing conditions.

METHODS

DNA extraction and PCR
Genomic DNA from patients with RTT was extracted from blood leukocytes using the QIAamp DNA Blood kit (Qiagen), following the manufacturer’s instructions.

PCR primers were designed to amplify MECP2 coding exons 2, 3, and 4. Primers for coding exons 2 and 3 correspond to flanking intron sequences. Exon 4 was amplified as four overlapping fragments that collectively span the 5’ intronic sequence and 3’ UTR sequences. Primers were synthesized with universal M13 tails (J21M13 or M13 reverse), to facilitate direct sequencing using dye-primer chemistry. PCR reactions were carried out in 50-µl reaction volumes containing 100 ng genomic DNA, 1X PCR buffer, 200 µM dNTP, 2 pmol of each primer, and 1.25 U Taq polymerase (Applied Biosystems).

DHPLC Analysis
Heteroduplex formation was induced by heat denaturation of PCR products at 94°C for 5 min, followed by gradual reannealing from 94°C to 25°C over 45 min. DHPLC analysis was performed with the WAVE DNA-fragment analysis system (Transgenomic). PCR products were eluted with a linear acetonitrile gradient. The values of the buffer gradients (buffer A, 0.1 M triethylammonium-acetate; buffer B, 0.1M triethylammoniumacetate/25% acetonitrile), start and end points of the gradient, and melting temperature predictions were determined by WAVEMAKER software (Transgenomic). Optimal run temperatures were empirically determined; mobile-phase temperatures were assessed within a 5°C window above and below the suggested run temperature on the basis of each fragment’s characteristic melting profile. Data analysis was based on visual inspection of the chromatograms, and comparison with normal controls was included in each run. Heterozygous profiles were detected as distinct elution peaks from homozygous wild-type peaks.

Direct Sequencing Analysis
The two-step diagnostic strategy calls for sequence analysis of samples that are positive by DHPLC analysis. PCR products used for sequencing analysis were purified using the QIAquick PCR purification kit (Qiagen) and bidirectionally sequenced using the ABI PrismBigDye Primer Cycle Sequencing Ready Reaction kit (Applied Biosystems). Samples were analyzed on an ABI PRISM 310 genetic analyzer (Applied Biosystems), according to the manufacturer’s instructions. Patient sequence data from both orientations were aligned for comparison with corresponding wild-type sequence using SEQUENCHER 3.0 analysis software.
Supplemental File 2

A

|                  | RTT-MSC | CTRL-MSC |
|------------------|---------|----------|
| WT MECP2         | 48.3±7.0| 46.4±7.1 |
| Mutated MECP2    | 35.1±4.7(*)| -        |

B

![Expression analysis of neural differentiation markers by RT-PCR.](chart)

C

|                  | RTT-MSC | CTRL-MSC |
|------------------|---------|----------|
| Progenitor Cell  |         |          |
| (NeuN -)         |         |          |
| WT MECP2         | 16.1±2.0| 17.0±3.3 |
| Mutated MECP2    | 30.0±4.0(*)| -        |
| Neuron-like Cell |         |          |
| (NeuN +)         |         |          |
| WT MECP2         | 13.8±3.1| 15.1±1.9 |
| Mutated MECP2    | 33.3±3.7(*)| -        |

A - NeuN staining. The table shows the mean expression percentages of NeuN positive cells (± SD, n = 3). The symbol (*) indicates statistically differences (p<0.05) among mutated-MECP2 and wild type cells in RTT patient with respect to control MSCs.

B – Expression analysis of neural differentiation markers by RT-PCR. The mRNA levels were normalized to HPRT as an internal control. The histogram shows the mean expression values (± SD, n = 3) of NSE and MAP2 in both proliferating and in differentiated MSCs. Statistical analysis was carried out comparing the same cell type among RTT patient with respect to control MSCs. The symbol (**) indicates highly statistically differences (p<0.01). The comparative cycle threshold (Ct) method was employed for quantitative analysis.

C - Senescence assay in differentiated MSCs. The table shows the percentage of senescent cells for each cell type (± SD, n = 3). Statistical analysis was carried out comparing the same cell type among mutated-MECP2 and wild type cells in RTT patient with respect to control MSCs. The symbol (*) indicates statistically differences (p<0.05).
A – MECP2 silencing in proliferating neuroblastoma cells. To induce shRNA transcription, the synthetic RSL1 ligand was added to SK-N-BE(2)-C cell cultures that had been co-transfected with pNEBR-R1 (regulator plasmid) and either pNEBR-X1-MECP2-4398, or pNEBR-X1-MECP2-1229, or pNEBR-X1-CTRL. MECP2 silencing was assessed by RT-PCR. The mRNA levels were normalized to HPRT as an internal control. The histogram shows the mean expression values of mRNA levels (± SD, n = 3). The comparative cycle threshold (Ct) method was used for the quantitative analysis.

B - NeuN staining to assess neural differentiation in cells with silenced MECP2. The table shows the mean expression values of NeuN-positive and negative cells (± SD, n = 3) (*p<0.05).

C – Senescence and annexin assays in MECP2-silenced neuroblastoma cells that were induced to differentiate into neuronal-like cells. The differentiation procedure was performed both in shMECP2-NBs and in shCTRL-NBs.
The table shows the percentage of senescent and apoptotic cells for each cell type (± SD, n = 3). Statistical analysis was carried out comparing the same cell type among samples with silenced MECP2 and control. The symbol (*) indicates statistically differences (p<0.05).
| GENE | PRIMER | SEQUENCE | ANNEALING |
|------|--------|----------|-----------|
|      |        |          | T (°C)    |
|      |        |          | LENGTH (bp) |
|      |        |          | GENE ID |

| GENE | PRIMER | SEQUENCE | ANNEALING |
|------|--------|----------|-----------|
| MECP2 | 6308 | 5'-TGAGATGGCTGGTGACATTACAG-3' | 61 |
|       | 6408 | 5'-TCCACCTTCATCACTCCCA-3' | 61 |
| MECP2 | 376  | 5'-GAAGGGAGGGAGAGGAGAC-3' | 58 |
|       | 376  | 5'-GAACCTGACCACTCTTGATGT-3' | 58 |
| HPRT  | 223  | 5'-TGAACTCTTGGCTGAGATGG-3' | 59 |
|       | 323  | 5'-CCACAGGTCAGCAGAAAGAATTT-3' | 59 |
| ACHE  | 1518 | 5'-TTTTAGACACGGTGGCTCCCA-3' | 61 |
|       | 1650 | 5'-GCGAAGATTTTCTCTCTGCC-3' | 61 |
| TH    | 767  | 5'-CTGATTCCTGAGATGGCCTTC-3' | 62 |
| DBH   | 690  | 5'-ATATGCTTCCCCGTAGC-3' | 62 |
| DAT   | 713  | 5'-TGCAACAACTCTCGGAACAGC-3' | 60 |
|       | 834  | 5'-CCACCTTCATCACTCCCA-3' | 60 |
| NSE   | 1633 | 5'-GCCCGAATCTCCCTGTGATTG-3' | 61 |
|       | 1741 | 5'-AACTGCAACCCCAATAGA-3' | 61 |
| MAP2  | 565  | 5'-TGCAACACTCATCACCCTGA-3' | 60 |
|       | 675  | 5'-CTCCTACATGACACCTTGTCA-3' | 60 |

Supplemental File 4
|                      | RTT-MSC       | CTRL-MSC  |
|----------------------|---------------|-----------|
| WT MECP2             | 41.4±6.2      | 43.7±7.8  |
| Mutated MECP2        | 30.5±3.9 (*)  | -         |

Supplemental File 5
|                          | RTT-MSC   | CTRL-MSC |
|--------------------------|-----------|----------|
| **Progenitor Cell (NeuN -)** |           |          |
| WT MECP2                 | 15.4±1.7  | 14.2±1.8 |
| Mutated MECP2            | 10.2±1.3(†) | -        |
| **Neuron-like Cell (NeuN +)** |         |          |
| WT MECP2                 | 10.7±1.8  | 11.3±1.5 |
| Mutated MECP2            | 9.0±1.3   | -        |

Supplemental File 6
Legend of supplemental file 8, 9 and 10
BrdU assay. The micrographs show a representative field of BrdU (brown) and MECP2 (purple) staining in MSC cultures. Double labeled cells appear blackish. PR = purple; BR = brown; BL = blackish.
**SUPPLEMENTAL FILE 11**

**Detailed immunostaining procedures**

*BrdU and MECP2 immunodetection (see figure 1B)*
For BrdU immunostaining, the cells were grown on glass coverslips and incubated for 10 hours with 10 μM BrdU (Sigma Aldrich, MO, USA). Briefly, the cells were rinsed with PBS, fixed with 100% methanol at 4°C for 10 min, air-dried, and then incubated with 2 N HCl for 30 min at 37°C. HCl was neutralized with 0.1 M borate buffer (pH 8.5). Following additional PBS washes, the slides were incubated with an anti-BrdU rabbit polyclonal antibody (1:200) (Bioss Inc., MA, USA). After 60 min at RT, the cells were washed with PBS and incubated with goat anti-rabbit secondary antibodies conjugated to peroxidase (Santa Cruz Biotech, CA, USA) for 60 min at RT. Finally, after additional washes in PBS, the slides were treated with DAB substrate (Roche, Germany). Following BrdU staining, cells were washed three-five times with PBS and were incubated over-night at 4°C with anti-MECP2 (clone Mec-168) primary antibody (1:200) (Abcam, UK). Subsequently, cells were washed with PBS and then incubated with goat anti-mouse secondary antibodies, conjugated to alkaline phosphatase (Santa Cruz Biotech, CA, USA) and then treated with the VECTOR Red Alkaline Phosphatase Substrate Kit (Vector Laboratories, CA, USA). The percentage of MECP2-positive and/or BrdU-positive cells was calculated by counting at least 500 cells in different microscopic fields.

BrdU-positive cells appeared brown and MECP2-positive cells were red purple. Double labeled cells appear blackish.

*Annexin and MECP2 immunodetection (see figure 1C)*
Apoptotic cells were detected using fluorescein-conjugated Annexin V (Roche, Italy) on unfixed cells according to the manufacturer’s instructions. Following annexin staining, cells were washed three-five times with PBS and were fixed with 4% paraformaldehyde for 15 min. Cells then were incubated over-night at 4°C with anti-MECP2 (clone Mec-168) primary antibody (1:200) (Abcam, UK). Subsequently, cells were washed with PBS and then were incubated with goat anti-mouse secondary antibodies, conjugated to Texas Red (Jackson Laboratories, MA, USA), and the nuclei were counterstained with Hoechst 33342.

In each experiment, at least 1,000 cells were counted in different fields to calculate the percentage of MECP2-positive and MECP2-negative dead cells in each culture. Annexin V positive cells appeared green and MECP2-positive cells were red. Nuclei were blue stained.

*Senescence-associated beta-galactosidase and MECP2 immunodetection (see figure 1D)*
The cells were fixed for 5 min with a solution of 2% formaldehyde and 0.2% glutaraldehyde. The cells were then washed with PBS and incubated at 37°C for at least 2 hours with a staining solution, according to the manufacturer’s protocol (Roche, Italy).

Following senescence staining, cells were washed three-five times with PBS and were incubated over-night at 4°C with anti-MECP2 (clone Mec-168) primary antibody (1:200) (Abcam, UK). Subsequently, cells were washed with PBS and then incubated with goat anti-mouse secondary antibodies, conjugated to peroxidase (Santa Cruz Biotech, CA, USA) and then treated with the DAB substrate (Vector Laboratories, CA, USA). The percentage of MECP2-positive and/or beta-galactosidase-positive cells was calculated by counting at least 500 cells in different microscopic fields.

Beta-galactosidase-positive cells appeared blue and MECP2-positive cells were brown.

*Neuronal Nuclei (NeuN) and MECP2 detection (see figure 2B)*
Cells were fixed for 15 min with 4% paraformaldehyde. NeuN was detected by immunocytochemistry using the anti-NeuN primary rabbit polyclonal antibody (Millipore Italy,
Italy) according to the manufacturer’s protocol. Cells were incubated with goat anti-rabbit secondary antibodies conjugated to FITC (Jackson Laboratories, MA, USA).

Following NeuN staining, cells were washed with PBS and fixed again for 5 min with 4% paraformaldehyde.

Cells then were incubated over-night at 4°C with anti-MECP2 (clone Mec-168) primary antibody (1:200) (Abcam, UK). Subsequently, cells were washed with PBS and then were incubated with goat anti-mouse secondary antibodies, conjugated to Texas Red (Jackson Laboratories, MA, USA), and the nuclei were counterstained with Hoechst 33342.

In each experiment, at least 1,000 cells were counted in different fields to calculate the percentage of MECP2-positive and NeuN-positive cells in each culture.

NeuN-positive cells appeared green and MECP2-positive cells were red. Nuclei were blue stained.

Annexin, MECP2 and NeuN immunodetection (see figure 2C)
Apoptotic cells were detected using fluorescein-conjugated Annexin V (Roche, Italy) on unfixed cells according to the manufacturer’s instructions. Following annexin staining, cells were washed three-five times with PBS and were fixed with 4% paraformaldehyde for 15 min. Cells then were incubated over-night at 4°C with anti-MECP2 (clone Mec-168) primary antibody (1:200) (Abcam, UK). Subsequently, cells were washed with PBS and then were incubated with goat anti-mouse secondary antibodies, conjugated to Texas Red (Jackson Laboratories, MA, USA).

Following MECP2 staining, cells were washed with PBS and fixed again for 5 min with 4% paraformaldehyde. NeuN was detected by immunocytochemistry using the anti-NeuN primary rabbit polyclonal antibody (Millipore Italy, Italy) according to the manufacturer’s protocol. Cells were incubated with goat anti-rabbit secondary antibodies conjugated to AMCA (Jackson Laboratories, MA, USA). At the end of immunodetection procedures, nuclei were counterstained with hematoxylin.

In each experiment, at least 1,000 cells were counted in different fields to calculate the percentage of MECP2-positive, NeuN positive and annexin positive-cells.
Annexin V positive cells appeared green, MECP2-positive cells were red and NeuN cells were blue stained. Nuclei appeared purple in light microscopy fields.

Senescence-associated beta-galactosidase, MECP2 and NeuN immunodetection (see figure 2D)
The cells were fixed for 5 min with a solution of 2% formaldehyde and 0.2% glutaraldehyde. The cells were then washed with PBS and incubated at 37°C for at least 2 hours with a staining solution, according to the manufacturer’s protocol (Roche, Italy).

Following senescence staining, cells were washed three-five times with PBS and were incubated over-night at 4°C with anti-MECP2 (clone Mec-168) primary antibody (1:200) (Abcam, UK). Subsequently, cells were washed with PBS and then incubated with goat anti-mouse secondary antibodies, conjugated to peroxidase (Santacruz Biotech, CA, USA) and then treated with the DAB substrate (Vector Laboratories, CA, USA).

Following MECP2 and beta-galactosidase staining, cells were washed with PBS and incubated with the anti-NeuN primary rabbit polyclonal antibody (Millipore Italy, Italy) according to the manufacturer’s protocol. Then cells were incubated with goat anti-rabbit secondary antibodies, conjugated to peroxidase (Santacruz Biotech, CA, USA) and treated with the ImmPACT VIP substrate (Vector Laboratories, CA, USA).

In each experiment, at least 1,000 cells were counted in different fields to calculate the percentage of MECP2-positive, NeuN positive and beta-galactosidase positive-cells.
Beta-galactosidase-positive cells appeared blue, MECP2-positive cells were brown and NeuN cells were purple. MECP2 and NeuN positive cells were blackish.

Annexin and NeuN immunodetection (see figure 3E and 4D)
Apoptotic cells were detected using fluorescein-conjugated Annexin V (Roche, Italy) on unfixed cells according to the manufacturer’s instructions. Following annexin staining, cells were washed three-five times with PBS and were fixed with 4% paraformaldehyde for 15 min.
NeuN was detected by immunocytochemistry using the anti-NeuN primary rabbit polyclonal antibody (Millipore Italy, Italy) according to the manufacturer’s protocol. Cells were incubated with goat anti-rabbit secondary antibodies conjugated to Texas red (Jackson Laboratories, MA, USA). At the end of immunodetection procedures, nuclei were counterstained with Hoechst 33342. In each experiment, at least 1,000 cells were counted in different fields to calculate the percentage of NeuN positive and annexin positive-cells. Annexin V positive cells appeared green, NeuN-positive cells were red. Nuclei were blue stained.

**Senescence-associated beta-galactosidase and NeuN immunodetection (see figure 3D and 4C)**

The cells were fixed for 5 min with a solution of 2% formaldehyde and 0.2% glutaraldehyde. The cells were then washed with PBS and incubated at 37°C for at least 2 hours with a staining solution, according to the manufacturer’s protocol (Roche, Italy).

Following senescence staining, cells were washed three-five times with PBS and were incubated over-night at 4°C the anti-NeuN primary antibody (Millipore Italy, Italy) according to the manufacturer’s protocol. Subsequently, cells were washed with PBS and then incubated with goat anti-rabbit secondary antibodies, conjugated to peroxidase (Santacruz Biotech, CA, USA) and then treated with the DAB substrate (Vector Laboratories, CA, USA).

The percentage of NeuN-positive and/or beta-galactosidase-positive cells was calculated by counting at least 500 cells in different microscopic fields. Beta-galactosidase-positive cells appeared blue and NeuN-positive cells were brown.