**MECP2 mutations affect ciliogenesis: a novel perspective for Rett syndrome and related disorders**

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Abstract

Mutations in MECP2 cause several neurological disorders of which Rett syndrome (RTT) represents the best-defined condition. Although mainly working as a transcriptional repressor, MeCP2 is a multifunctional protein revealing several activities, the involvement of which in RTT remains obscure. Besides being mainly localized in the nucleus, MeCP2 associates with the centrosome, an organelle from which primary cilia originate. Primary cilia function as “sensory antennae” protruding from most cells, and a link between primary cilia and mental illness has recently been reported. We herein demonstrate that MeCP2 deficiency affects ciliogenesis in cultured cells, including neurons and RTT fibroblasts, and in the mouse brain. Consequently, the cilium-related Sonic Hedgehog pathway, which is essential for brain development and functioning, is impaired. Microtubule instability participates in these phenotypes that can be rescued by HDAC6 inhibition together with the recovery of RTT-related neuronal defects. Our data indicate defects of primary cilium as a novel pathogenic mechanism that by contributing to the clinical features of RTT might impact on proper cerebellum/brain development and functioning, thus providing a novel therapeutic target.

Keywords MeCP2; primary cilium; Rett syndrome; sonic hedgehog; tubacin treatment

Subject Categories Genetics, Gene Therapy & Genetic Disease; Neuroscience

DOI 10.15252/emmm.201910270 | Received 3 January 2019 | Revised 19 March 2020 | Accepted 26 March 2020 | Published online 8 May 2020

EMBO Mol Med (2020) 12: e10270

Introduction

The Methyl-CpG-binding Protein 2 (MECP2) gene, localized on the X chromosome, codes for a multifunctional epigenetic regulator with a prominent role in brain. Accordingly, MECP2 mutations are linked to several neurological conditions characterized by cognitive impairment and intellectual disability (Ezeonwuka & Rastegar, 2014). In particular, loss-of-function mutations are mainly associated with Rett (RTT) syndrome, a severe neurodevelopmental disease that principally affects females (Amir et al., 1999; Chahrour & Zoghbi, 2007). Less frequently, MECP2 mutations cause autism, schizophrenia, mental retardation, Angelman-like syndrome in both genders and neonatal encephalopathy in males (Ezeonwuka & Rastegar, 2014). In parallel, a non-physiological increase in MeCP2 expression is responsible for the recently identified MECP2 duplication syndrome, mainly affecting males (Ramocki et al., 2010). In accordance with its ubiquitous expression, MECP2 has also been linked to non-neurological diseases, such as lupus erythematosus, rheumatoid arthritis and cancer (Ezeonwuka & Rastegar, 2014).

Originally isolated as the first protein able to specifically bind methylated cytosines, MeCP2 is generally described as an epigenetic transcriptional regulator that represses transcription of methylated DNA. This repressive activity is mainly mediated by the ability of MeCP2 to recruit corepressor complexes able to modify chromatin structure (Clouaire & Stancheva, 2008). In addition to its proposed role in gene silencing and chromatin architecture, several other functions have more recently been ascribed to MeCP2. Indeed, nowadays MeCP2 appears as a multifunctional protein that manifests different activities depending on its partners and post-translational modifications (Young et al., 2005; Chahrour et al., 2008; Ghosh et al., 2010; Skene et al., 2010; Bedogni et al., 2014; Bellini et al., 2014).

Accordingly, we have recently demonstrated that MeCP2 is functionally associated with centrosomes and, similarly to many...
centrosomal proteins, its deficiency causes aberrant spindle geometry, defects in cell proliferation, microtubule nucleation and prolonged mitosis. This novel function reconciled previous data regarding a role of MeCP2 in cell growth, cytoskeleton stability, and axonal transport (Bergo et al., 2015). While in cycling cells, the centrosome organizes the interphase microtubule network and the mitotic spindle, in most quiescent or post-mitotic non-cycling cells, it participates in the nucleation of primary cilia (Stearns, 2001). We thus decided to investigate whether MeCP2 defects could play a role also in primary cilium formation and functioning.

Primary cilium is a non-motile microtubule-based organelle that grows from a centrosome-derived structure, termed the basal body, and protrudes from the cell surface of almost every quiescent or differentiated mammalian cell, including neurons (Guemez-Gamboa et al., 2014). It consists of an axoneme, formed by a radial array of nine doublet microtubules of polymerized α/β-tubulin heterodimers, and the process of intraflagellar transport (IFT) is responsible for building and maintaining cilium structure and function (van Reeuwijk et al., 2011; Pala et al., 2017; Youn & Han, 2018). Primary cilium is a sensory organelle that receives and transduces many extracellular signals, thereby influencing a wide variety of cellular processes, such as proliferation, differentiation, migration, and apical–basal polarity in cortical development and neuronal growth (van Reeuwijk et al., 2011; Pala et al., 2017; Youn & Han, 2018; Park et al., 2019). In accordance with its main function, several receptors for signaling cascades, such as Sonic Hedgehog (Shh), PDGF, mTOR, Wnt, Notch, planar cell polarity (PCP) and Hippo, accumulate in the membrane of primary cilium (Goetz & Anderson, 2010; Pala et al., 2017; Wheway et al., 2018). The importance of this organelle in normal physiology is exemplified by a growing list of genetic disorders, known as “cilipathies”, caused by dysfunctional ciliary assembly, anchoring and/or signaling, characterized by a high variability in clinical presentation (Novarino et al., 2011; Ware et al., 2011; Lee & Gleeson, 2014; Valente et al., 2014; Reiter & Leroux, 2017). Interestingly, among the variety of clinical features, some symptoms such as developmental delay, cognitive disabilities, ataxia, tendency for obesity, bone loss and defects in respiration rhythms are also manifested by RTT patients (Kyle et al., 2011). Furthermore, a recent study has indicated that among 41 genes connected to diverse neuropsychiatric disorders, 23 are involved in primary cilium assembly and disassembly (Marley & von Zastrow, 2012).

Cilium assembly/disassembly depends on a great number of different factors that have to be optimally tuned in order to generate cilia of the right size and length, thereby ensuring proper functioning (Keeling et al., 2016). Among these factors, acetylation of lysine (K) 40 of α-tubulin is pivotal for maintaining primary cilium; indeed, its HDAC6-dependent deacetylation leads to the disassembly of the organelle (Fornioli-Conti et al., 2016). Importantly, MECP2-deficient cells, including fibroblasts from RTT patients, are characterized by the upregulation of HDAC6 and, consequently, reduced acetylation of polymerized α-tubulin and microtubule instability (Delépine et al., 2013, 2016; Gold et al., 2015).

Considering all above, for the first time, we have investigated and identified primary cilium dysfunctions in vitro in all tested cells, including fibroblasts from RTT patients, and in vivo in Mecp2 null and heterozygous brains, demonstrating a causal connection between MeCP2 expression and cilogenesis. Importantly, these defects reflect, both in vitro and in vivo, a functional impairment of the ciliary-related Shh signaling pathway. Stabilization of α-tubulin, through a selective inhibition of HDAC6, can revert the observed morphological and functional ciliary alterations, in concomitance with a recovery of RTT-related phenotypes in Mecp2 null neurons.

Results

Primary cilium formation is facilitated by MeCP2

As mentioned above, we have recently demonstrated a molecular and functional association between MeCP2 and the centrosome, the cellular organelle that templates the assembly of primary cilium (Bergo et al., 2015). Almost every quiescent or post-mitotic mammalian cell presents a primary cilium; we thus investigated whether MeCP2 deficiency affects the presence and/or length of the primary cilium on the cell surface.

First, we compared wild-type (WT) and Mecp2 null mouse quiescent embryonic fibroblasts (MEFs). Ciliated cells were detected by immunoﬂuorescence staining for acetylated α-tubulin and γ-tubulin, two microtubule proteins that are enriched, respectively, in the axoneme and the basal body of the cilium, where they are critical for maintaining its structure (Fig 1A). As shown in Fig 1B, the percentage of ciliated cells was significantly decreased by 38% compared to WT cells (***P < 0.01, Mann–Whitney test). Since almost 53% of Mecp2 null cells showed a primary cilium, we measured its length. Nascent or extended acetylated α-tubulin emerging from the γ-tubulin-positive centriole was measured, revealing that Mecp2 null MEFs show a statistically signiﬁcant reduction in cilium length (***P < 0.001, Student’s t-test; Fig 1C). Similar results were obtained analyzing MEFs from a Mecp2<sup>y120D/y</sup> knock-in mouse mimicking a pathogenic human mutation (*P < 0.05, Mann–Whitney test; Fig EV1; Gandaglia et al., 2018). To support these data, we repeated the same analyses in hTERT-RPE-1 (immortalized retinal pigment epithelial) cells interfered with a specific MeCP2-siRNA, reducing MeCP2 expression to less than half, as revealed by Western blot (Fig 1E). Accordingly, immunoﬂuorescence analysis proved that almost 40% of cells show a positive MeCP2 signal (***P < 0.001, Student’s t-test; Fig 1F). Primary cilia were stained and analyzed (Fig 1D). Quantitative analyses indicated that the percentage of ciliated cells was signiﬁcantly reduced compared to control (**P < 0.01, Mann–Whitney test; Fig 1G) and, when present, cilium length was signiﬁcantly decreased in silenced cells (***P < 0.001, Student’s t-test; Fig 1H). Since Rett syndrome is a neurological disorder affecting both neurons and astrocytes, we also analyzed cilogenesis in these cells. Primary neurons derived from WT or Mecp2 null embryonic cortices were fixed at different maturation stages and cilium was detected by immunostaining for adenylate cyclase type 3 (AC3), a protein expressed in the primary cilium of neurons (Bishop et al., 2007; Fig 1I). Indeed, it is well recognized that the antibody against acetylated α-tubulin, which is commonly used to mark the primary cilium in several non-neuronal cell types, in neurons causes a diffuse staining throughout the cell body and neurites, thus preventing its use as valid cilium marker (Berbari et al., 2013). The staining was conducted at DIV3, DIV7 and DIV14. Results revealed an impairment in the percentage of ciliated cells and in cilium length.
Figure 1.

A

**MEFs**

|         | WT | Mecp2 null |
|---------|----|------------|
| actTUB-TUB |    |            |

B

**% ciliated cells**

|         | WT | Mecp2 null |
|---------|----|------------|
|         |    |            |

C

**Cilium length (μm)**

|         | WT | Mecp2 null |
|---------|----|------------|
|         |    |            |

D

**hTERT-RPE-1**

|         | CTRL | siMecp2 |
|---------|------|---------|
| actTUB-TUB |     |         |

E

**Western Blot**

- MeCP2 75kDa
- GAPDH 37kDa

F

**% Mecp2 cells**

|         | CTRL | siMecp2 |
|---------|------|---------|
|         |      |         |

G

**% ciliated cells**

|         | CTRL | siMecp2 |
|---------|------|---------|
|         |      |         |

H

**Cilium length (μm)**

|         | CTRL | siMecp2 |
|---------|------|---------|
|         |      |         |

I

**CORTICAL NEURONS**

|         | WT | Mecp2 null |
|---------|----|------------|
| DIV7    |    |            |
| DIV14   |    |            |

J

**% ciliated cells**

|         | WT | Mecp2 null |
|---------|----|------------|
| DIV3    |    |            |
| DIV7    |    |            |
| DIV14   |    |            |

K

**Cilium length (μm)**

|         | DIV3 | DIV7 | DIV14 |
|---------|------|------|-------|
|         |      |      |       |

L

**CORTICAL ASTROCYTES**

|         | WT | Mecp2 null |
|---------|----|------------|
| actTUB |    |            |

M

**% ciliated cells**

|         | WT | Mecp2 null |
|---------|----|------------|
|         |    |            |

N

**Cilium length (μm)**

|         | WT | Mecp2 null |
|---------|----|------------|
|         |    |            |
Figure 1. MeCP2 deficiency affects primary cilium formation.

A Representative immunofluorescence of primary cilium in WT or Mecp2 null MEF cells. Cells were starved for 48 h before staining with anti-γ-tubulin (green) and anti-acetylated α-tubulin (red) antibodies. Merge of all channels and DAPI staining (blue) is depicted for both genotypes. Scale bar 20 μm, and 10 μm in the enlarged image. Arrows indicate not ciliated cells.

B, C Cilia were counted and analyzed with ImageJ to quantify the percentage of ciliated cells (n = 134 WT and n = 140 Mecp2 null cells) (B) and the cilium length (n = 104 WT and n = 43 Mecp2 null cells) (C). The graphs show the average of three independent experiments (mean ± SE, **p < 0.01, Student’s t-test).

D Representative immunofluorescence of primary cilium in hTERT-RPE-1 cells silenced with MECPP2 or control siRNAs. Cells were starved for 48 h before staining with anti-γ-tubulin (green) and anti-acetylated α-tubulin (red) antibodies. Scale bar 10 μm, and 5 μm in the enlarged image. Arrows indicate not ciliated cells.

E Representative Western blotting showing the MeCP2 expression in CTRL and silenced hTERT-RPE-1 cells, with respect to GAPDH, used as loading control.

F The percentage of MeCP2-positive cells was calculated by analyzing n = 1,000 CTRL and silenced hTERT-RPE-1 cells (mean ± SE. ***p < 0.001, Student’s t-test).

G, H Cilia were counted and analyzed with ImageJ to quantify the percentage of ciliated cells (n = 191 CTRL and n = 197 siMeCP2 cells) (G) and the cilium length (n = 50 CTRL and n = 50 siMeCP2 cells) (H). The graphs show the mean ± SE of three independent experiments (**p < 0.001, Mann–Whitney test (G) or Student’s t-test (H)).

I Representative immunofluorescence of primary cilium in WT and Mecp2 null primary cortical neurons at DIV7 and DIV14. Scale bar = 20 μm, and 5 μm in the enlarged image. Arrows indicate not ciliated cells.

J, K Neurons were fixed at DIV5, DIV7, and DIV14, and primary cilium was detected by immunostaining with anti-AC3 antibody. Percentage of ciliated cells (l) and cilium length (K) were analyzed. Histograms represent the average of three independent experiments, analyzing neurons derived from 3 different experiments (n = 136 WT DIV3; n = 49 KO DIV3; n = 177 WT DIV7; n = 82 KO DIV7; n = 59 WT DIV14; and n = 37 KO DIV14) (mean ± SE, **p < 0.001, two-way ANOVA followed by Bonferroni post hoc test).

L Primary cortical astrocytes were fixed at DIV15 and stained with anti-acetylated α-tubulin (red) and anti-γ-tubulin (green) antibodies. Scale bar = 20 μm, and 10 μm in the enlarged image. Arrow indicates not ciliated cell.

M, N Percentage of ciliated cells (M) and cilium length (N) were analyzed (n = 26 WT and n = 34 Mecp2 null astrocytes). Histograms represent the average of three independent experiments (mean ± SE, *p < 0.05, Student’s t-test).

Source data are available online for this figure.

(*p < 0.05, two-way ANOVA followed by Bonferroni post hoc test), indicating a defective ciliogenesis in Mecp2 null neurons compared to controls, in front of a progressive increase of ciliogenesis in both WT and null neurons along neuronal maturation (F(2, 584) = 44.06 for % ciliated cells; F(2, 551) = 30.68 for cilium length) (Fig 1J and K). In a similar way, cortical astrocytes were analyzed by immunostaining cilia for acetylated α-tubulin and γ-tubulin (Fig 1L–N) and a significant reduction in both the percentage of ciliated cells and in cilium length was demonstrated (*p < 0.05, Student’s t-test).

To confirm that MeCP2 expression is involved in the observed phenotypes, we performed a rescue experiment on Mecp2 null primary neurons (Fig 2). WT and Mecp2 null cortical neurons were infected at DIV0 with lentiviruses expressing GFP or MeCP2iresGFP, and the expression of MeCP2 was verified by Western blot (Fig 2B). At DIV7, neuronal cilium was immunostained for AC3 (Fig 2A) and the percentage of ciliated cells and cilium length were determined only on GFP-positive cells. We confirmed the impairment in primary cilium formation in Mecp2 null neurons and demonstrated a significant recovery of the phenotype following MeCP2 expression (***p < 0.001, two-way ANOVA followed by Bonferroni post hoc test) (Fig 2C). Similarly, MeCP2 transduction fully recovered cilium length in Mecp2 null neurons (**p < 0.001; **p < 0.01, two-way ANOVA followed by Bonferroni post hoc test) (Fig 2D).

**Mecp2 deficiency affects the cilium-associated Sonic Hedgehog (Shh) signaling pathway**

Since our data demonstrate that MeCP2 deficiency is associated with morphological alterations of the primary cilium, we proceeded investigating ciliary functions. We focused our analysis on the Shh cascade, because this signaling pathway is generally recognized as the one that is mainly associated with primary cilia (Wheway et al., 2018). Shh activation can be detected by measuring the levels of various components of its pathway along the primary cilium or in a ciliary subdomain. In particular, the binding of the ligand Shh to its receptor, patched homolog 1 (Ptc1), triggers the accumulation of Smoothened (Smo) within the ciliary membrane with the consequent activation of Gli transcription factors (Fig 3A). We thus analyzed Smo localization in the primary cilium of starved hTERT-RPE-1 cells silenced for MeCP2 and treated with SAG, a Smo agonist able to activate the Shh pathway (Fig 3B). To assess the percentage of Smo-positive cilia, cells were stained for Smo and acetylated α-tubulin; the results demonstrated that MeCP2 depletion significantly impairs the ciliary accumulation of Smo upon Shh stimulation (**p < 0.001, two-way ANOVA followed by Bonferroni post hoc test). Similar results were obtained in Mecp2 null MEFs following SAG exposure and a significant difference between cells was evident when the higher dose of SAG was used (**p < 0.05, two-way ANOVA followed by Bonferroni post hoc test; Fig 3C).

To further support a functional defect of the Shh signaling pathway in Mecp2-defective cells, the protein and mRNA levels of the downstream transcription factor Gli1 were measured in cultured MEFs and neurons. Western blots from SAG-treated and untreated cells proved that the lack of MeCP2 impairs Gli1 activation (Fig 3D and E). Indeed, while a significant increase in Gli1 expression was measured both in WT MEFs and in neurons (***p < 0.001, two-way ANOVA followed by Bonferroni post hoc test), the activation of the Shh pathway did not lead to a significant Gli1 induction in Mecp2 null cells. Accordingly, Gli1 levels were significantly reduced in SAG-treated Mecp2 null cells with respect to WT (**p < 0.05; **p < 0.01, two-way ANOVA followed by Bonferroni post hoc test). By analyzing Gli1 mRNA, we confirmed the defective response to SAG in both Mecp2 null cells, although cortical neurons exhibited a trend that did not reach statistical significance (Fig 3F and G). Of note, the low expression of Gli1 mRNA in basal conditions did not permit quantitative analysis.
Mecp2 deficiency is associated with a significant shortening of primary cilia in the cerebral cortex and cerebellum

Disruption of neuronal primary cilia can induce defects in neuronal connectivity and reduce intellectual functions (Lee & Gleeson, 2014; Reiter & Leroux, 2017). Thus, we investigated whether Mecp2 deficiency could also affect ciliogenesis in vivo. Considering the involvement of cerebral cortex in RTT, we initially focused on the Mecp2 null developing cortex (P14). As shown in Fig 4A–C, in knock-out cortices, cilium length was significantly reduced compared to WT controls (*P < 0.05; Mann–Whitney test). To identify the most affected cortical layers, the analysis was conducted separately for each layer; besides a significant genotype effect (F(1, 20) = 45.61, P < 0.001), post hoc analysis indicated a significant reduction in cilium length in layers 1, 2/3, and 4 (**P < 0.01; ***P < 0.001; *P < 0.05, two-way ANOVA followed by Bonferroni post hoc test; Fig 4C).

Although primary cilium is present in most neurons, its role for cerebral cortex development and functioning still remains debated; on the contrary, the primary cilium and the associated Shh pathway are recognized as master players for cerebellum development. In particular, in the cerebellum, Shh activation starts at E17.5, peaks at P5-P8, and then progressively declines (De Smaele et al., 2008; Vaillant & Monard, 2009). We thus analyzed cilium length in P7...
cerebella, including in the study both MeCP2 null and heterozygous tissues and discriminating the external (EGL) and the internal (IGL) granular layers (Fig 4D). In good accordance with the more mature state, the WT IGL revealed a longer primary cilium than the WT EGL, while the gender did not play any effect. Importantly, a significant reduction of cilium length was measured in the two layers of both MeCP2 null and heterozygous cerebella (**P < 0.01; ***P < 0.001, Mann–Whitney test), although the magnitude of the effect was higher in knock-out tissues (Fig 4E and F). To support the observed morphological defects, we analyzed Gli1 expression...
in the MeCP2 null cerebellum, which manifests a more severe phenotype. Our results indicated a significant reduction of both Gli1 protein and mRNA (*P < 0.05, Student’s t-test; Fig 4G and H). Gli1 deficiency was supported by a transcriptional reduction of its direct targets, Cyclin D1 (*P < 0.05, Student’s t-test) and Cyclin D2 (revealing only a trend of reduction) (Fig 4H; Kenney & Rowitch, 2000).

Microtubule instability participates in the observed ciliary defects

As already mentioned, ciliary assembly and elongation are negatively influenced by microtubule instability, a defect that is known to occur when MeCP2 is deficient (Delépine et al., 2013, 2016). Previously, we have demonstrated that MeCP2 depletion affects the microtubule nucleation potential of the centrosome (Bergo et al., 2015); accordingly, RTT patients’ fibroblasts are characterized by defective microtubule polymerization and increased HDAC6 levels (Gold et al., 2015). By deacetylating α-tubulin, HDAC6 acts as a key driver of primary cilium disassembly and has already been proposed as a possible therapeutic target for ciliopathies (Yu et al., 2016).

Considering all above, we initially used tubacin, a potent, selective and cell-permeable HDAC6 inhibitor, to determine whether microtubule stabilization might recover the observed ciliary phenotypes. After starvation, WT and MeCP2 null MEFs were treated with vehicle (DMSO) or tubacin (1 μM) for 48 h and then stained for acetylated α-tubulin to detect cilia. As depicted in Fig 5A, tubacin significantly restored the percentage of ciliated MeCP2 null cells (*P < 0.05, two-way ANOVA followed by Bonferroni post hoc test) and post hoc analysis confirmed the already-known ciliary defects in MeCP2 null cells (*P < 0.05; **P < 0.01, two-way ANOVA followed by Bonferroni post hoc test). Importantly, similar results were obtained when MeCP2 null cortical neurons were treated with tubacin (Fig 5B) (*P < 0.05; **P < 0.01, two-way ANOVA followed by Bonferroni post hoc test).

To determine whether the observed morphological rescue could be associated with a functional recovery, we investigated the Shh pathway after tubacin treatment in MeCP2 silenced hTERT-RPE-1 cells. In particular, we analyzed whether the drug could rescue Smo activity in MeCP2 silenced hTERT-RPE-1 cells. Considering all above, we initially used tubacin, a potent, selective and cell-permeable HDAC6 inhibitor, to determine whether microtubule stabilization might recover the observed ciliary phenotypes. After starvation, WT and MeCP2 null MEFs were treated with vehicle (DMSO) or tubacin (1 μM) for 48 h and then stained for acetylated α-tubulin to detect cilia. As depicted in Fig 5A, tubacin significantly restored the percentage of ciliated MeCP2 null cells (*P < 0.05, two-way ANOVA followed by Bonferroni post hoc test) and post hoc analysis confirmed the already-known ciliary defects in MeCP2 null cells (*P < 0.05; **P < 0.01, two-way ANOVA followed by Bonferroni post hoc test). Importantly, similar results were obtained when MeCP2 null cortical neurons were treated with tubacin (Fig 5B) (*P < 0.05; **P < 0.01, two-way ANOVA followed by Bonferroni post hoc test).

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Figure 4.
activation of the Shh pathway in silenced cells that was rescued by tubacin treatment \((**P < 0.01, \text{three-way ANOVA followed by Tukey's post hoc test})\).

To reinforce our results, we investigated whether in concomitance with restored ciliogenesis, we could observe an amelioration of neuronal alterations that are commonly detected in Mecp2 null cells and that are relevant for Rett syndrome. WT and Mecp2 null neurons were thus treated with tubacin or TC-S 7010, a specific inhibitor of the centrosomal kinase Aurora A. In neurons, Aurora A acts by phosphorylating and activating HDAC6 at the basal body, thus promoting cillum disassembly (Pugacheva et al., 2007). Analysis of the cilium length in cortical neurons proved that both the Aurora A inhibitor (7 nM, 48 h) and tubacin (1 μM, 48 h) effectively mediated primary cilium elongation in Mecp2 null cells (Fig EV2). By Western blot, we reported that TC-S 7010, but not tubacin, maintains the unaffected levels of cytoplasmic acetylated α-tubulin, confirming the more specific action of this inhibitor at the primary cilium level (Pugacheva et al., 2007; Fig EV3).

Recent studies suggest that proper formation of neuronal dendrites requires structurally normal primary cilia and a correct
localization of signaling proteins into the cilium, thus linking this organelle to the ability of primary neurons to form correct network connections (Guadiana et al., 2013). Therefore, by Sholl analysis we investigated whether tubacin and TC-S 7010 could ameliorate the typical reduction in dendritic complexity in MeCP2 null neurons (DIV7) (**P < 0.01, Mann–Whitney test; Bedogni et al., 2016). Figure 6A, B proves that both molecules significantly increased dendritic branching, thus recovering defective morphology (*P < 0.05; **P < 0.01, one-way ANOVA followed by Dunn’s test).

We next analyzed whether HDAC6 inhibition was also effective in ameliorating the synaptic phenotypes of null neurons (Fig 6C–F). As expected, the analysis of synaptic puncta density in MeCP2 null DIV14 cortical neurons reported a significant reduction of both pre-synaptic Synapsin1/2 and post-synaptic Shank2 puncta (**P < 0.01; ***P < 0.001, Mann–Whitney test; Fig 6D and E). Interestingly, while TC-S 7010 reverted the defects both at the pre- and the post-synaptic compartments (***P < 0.001, one-way ANOVA followed by Dunn’s test), tubacin restored only the density of pre-synaptic puncta (*P < 0.05, by one-way ANOVA followed by Dunn’s test). As a measure of functional synapses, we next analyzed the co-localization of Synapsin1/2 with Shank2 puncta. As expected, our data highlighted a defective co-localization in MeCP2 null neurons compared

Figure 6.
to WT cells (**p < 0.001, Mann–Whitney test), which was rescued only by TC-S 7010 (*p < 0.05, one-way ANOVA followed by Dunn’s test; Fig 6F).

**Discussion**

Rett syndrome is a complex neurodevelopmental disorder characterized by a plethora of clinical alterations affecting both the central and the peripheral system, leading to the manifestation of several symptoms. No treatment is available to combat the primary pathology for any of the MECP2-pathies and patients are treated only to alleviate clinical symptoms. The lack of a full comprehension of the molecular mechanisms causing MECP2-related pathologies, including Rett syndrome, certainly slows down the identification of effective therapies.

MeCP2 was originally isolated as an epigenetic reader of methylated DNA that represses gene expression mainly through chromatin compaction (Jones et al, 1998; Nan et al, 2007; Skene et al, 2010). However, the proposed gene regulatory role has been expanded beyond gene silencing and chromatin architecture to include transcriptional activation, regulation of mRNA splicing, non-coding RNA maturation and protein synthesis modulation (Bedogni et al, 2014; Cheng & Qiu, 2014). While these studies indicate that MeCP2 is a multifunctional protein, they also highlight that we still do not know how many functions are associated with MeCP2 and which ones are relevant to MECP2-related disorders. However, it is commonly accepted that the majority of clinical symptoms result from the effect of MeCP2 on gene expression (Bellini et al, 2014). The recently identified function of MeCP2 at the centrosome diverges from the above-described roles of the methyl binding protein, since it does not directly impact on gene transcription (Bergo et al, 2015). Indeed, most functions of the centrosome rotate around its capacity to nucleate and organize microtubules; in neurons, which represent the cells mostly involved in Rett syndrome, the centrosome plays several functions. It is essential for axon formation (Ge et al, 2010) and is generally considered crucial for cell migration and polarization (Kuijpers & Hoogenraad, 2011). Furthermore, in these terminally differentiated cells, the mother centriole of the centrosome migrates toward the cell surface and converts into a basal body, thereby organizing the assembly of the primary cilium (Stearns, 2001).

Considering our previous data suggesting a centrosomal function of MeCP2 (Bergo et al, 2015), we found interesting to investigate whether primary cilium might suffer from structural and/or
functional alterations in Mecp2-deficient cells. Indeed, primary cilia regulate a variety of physiological functions, including metabolism, cell division and differentiation whose relevance is highlighted by a long list of heterogeneous disorders, generally called “ciliopathies” and characterized by defective primary cilia. Notably, minor deviations from the normal range of cilium length characterizing each cell type can lead to pathogenic phenotypes (Avasthi & Marshall, 2012).

In the mature brain, virtually each neuron contains this cellular appendage. Although we still lack a clear comprehension of the main roles of primary cilium in adult neurons, it is generally accepted that their dysfunction is associated with obesity, cognitive impairment and developmental disorders (Sterpka & Chen, 2018). On the contrary, little is known about the functions and consequences of ciliary deficiency in astrocytes (Sterpka & Chen, 2018). Microglia do not display primary cilia (Sipos et al, 2018) and we still lack evidence of their presence on mature oligodendrocytes, although they are present on young oligodendrocyte precursor cells (Falcón-Urrutia et al, 2015).

Our data demonstrate a structural and functional impairment of primary cilium in any tested MeCP2-defective cell, ranging from silenced human cell lines, Mecp2 null neurons, astrocytes, and MeCP2 knock-out or knock-in MEFs. Furthermore, by exogenously expressing MeCP2 in null neurons, we have proved a direct link between the expression of the methyl binding protein and accurate ciliogenesis. Importantly, the presence of primary cilium defects was confirmed in heterozygous RTT patients’ fibroblasts characterized by premature stop codons or a frameshift mutation occurring in the TRD and in Mecp2Y120D/y MEFs, harboring a missense mutation that mainly affects the capacity of the protein to tightly associate with DNA and chromatin (D’Annessa et al, 2018; Gandaglia et al, 2018). By inspecting whether the reduction in the primary cilium length is proportionally related to a reduction in soma size, we found that Mecp2 null neurons exhibit the well-known reduction in the soma area that is not present in RTT patients’ fibroblasts; however, in both cell types...
no correlation exists between primary cilium length and soma size (Appendix Fig S1).

We hypothesize that the transcriptional functions of MeCP2 contribute to the observed ciliary defects, although an involvement of its centrosomal functions cannot be excluded. Indeed, we have recently shown that in the absence of MeCP2, microtubule polymerization from centrosomes is impaired (Bergo et al., 2015). In good accordance, a reduction in microtubule stability associated with decreased α-tubulin acetylation, caused by increased levels of HDAC6, was reported (Xu et al., 2014; Gold et al., 2015; Delépine et al., 2016; Landucci et al., 2018). Real-time RT–PCR suggested that a post-transcriptional mechanism is involved in the observed HDAC6 upregulation (Delépine et al., 2016) and our unpublished data confirm that Hdac6 mRNA levels are not perturbed in the Mecp2 null brain. Of note, although the molecular mechanisms causing this aberrant HDAC6 activity remain unknown, it has already been reported that miRNA dysregulation can lead to HDAC6 overexpression (Mansini et al., 2018) and MeCP2 deficiency affects the transcription of several miRNAs (Ip et al., 2018 and references therein). These results suggest a possible mechanism involved in the observed ciliary defect. In fact, it is generally recognized that by directly affecting microtubule stability, HDAC6 can control ciliary length (De Diego et al., 2014). By demonstrating that HDAC6 inhibition rescues ciliogenesis in Mecp2-deficient cells with a concomitant amelioration of their typical neuronal dysfunctions, we proved that microtubule instability participates in the observed phenotypes, thereby offering a possible therapeutic target. Further, we demonstrated that TC-S 7010, that in post-mitotic cells preferentially affects HDAC6 activity in the primary cilium through the inhibition of Aurora A (Pugacheva et al., 2007; Korobeynikov et al., 2018), is significantly more effective than tubacin, which inhibits cytoplasmic HDAC6. Altogether these results support the validity of a pharmacological treatment acting selectively on the stability of primary cilium and indicate that the stabilization of primary cilium can lead to an improvement in neuronal complexity and synaptic alterations, in accordance with the role of this organelle in neuronal development and synaptic plasticity (Rhee et al., 2016). Although the exact mechanisms associating primary cilium with dendrites are not fully understood, recent evidence suggests that neuronal dendritogenesis depends on the formation of structurally and functionally normal cilia (Guadagnu et al., 2013). However, although we suggest that the observed amelioration of the number of synapses and dendrites is associated with rescued ciliogenesis, we cannot exclude the intervention of another mechanism independent of cilia formation.

We reinforced our in vitro observations demonstrating defective ciliogenesis in Mecp2 null and heterozygous mouse brains. Indeed, we have reported ciliary defects in Mecp2 null cortices (P14) and in Mecp2 null and heterozygous developing cerebella (P7). The occurrence of the defect also in heterozygous brains is particularly relevant to Rett syndrome. Further, most of the cells has reduced cilium length without showing a bimodal distribution, although almost 40% of them express Mecp2, thereby suggesting the involvement of a non-cell-autonomous mechanism.

Since the Shh signaling pathway still represents the best-established primary cilium-associated pathway, we analyzed whether MeCP2 dysfunctions might affect it. Our in vitro results proved a functional impairment of the primary cilium in human and murine MeCP2-defective samples. Further, our in vivo data indicate that Mecp2 null cerebella are characterized by reduced levels of Gli1 and a defective expression of other members of the same pathway, opening up new perspectives for the comprehension of the pathogenic mechanisms contributing to RTT and their treatment.

Conditional disruption of ciliogenesis in the mouse cortex and hippocampus impacts on learning and memory and modifies paired-pulse response of hippocampal neurons (Berbari et al., 2013), and many genes associated with psychiatric disorders affect ciliogenesis in vitro (Marley & von Zastrow, 2012). Furthermore, multiple ciliopathies, such as Joubert syndrome and Bardet-Biedl syndrome, present both neurodevelopmental and cognitive deficits. As a matter of facts, loss-of-function mutations in ciliary genes often results in cerebellar hypoplasia, and mice with conditional mutations disrupting the Shh pathway exhibit defects in cerebellar foliation and granule cell proliferation (Corrales et al., 2006). Interestingly, previous studies of magnetic resonance imaging (MRI) have reported a reduction in cerebellar volume both in RTT patients and in Mecp2 null mouse models (Allemand-Grand et al., 2017; Shiohama et al., 2019). These results, together with the well-recognized association of cerebellar dysfunctions with autistic-like behaviors and ataxia (Tsai et al., 2018), prompt a detailed description of the development of Mecp2 null cerebellum and whether Mecp2 null brains manifest alterations in cortico-cerebellar motor circuitries. In the future, we will also investigate in mouse models of RTT the therapeutic potential of rescuing ciliogenesis or the associated signaling pathways.

To conclude, our data demonstrate for the first time that MeCP2 influences proper formation and functioning of primary cilium, which in turn might contribute to the occurrence of central and metabolic symptoms typical of Rett syndrome. Thus, although we do not want to define Rett syndrome as a ciliopathy, we consider important to highlight that it shares several symptoms with these disorders, such as neurodevelopmental alteration, intellectual disability, autistic features, ataxia, seizures, respiratory abnormalities, obesity, hypotonia and skeletal bone defects (Hagberg et al., 2002; Novarino et al., 2011). We therefore suggest the importance of future studies testing whether novel pharmacological approaches effective for ciliopathies could be re-directed on Rett syndrome.

Materials and Methods

Drugs

Shh small molecule agonist (SAG) was purchased from Sigma-Aldrich (SML1314) and dissolved in sterile water. MEFs and hTERT-RPE-1 cells were treated with SAG (100 nM for MEFs and 200 nm and 500 nM for hTERT-RPE-1 cells) for 24 h after serum starvation with low serum medium (0.5% fetal bovine serum). Neurons were treated with 200 nM and 400 nM SAG for 48 h. Tubacin (SML0065, Sigma-Aldrich) was dissolved in DMSO. MEFs, neurons, and hTERT-RPE-1 cells were treated with 1 μM tubacin for 48 h, whereas fibroblasts were treated with 250 nM tubacin for 24 h in low serum medium. Aurora A inhibitor I (TC-S 7010, Selleckchem) was dissolved in DMSO, and a dose of 7 μM was used on neurons for 48 h.
Plasmids
To silence MECP2, hTERT-RPE-1 cells were transfected with the already-described siMeCP2#DH4 (sense, 5'-GGAAGGACUGAA GACCUGUU-3') or, as corresponding control, the scrambled siRNA (sense, 5'-UAGCGACUAAACACAUAAC-3'), both purchased from Dharmacon (Bergo et al, 2015). Neurons (DIV0) were infected with the already-described lentiviruses expressing MeCP2iresGFP or exclusively GFP as control (Stefanelli et al, 2016).

Animals
As lengthily described (Cobolli Gigli et al, 2016), the Mcp2 null mouse strain, originally purchased from the Jackson Laboratories, was backcrossed and maintained on a clean CD1 background. These mice recapitulate the typical phenotype of C57BL/6 mice, with the advantage of having a larger progeny and minor risk of litter cannibalization (Cobolli Gigli et al, 2016). Mcp2 null mouse genotype was determined by PCR protocol on genomic DNA purified from tails using the following primers: 5'-ACCTAGCTCCTGTACTTT-3' as forward primer for null allele; 5'-GACTGAAGTACAGATGG TTGTG-3' as forward primer for wild-type allele; and 5'-CCACCCTC CAGTTTTGTTTA-3' as common reverse primer. Mcp2(Y120D/y) mouse genotype was assessed using the following primers: 5'-CAG GCCCTCAGAACAAGC-3' as common forward primer; 5'-GCAGAGG GGGT-3' as reverse primer for knock-in allele. For mouse embryonic fibroblasts (MEFs) and neuronal/astrocytic cultures, wild-type (WT) and Mcp2 null embryos or pups were generated by mating Mcp2 heterozygous females with WT male mice. The day of vaginal plug was considered E0.5 and animals were housed in a temperature- and humidity-controlled environment in a 12-h light/12-h dark cycle with food and water ad libitum. All procedures were performed in accordance with the European Union Communities Council Directive (2010/63/EU) and Italian laws (D.L.26/2014). Protocols were approved by the Italian Ministry for Scientific Research and by the San Raffaele Scientific Institutional Animal Care and Use Committee in accordance with the Italian law.

Cell cultures
Mouse embryonic fibroblasts (MEFs)
Mouse quiescent embryonic fibroblasts were prepared from wild-type and Mcp2 null E13.5 embryos, as previously described (Bergo et al, 2015). Harvested embryos were placed in a 10-cm dish with PBS. Heads were collected for genotyping and the inner organs were discarded. Each embryo was minced with forceps and incubated at 37°C for 30 min in 0.25% trypsin/EDTA (Life Technologies) in PBS. After trypsin removal, the tissue was re-suspended in Dulbecco’s modified Eagle’s medium (DMEM; Sigma-Aldrich) with 10% fetal bovine serum (FBS; Gibco) and antibiotics by gentle trituration with a Pasteur pipette, and the supernatant was transferred to T25 flasks. MEFs were maintained in DMEM supplemented with 10% FBS, l-glutamine (Sigma-Aldrich), and 1% penicillin/streptomycin (P/S; Sigma-Aldrich) and grown at 37°C with 5% CO2. MEFs were starved for 24 or 48 h with low serum medium (0.5% FBS) and eventually treated with drugs (SAG and/or tubacin).

Immortalized human retinal pigment epithelial (hTERT-RPE-1) cells
hTERT-RPE-1 (kindly provided by Dr. Storchova, Max-Planck Institute) cells were maintained in DMEM/F12 (Dulbecco’s modified Eagle’s medium: 1:1 mixture of DMEM and Ham’s F-12 Nutrient Mixture; 15 mM HEPES; Sigma-Aldrich); medium was supplemented with 10% FBS, l-glutamine and 1% P/S. Cells were grown at 37°C with 5% CO2. For siRNA transfection, 20 nM siRNA oligonucleotide targeting MECP2 or control siRNA was transfected into RPE-1 cells using Lipofectamine™ RNAmax (Invitrogen). Silenced cells were processed 96 h post-transfection. After starvation, cells were treated with 1 μM tubacin for 24 h and then exposed to 200 nM SAG.

Cortical neuronal cultures
Primary cortical neurons were prepared from WT and Mcp2 null mouse embryos at 15.5 days. Embryos were sacrificed by decapitation and brains were removed under a microscope and immersed in ice-cold Hank’s Buffered Salt Solution (HBSS; Life Technologies). Meninges were removed, and cerebral cortex was rapidly dissected and brains were removed under a microscope and immersed in ice-cold HBSS. Tissues were incubated with 0.25% trypsin/EDTA for 7 min at 37°C and the digestion was blocked with 10% FBS in DMEM (Life Technologies). Cortices were then mechanically dissociated by pipetting in Neurobasal medium, containing 10% FBS, 2% B27 (Life Technologies), 1% L-glutamine (Sigma-Aldrich) and grown at 37°C. The resulting cells were centrifuged at 400 g for 200 rpm for 8 h at 37°C to eliminate residual microglia, and the medium was replaced with fresh culture medium. Cells were incubated in a humidified incubator at 37°C and 5% CO2 untilDIV11 when confluence was reached. Astrocytes were detached by 0.25% trypsin/EDTA in HBSS and seeded onto glass coverslips.
at a density of 20,000 cells/cm² for immunofluorescence analysis. A starvation protocol was applied, by incubating cells with medium without FBS for 24 h. Glial cultures contained 95% GFAP⁺ astrocytes and 5% Iba1⁺ microglia and no neuron, as indicated by the lack of MAP2 reactivity.

**Human fibroblasts**

Human control fibroblasts from healthy individuals (GM00037, GM00969, and GM01651) and fibroblasts carrying 705delG (GM07982) and Q244X (GM16548) mutations were provided by Coriell Institute of Medical Research. Human fibroblasts carrying R255X mutation (R_FFF2238) were obtained from Telethon Network of Genetic Biobank. Cells were maintained in DMEM, containing 10% FBS, 1% l-glutamine, and 1% P/S, and they were grown at 37°C with 5% CO₂. Fibroblasts were starved for 24 h with low serum medium (0.5% FBS) and then used for immunofluorescence and Western blot.

**Immunoblot analysis**

hTERT-RPE-1, MEFs, and primary cortical neurons were lysed in ice-cold lysis buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% NP-40, and 1X complete EDTA-free protease inhibitor cocktail [Roche Diagnostic]) for 30 min on ice and then centrifuged for 30 min at 10,000 g at 4°C. Cerebella were lysed in ice-cold lysis buffer for 30 min, sonicated on ice for 10 s at 30 amplitude, and then clarified by centrifugation for 30 min at 10,000 g (4°C). Protein concentrations were measured by the Bradford assay (Bio-Rad). Equal amounts of protein lysates were separated on polyacrylamide gel for SDS–PAGE and proteins were blotted onto a nitrocellulose membrane using a semidry transfer apparatus (Trans-blot SD; Bio-Rad). Membranes were incubated 1 h in blocking solution (Tris-buffered saline containing 5% nonfat milk and 0.1% Tween-20, pH 7.4) and then incubated overnight (4°C) with the primary antibody diluted in blocking solution. The following primary antibodies were used: anti-MeCP2 (M9317, Sigma-Aldrich, 1:1,000), anti-Gli1 (L42B10; Cell Signaling, 1:1,000), anti-acetylated α-tubulin (32-2700; Invitrogen, 1:2,000), anti-α-tubulin (T5168; Sigma-Aldrich, 1:10,000), and anti-GAPDH (SAB1405848; Sigma-Aldrich, 1:10,000). After three washes in TBS-T (Tris-buffered saline containing 0.1% Tween-20, pH 7.4), blots were incubated with HRP-conjugated secondary antibody (1:10,000 in 5% milk in TBS-T) for 1 h at room temperature. The immunocomplexes were visualized by using the ECL substrate (GeneSpin) and Uvitec system. Band density measurements were performed using Uvitec software. Results were normalized to α-tubulin (MEFs, neurons, and cerebella), to GAPDH (hTERT-RPE-1) or to total protein content (neurons) visualized by a TGX stain-free method (Bio-Rad).

**RNA extraction and quantitative real-time PCR**

Total RNA from MEFs, primary neurons and cerebella was extracted using PureZOL (Bio-Rad) and quantified using a NanoDrop spectrophotometer. After having assessed RNA integrity by agarose electrophoresis, RNA was reversely transcribed using the RT2 First Strand Kit (Qiagen) as instructed by the manufacturer. The resulting cDNA was used as a template for qRT–PCR with SYBR Green Master Mix (Applied Biosystems) with designated primers for Gli1: Forward Primer: GGAAGGGGACATGTCTAGC; Reverse Primer: AATGGCA CAGAATTCTTTC; CyclinD1: Forward Primer: CCCCAAA CAACTTCCTTCTCCT; Reverse Primer: CCAGCTTCTTCCCTCACTT; and CyclinD2: Forward Primer: GAGTTGGGAACCTGAGTTT; Reverse Primer: CGCACAGGAGTGAAGGT. Melting curve showed a single product peak, indicating good product specificity. HPRT and RPL-13 served as internal standards, and fold change in gene expression was calculated using the 2(−delta C) method.

**Immunofluorescence**

Cells seeded on glass coverslips were fixed with 4% paraformaldehyde in PBS for 30 min at room temperature and then washed three times with 10 mM PBS. For the analysis of synaptic puncta, neurons (DIV14) were fixed for 8 min with 4% paraformaldehyde dissolved in PBS with 10% sucrose. Cells were permeabilized at 4°C in PBS containing 0.1% Triton X-100 for 3 min and blocked with 10% FBS in PBS for 15 min. Then, cells were incubated with primary antibody overnight (4°C). Immunofluorescence staining was done with anti-MeCP2 (M9317, Sigma-Aldrich, 1:100), anti-γ-tubulin (T5326, Sigma-Aldrich, 1:500), anti-acetyl-α-tubulin (lys40, 5335, Cell Signaling, 1:500), anti-Smoothened (ab38686, Abcam, 1:200), anti-adenylate cyclase type 3 (AC3, ab125093, Abcam, 1:1,000), anti-MAP2 (8707, Cell Signaling, 1:1,000), anti-Synapsin1/2 (ab106006, Synaptic System, 1:500), and anti-Shank2 (ab162211, Synaptic System, 1:300). Cells were then incubated with Alexa Fluor 488-, Alexa Flour 546-, or Alexa Fluor 647-conjugated secondary antibodies (Life Technologies) for 1 h and then washed with PBS. DNA was stained with DAPI solution (1:1,000 in PBS) for 10 min, and slides were mounted with Fluoromount-G reagent (eBioscience). For the quantitative evaluation of the percentage of MeCP2-expressing cells, ciliated cell, cilium length, and ciliary Smo localization, images were acquired from at least three coverslips for each experimental group with an epi-fluorescence microscope by Nikon and analysis was conducted using ImageJ software. To analyze dendritic complexity, isolated MAP2-positive neurons at DIV7 were acquired under an epi-fluorescence microscope by Nikon with a 40× objective and neuronal arborization was analyzed by using a Sholl analysis plug-in distributed with ImageJ software. For the analysis of synaptic puncta, 144.88 × 144.88 μm² Z-stack images (1,024 × 1,024 pixel resolution, 8-bit grayscale depth) were acquired at 1× digital zoom using a 63× oil-immersion objective by laser-scanning confocal microscope (Zeiss LSM 800, Carl Zeiss) with a step size of 0.3 μm. For each dataset acquisition, parameters (offset background, digital gain, and laser intensity) were maintained constant among different experiments. By ImageJ software, maximum intensity projection images were converted to binary images and processed with a fixed threshold for each channel acquired. The puncta density was calculated by counting only puncta lying along manually selected ROIs within 20 μm of 3 primary branches/neuron. Only puncta with a minimum size of 0.16 μm² were counted using Analyze Particles. To assess puncta co-localization of pre- and post-synaptic markers, the ImageJ Plugin Colocalization highlighter was run on each Z-stack image acquired. Co-localized puncta were quantified in manually selected ROIs of the binary mask created from the maximum intensity projection.

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**Immunofluorescence on brain sections**

Mice were anesthetized by using a mixture of Rompun/Zoletil and transcardially perfused with 4% paraformaldehyde in PBS. Brains were post-fixed overnight at 4°C and slowly dehydrated by using increasing concentrations of sucrose in PBS at 4°C. Brains were embedded with Tissue-TEK (O.C.T. Sakura Finetek), frozen in isopentane, and stored at −20°C. Sectioning was then performed with a cryostat. Antigen retrieval was applied before immunostaining, and then, sections were permeabilized using 0.3% Triton X-100 in PBS for 30 min and quenched with 0.1 M glycine for 30 min. Sections were incubated with mouse anti-Arl13b (ab136648, Abcam, 1:1,000) primary antibodies, overnight at 4°C, and then with secondary antibodies for 1 h at RT in a solution of 0.2% gelatin, 300 mM NaCl, and 0.3% Triton X-100 in PBS. DNA was stained in the last wash using DAPI for 30 min at RT and mounted using ProLong antifade reagent (Thermo Scientific). Imaging was performed using a Leica TCS SP5-AOBS 5-channel confocal system (Leica Microsystems) equipped with a 405-nm diode, an argon ion, and a 561-nm DPSS laser. Fixed cells were imaged using a PLAPO 40X/1.2 NA oil-immersion objective. All the images were analyzed by using Bitplane Imaris software.

**Statistical analysis**

Data are expressed as mean ± SEM and were analyzed using GraphPad Prism software 7.0. The percentage of ciliated cells and cilia length in cultured cells and morphological data in neurons were obtained from cells derived from three separate experiments. For the pharmacological experiments, cells derived from the same batch were subdivided among all the experimental groups. For ex vivo analyses, we used animals derived from at least three different litters, in order to exclude maternal effect on the observed phenotypes. Image analyses were performed by a researcher blind to the genotype and/or treatment. In order to use the correct statistical test, we first evaluated the normal distribution of data by applying the D’Agostino–Pearson normality test. Student’s t-test or Mann-Whitney test was used for the statistical analysis when WT samples were compared to Mecp2 mutant samples. One-way ANOVA was used to statistically compare the effects of tubacin and TC-S 7010 on Mecp2 null neurons. Data from the experiment on neuronal cultures at different DIVs, from the experiment on infected neurons, and from Smo-localization experiments and pharmacological experiments were analyzed by two-way ANOVA. Three-way ANOVA was used to statistically analyze Smo-localization data in hTERT-RPE-1 cells following pharmacological treatment. When there was a significant effect of treatment or genotype, or a significant interaction between the variables (two-way or three-way ANOVA), appropriate post hoc test was applied. A P-value < 0.05 was considered significant.

**Expanded View** for this article is available online.

**Acknowledgements**

This work was mainly supported by the Italian parents’ association “ProRETT Ricerca” to N.L. We are grateful to all members of N.L. and C.K.N laboratories for helpful discussions. The RTT fibroblasts were obtained by cell lines and DNA bank of Rett syndrome, X-linked mental retardation and other genetic diseases, member of the Telethon Network of Genetic Biobanks (project number no. GTB12001), funded by Telethon Italy, EuroBioBank network and the “Associazione italiana Rett O.N.L.U.S.”

**Author contribution**

AF, ES, MP, EA, MMV, BL, AB, FB, and VDC conducted the experiments. AF and NL designed the study, prepared the figures, and wrote the manuscript. ES, MP, FDC, and CK-N revised the manuscript and assisted in interpreting and discussing some results.

**Conflict of interest**

The authors declare that they have no conflict of interest.

**For more information**

(i) http://biobanknetwork.telethon.it/
(ii) http://www.eurobiobank.org/
(iii) https://www.coriell.org/Browse/Biobanks
(iv) https://prorett.org/, https://www.rettsyndrome.org/
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