Reduced AKT/mTOR signaling and protein synthesis dysregulation in a Rett syndrome animal model

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Rett syndrome (RTT) is a neurodevelopmental disorder with no efficient treatment that is caused in the majority of cases by mutations in the gene methyl-CpG binding-protein 2 (\textit{MECP2}). RTT becomes manifest after a period of apparently normal development and causes growth deceleration, severe psychomotor impairment and mental retardation. Effective animal models for RTT are available and show morphofunctional abnormalities of synaptic connectivity. However, the molecular consequences of MeCP2 disruption leading to neuronal and synaptic alterations are not known. Protein synthesis regulation via the mammalian target of the rapamycin (mTOR) pathway is crucial for synaptic organization, and its disruption is involved in a number of neurodevelopmental diseases. We investigated the phosphorylation of the ribosomal protein (rp) S6, whose activation is highly dependent from mTOR activity. Immunohistochemistry showed that rpS6 phosphorylation is severely affected in neurons across the cortical areas of \textit{Mecp2} mutants and that this alteration precedes the severe symptomatic phase of the disease. Moreover, we found a severe defect of the initiation of protein synthesis in the brain of presymptomatic \textit{Mecp2} mutant that was not restricted to specific subsets of transcripts. Finally, we provide evidence for a general dysfunction of the Akt/mTOR, but not extracellular-regulated kinase, signaling associated with the disease progression in mutant brains. Our results indicate that defects in the AKT/mTOR pathway are responsible for the altered translational control in \textit{Mecp2} mutant neurons and disclosed a novel putative biomarker of the pathological process. Importantly, this study provides a novel context of therapeutic interventions that can be designed to successfully restrain or ameliorate the development of RTT.

INTRODUCTION
Rett syndrome (RTT; MIM312750) is a pediatric neurological progressive disorder leading to severe mental retardation, psychomotor impairment and autistic behavior that affects about 1:10 000 girls worldwide (1\textendash3). Children with RTT have apparently normal development until 6 months of life, after which they undergo a rapid regression marked by a
deceleration of head growth, the onset of stereotyped hand movements, irregular breathing and seizures (4). Loss-of-function mutations in the X-linked methyl-CpG-binding protein 2 (MECP2) gene, a transcriptional regulator that acts through epigenetic mechanisms on chromatin structure (5), cause the 95% of RTT cases. Importantly, mutations of this gene are also found in patients with other neurological conditions such as Angelman-like syndrome, motor and learning disabilities, seizures, bipolar disease, juvenile-onset schizophrenia, neonatal encephalopathy and autism (2,6,7). The finding that in mice targeted the mutation of MeCP2 in the central nervous system may produce a phenotype similar to the whole-body mutation has suggested that impairment of MeCP2 function in the brain is crucial for the pathogenesis of the disease (8,9). Indeed, the analysis of the available animal models for RTT has indicated that altered MeCP2 levels in neurons cause morphological defects in both dendritic complexity, spine number as well as alterations in synaptic transmission and plasticity (10–16). These results have suggested a model for the etiopathogenesis of RTT in which the behavioral alterations induced by the MeCP2 mutation could depend on the modifications of brain synaptic organization. However, the molecular events leading from the disruption of MeCP2 expression in the brain to the alterations of neuronal circuits are yet not known.

There is mounting evidence showing that aberrant neuronal protein synthesis as one underlying cause of the clinical features of autism spectrum disorders (17,18). Alterations of signaling cascades involved in the regulation protein synthesis, such as the mammalian target of rapamycin (mTOR) and phosphoinositide 3-kinase (PI3K) pathways, have been involved in neurodevelopmental diseases associated with severe mental retardation (19,20) such as Fragile X (21), tuberous sclerosis (22) and Phelan-McDermid syndrome (23). Interestingly, the regulation of protein synthesis via the mTOR/PI3K pathway is crucially involved in synaptic function, structure and plasticity (20,24–26). In post-mitotic neurons, mTOR activity and its downstream targets can control the size of the neuronal cell soma, axon pathfinding and regeneration, dendrite arborization, dendritic spine morphology and synaptic plasticity (20,25). Intriguingly, all these aspects are altered in MeCP2 mutants. These changes are not accompanied, surprisingly, by important changes in the transcriptome (1,27,28), suggesting that MeCP2 may subtly act at the post-transcriptional level. In spite of it, studies describing the efficacy of protein synthesis or the translation-related intracellular signaling in RTT are not yet available.

In this study, we investigate whether the activation of ribosomal protein (rp) S6, a component of the 40S ribosomal subunit, is normal during the post-natal brain development of MeCP2 mutant mice. In particular, we analyze the modifications occurring at Ser235/236 and Ser240/244. These residues can be phosphorylated by both the extracellular-regulated kinase (ERK) and the mTOR/PI3K pathway and correlate with cellular translational rates (29–31). Moreover, to test the hypothesis that translational deficits may be a cause of RTT neurological dysfunctions, we looked at polysomal complexity and polysome-associated mRNAs in the brain of MeCP2 mutants. Finally, we analyze the contribution of intracellular upstream signaling to dissect out the mechanism regulating protein synthesis in neurons completely lacking MeCP2.

RESULTS

Pervasive hypophosphorylation of rpS6 in MeCP2−/− brains precedes symptoms manifestation

mTOR/PI3K-dependent protein synthesis in neurons is crucial for the organization of neuronal and synapse structures (e.g. dendrites and dendritic spines) as well as for synaptic plasticity (24–26). In addition, dendritic protein synthesis induced by activation and plasticity of synaptic connections requires the action of ERK-dependent signaling (24,32,33). To address weather possible defects of these intracellular pathways might occur in the brain of MeCP2 knock-out (KO) mice (MeCP2−/−) (8), we examined the immunolocalization of phosphorylated (p) rpS6, a converging target of both ERK and mTOR/PI3K activity (31). We analyzed the localization of activated rpS6 in the primary sensory (S1) cortex and in the CA1 region of the hippocampus, where neuronal and synaptic alterations have previously been found in mice models of RTT (10,12,15,34–37). The analysis was performed in MeCP2−/− mice at 8 weeks of age (P56) showing clear symptomatic signs (i.e. locomotor impairments, hind limb clasping, perseverative scratching and breathing irregularities). Sections were probed with two specific antibodies. The first recognizes rpS6 only when is activated at Ser235/236, two residues that can be phosphorylated by both ERK and mTOR/PI3K activities. The second antibody recognizes the activated form of rpS6 at Ser240/244 sites, a modification that can be induced by the action of the mTOR/PI3K pathway and is ERK-independent.

As illustrated in Figure 1A, we observed a robust decrease in p-rpS6 immunoreactivity throughout the S1 cortex of MeCP2 mutants compared with wild-type (WT) littermates. In general, rpS6 activation was more intense in layer 5 of the cortex of WT animals. A close inspection of the immunolabeled sections revealed that in individual cortical neurons, rpS6 can be activated both in the soma and in the most proximal portion of the apical dendrite. As can be inferred in Figure 1A insets, rpS6 staining showed differences among the cells. Remarkably, quantification of the immunosignal intensity (Fig. 1D), calculated as optical density (OD) values of the peroxidase staining, confirmed a significant decrease in rpS6 activation at both Ser235/236 and Ser240/244 in the MeCP2 mutants. We found a 32.2% reduction in p-rpS6 (Ser235/236) activation throughout the cortex of KO mice in comparison with WT littermates (n = 3, P < 0.005), whereas the activation of p-rpS6 (Ser240/244) was 30.2% less in the mutants than in control animals (n = 3, P < 0.05). In contrast, we detected neither differences in localization (Fig. 1A) nor significant changes in immunolabeling intensity (Fig. 1B) for the total rpS6 protein between WT and MeCP2−/− mice. Thus, because of reduced phosphorylation, rpS6 cannot properly function in symptomatic MeCP2 KO male mice.

To address whether dysfunctional rpS6 phosphorylation in the cortex may either anticipate or correlate with the
disease progression, we next examined KO animals at earlier post-natal ages (i.e. P14 and P28). As illustrated in Figure 1C, at P28, i.e. before the appearance of evident locomotor deficits and hind limb clasping in Mecp2 KO mice, mutants show a significative reduction in p-rpS6 immunoreactive signals (p-rpS6-Ser235/236: 23.6% reduction in KO mice versus WT littermates, n = 3 WT and KO, P < 0.005; p-rpS6-Ser240/244: 41.4% reduction in KO mice versus WT littermates, n = 3 WT and KO, P < 0.001). Intriguingly, at an earlier post-natal developmental stage (P14), when mutants are asymptomatic, KO mice showed WT levels of rpS6 activation (Fig. 1B). At this early post-natal developmental stage, we detected no differences in rpS6 immunostaining intensity for any of the phosphorylation sites throughout the cortical layers of P28 (C) and P56 (D) KO mice compared with WT littermates. Total rpS6 expression is not altered in these animals. Data are expressed as the mean ± sem. *P < 0.05, **P < 0.01, ***P < 0.001 (scale bar, 100 μm).
both P14 and P28 (Fig. 1C and D). These data indicate that after a period of putative normal activity, rpS6 becomes hypofunctional in correlation with the onset of the symptoms.

To determine whether rpS6 phosphorylation may be similarly affected in other brain areas, we, next, performed a similar immunohistochemical analysis of p-rpS6 localization in the CA1 hippocampal area of MeCP2 mutants at the three ages examined previously. Interestingly, although CA1 pyramidal neurons in MeCP2 KO mice showed less rpS6 activation than WT animals (Supplementary Material, Fig. S1) the effect was less dramatic than what we observed in the S1 cortex. In particular, we found that there is a significant decrease in rpS6 activation on Ser240/244 in P28 KO mice (Supplementary Material, Fig. S1D; OD WT: 0.16 ± 0.02; OD KO: 0.08 ± 0.01; n = 3 WT and KO, P < 0.05).

It has been recently shown that glial cells lacking MeCP2 may cause defects in the dendritic morphology and complexity of co-cultured MeCP2-positive neurons thus implying a critical role of the glia in the neurological alterations associated with RS (38). To explore whether the rpS6 phosphorylation changes observed in MeCP2 (P28) mutants are associated with alterations occurring also in glial cells we analyzed rpS6 phosphorylation in dually labeled brain sections with antibodies against GFAP or NeuN. As illustrated in Figure S2, the number of GFAP+ glial cells was very low throughout the layers of the S1 cortex in both WT and mutant animals. Moreover, virtually no co-localization between GFAP+ cells and p-rpS6-Ser235/236 or p-rpS6-Ser240/244 immunosignal was detectable in sections from both genotypes (Supplementary Material, Fig. S2A and B) indicating that rpS6 shows was detectable in sections from both genotypes (Supplementary Material, Fig. S2A and B) indicating that rpS6 shows

correlation with the activation of the protein synthesis apparatus (30).

With this in mind, we decided to analyze protein synthesis in MeCP2 mutant mice at P56. Neuropathological signs are already developed at this stage exemplified by motor unbalance, learning impairments as well as cognitive deficits and anxious behavior (37) are already developed.

To have a read-out of protein synthesis, incorporation of [35S]-methionine into new proteins was examined in vitro cultured brain slices of WT (n = 3) and MeCP2 mutant brains (n = 3). This metabolic labeling showed a trend in reduction in methionine incorporation (P = 0.06) in the mutants (Supplementary Material, Fig. S3), prompting us to analyze the specific reduction in initiation of translation by polysomal profiles.

Cytoplasmic cell extracts from MeCP2 mutant and WT brain lysates were prepared and fractioned on a sucrose gradient. Figure 3A shows cell extracts profiles in which the polysomal fraction is evidently reduced in two different MeCP2 mutants (red line) when compared with WT (black line) brains (WT, n = 3; KO, n = 3). Therefore, the overall pool of active translating polyribosomes is abnormally diminished in mutant brains where the disease has progressed. Indeed, densitometric scanning of the P56 brain polysomal profiles (Fig. 3B) showed a significant decrease in the area of ribosomal peaks in the polysome fraction of MeCP2 null mice with respect to WT littermates (decrease of 8 ± 0.1% of WT, n = 3, P < 0.05). Consistently, there was an increase in the area of ribosomal peaks in the mRNA-free part of the gradient in MeCP2 null mice with respect to WT controls (increase of 7.9 ± 0.08% of WT, n = 3, P < 0.05).

This dysfunction of initiation of the translation rate might be caused by the general compromised state of the mutants at this late phase of the disease. In alternative, this defect might be an early event in the disease evolution that, nonetheless, remains detectable even at late stages of the pathology. To discriminate between these two possibilities, we performed polysomal profiles in young MeCP2 null animals at a presymptomatic stage (P26). Noteworthy, cell extracts profiles present a notable reduced polysomal fraction when comparing MeCP2 null with WT brains (WT, n = 3; KO, n = 3) (Fig. 3C).

The densitometric scanning of the P26 brain polysomal profiles (Fig. 3D) showed a significant decrease in the area of ribosomal peaks in the polysome fraction of MeCP2 null mice with respect to WT littersmates (decrease of 7.6 ± 0.04% of WT, n = 3, P < 0.01). Consistently, there was an increase in the area of ribosomal peaks in the mRNA-free part of the gradient in MeCP2 null mice with respect to WT controls (increase of 7.6 ± 0.03% of WT, n = 3, P < 0.01).
In line with this finding, the amount of Rack1, a major component of translating ribosome, was mainly reduced in the fast sedimenting fractions (7–10) where polyribosomes are located in the MeCP2 null with respect to the control brain lysates (Fig. 3D). Conversely, at this same stage initiation of translation was found only minimally affected in the liver of MeCP2 mutant with respect to WT animals (WT, n = 3; KO, n = 3), a peripheral organ with an intense metabolic activity (Fig. 3B). These data provide a first indication that defects in the initiation of translational are an early sign of the disease that accompanies or even precedes the initial neurological impairment in the MeCP2 null mice.

A failure in initiation might have a general impact on global mRNA translation or, alternatively, might affect specific transcripts in particular. To address this issue, RNA was isolated from each fraction of the ribosomal profiles of P26 Mecp2 mutant and WT brain lysates and transcript levels for a specific gene were assessed by quantitative RT-PCR (qRT-PCR). We selected two genes coding for molecules with a pivotal function in neuronal activity and plasticity such as CAMKIIα and PSD95. In addition, three genes not associated with a neuronal function were also analyzed (Rack1, hnRNP A2/B1 and GADPH). Finally, we assessed the levels of the 18S ribosomal RNA (rRNA) which is incorporated in 40S ribosomal subunit. In total agreement with polysomal profiles, in MeCP2 mutant brains, the 18S rRNA was reduced in the polysome fraction and enriched in the mRNA-free part of the gradient (Fig. 4A). A similar trend was observed for CAMKIIα and PSD-95 mRNAs as well as for Rack1, hnRNP A2/B1 and GADPH mRNAs (Fig. 4B–D). Indeed, polysomal incorporation of these transcripts was significantly lower in Mecp2 KO cortex and cerebellum but not in the hippocampus. No difference was present in total rpS6 expression. Error bars represent the standard error of the mean. *P < 0.05.
quantitated signals of the last six fractions containing active polysomes by the sum of the 11 fractions. In the case of control tissues, the PMP value was over 60% for all six genes, whereas it got reduced to ~50% in MeCP2 mutant brains (Fig. 4D). Taken together, these data demonstrate a comparable reduction in the translation rate of all the tested mRNAs.

**AKT/mTOR signaling is perturbed in Mecp2 mutant brain tissue**

mRNA translation is a highly regulated process and represents the final target of diverse signaling pathways that sense both the stimuli from the external environment as well as the metabolic needs. Therefore, we decided to investigate any changes in diverse intracellular pathways involved in protein synthesis. It is well recognized that rpS6 is the major substrate of p70 S6 kinase (S6K) which promotes the phosphorylation of consecutive serine residues at the rpS6 C-terminal part and thereby stimulating protein translation (30). Activation of the p70 S6K depends on multiple phosphorylation events occurring in a hierarchical manner within the C-terminal autoinhibitory domain (40). Interestingly, the S6K phosphorylation levels were reduced in brain lysates of P48 MeCP2 mutant with respect to WT
littermates (decrease of 35 ± 6% of WT; n = 3 WT and KO; P < 0.05) (Fig. 5A and B). In contrast, no appreciable change was detected in S6K protein abundance between MeCP2 null and WT brain lysates (Fig. 5A and B).

A large body of evidence has shown that the rpS6-S6K pathway is tightly controlled by the mTOR kinase activity which integrates signals from mitogenic growth factors, nutrients, stress and cellular energy level to promote protein synthesis (41,42). Therefore, we examined the abundance and phosphorylation of mTOR at Ser2448, a biochemical indicator of mTOR activation (41), in MeCP2 KO and WT brain lysates. The phosphorylation of mTOR at Ser2448 was reduced in whole-cell lysates of the brain of MeCP2 mutant mice (decrease of 27 ± 7% of WT; n = 3 WT and KO; P < 0.05) relative to that of WT littermates (Fig. 5C and D). RpS6 is known to be also activated by the RAS-ERK signaling cascade through phosphorylation by the p90 ribosomal S6K exclusively in Ser235/236 (31). Thus, we examined the levels of the activation of Erk1/2 kinases in brains lacking MeCP2. No difference in ERK2 phosphorylation was observed in the same mice (94.4 ± 17% of WT levels, n = 11, P = 0.56) (Fig. 6A and B).

The findings reported thus far suggest a rather specific defect in mTOR signaling in the brain of MeCP2 KO mice. At least two mTOR complexes exist, mTORC1, upstream of rpS6, and mTORC2. The phosphorylation status of S473 depends directly by mTORC2 activity and therefore represents an indication of the overall mTOR activity in the cell (43). Interestingly, we found a consistent reduction in AKT S473 phosphorylation in mutant MeCP2 compared with WT P46 brain lysates (decrease to 45.7 ± 9.5% of WT levels, n = 10, P = 0.037) (Fig. 6C and D).
Phosphorylation of rpS6 is abnormal in MeCP2^{+/−} female mice

MeCP2^{+/−} mutants are the most utilized animal model to evaluate harsh consequences of MeCP2 mutation. However, the greatest number of the affected RTT patients is MECP2-mutated heterozygote girls. MeCP2^{+/−} female mice possess various phenotypic characteristics of RTT patients, such as locomotor and autonomic alterations, abnormal cognitive and emotional behavior as well as a relatively normal lifespan.

We thus analyzed if the phosphorylation of rpS6 is impaired in the brain of 10 months old female heterozygous mutants that showed clear signs of the pathology (i.e. tremors, reduced size and severe locomotor defects). As shown in Figure 7A, we found that the intensity of immunostaining of the phosphorylated forms of rpS6 (Ser235/236 and Ser240/244) activation in the cortex of female mutants compared with WT female mice is drastically reduced in the brain of MeCP2^{+/−} mutants compared with WT female animals of the same age. The decrease in p-rpS6 levels was observed throughout the layers of the S1 cortex and in the CA1 area of the hippocampus (Fig. 7A). Indeed, OD analysis of the phosphorylated forms of rpS6 (Ser235/236 and Ser240/244 sites) was drastically reduced in the brain of MeCP2^{+/−} mice compared with WT female animals of the same age. The decrease in p-rpS6 levels was observed throughout the layers of the S1 cortex and in the CA1 area of the hippocampus (Fig. 7A). Indeed, OD analysis of the phosphorylated forms of rpS6 (Ser235/236 and Ser240/244 sites) was drastically reduced in the brain of MeCP2^{+/−} mice compared with WT female animals of the same age.

MeCP2 is not a ribosomal resident protein

To assess any direct involvement of MeCP2 in controlling protein synthesis, we investigated its association with the ribosomal machinery. In polysomal profiles, MeCP2 was mainly localized in fractions 1–5 partially co-fractionating with ribosomal subunits 40S, 60S and 80S as tested by immunoblotting with two different antibodies on each fraction of the gradient (see Materials and Methods). To determine whether the co-sedimentation of MeCP2 with ribosomes is stable, we repeated ribosomal profiles after treating cell extracts with EDTA (30 mM) which causes complete dissociation of the translating ribosomes into subunits and the release of polyribosomal mRNPs. In this case, ribosomal-associated proteins no longer were collected from the fast-sedimenting gradient fractions, but were retrieved from the fractions at the top of the sucrose gradient (Supplementary Material, Fig. S4). In line with this, Rack1, a protein stable associated with ribosomes, after EDTA treatment was mostly located in the cellular soluble content confirming the expected outcome. Nevertheless, under these conditions, the MeCP2 sedimentation pattern was neither shifted into the lighter fractions nor changed in other evident ways (Supplementary Material, Fig. S4). These results indicate that MeCP2 is associated with high-weighted molecular complexes but it is not stably associated with ribosomal complexes.
Mecp2 alleles caused by the random inactivation of one X-linked show the mosaic expression of null and wild-type. To test this hypothesis, we quantitatively analyzed p-rpS6 localization in the S1 cortex of heterozygote females only cells expressing the null allele would exhibit a phenotype. To non-cell autonomous effects. On the contrary, if deficits of MeCP2 non-cell autonomous effect on rpS6 phosphorylation

Finally, we assumed that if Mecp2 loss impacts on such a complex signaling pathway, its deficiency might lead to non-cell autonomous effects. On the contrary, if deficits of rpS6 activation were produced by a completely cell-autonomous mechanism in heterozygous females only expressing the null allele would exhibit a phenotype. To test this hypothesis, we quantitatively analyzed p-rpS6 localization in the S1 cortex of Mecp2 heterozygote females that show the mosaic expression of null and wild-type Mecp2 alleles caused by the random inactivation of one X-linked Mecp2 allele (44). Interestingly, the analysis of high magnification confocal images showed that p-rpS6 immunofluorescence was evidently reduced not only in MeCP2-negative neurons, but even in adjoining MeCP2 expressing cells (Fig. 8A). Indeed, the measurement of the mean p-rpS6 fluorescence intensity in the cell soma (Fig. 8B) revealed no differences between MeCP2+ and MeCP2− neurons (p-rpS6-Ser235/236, Mecp2+ versus MeCP2− cells, n = 4, P > 0.3; p-rpS6-Ser240/244, Mecp2+ versus MeCP2− cells, n = 4, P > 0.1). Note that in MeCP2− cortical neurons of Mecp2 heterozygote females, the immunofluorescence signal for MeCP2 precisely co-localized with bright puncta of DAPI counterstain in the nucleus (Fig. 8C). This finding indicates that even a mosaic loss of MeCP2 in heterozygote female tissues might exert a more general impairment in signaling pathways and its downstream effectors.

DISCUSSION

In this study, we identified how the phosphorylation of rpS6 is broadly reduced across different brain areas and at different stages in mice lacking Mecp2. Surprisingly, this defect is detectable extremely early during post-natal development soon before neurological signs of the disease are evidently manifested. RpS6 abnormal low levels of phosphorylation are noticeable in both Mecp2 null males and heterozygote females and the dynamic of its reduction follows the worsening of the neurological conditions. Therefore, p-rpS6 might represent a valuable biomarker of the disease onset and its development. This is particularly significant for RTT that currently lacks any reliable molecular marker to follow its neuro-pathological progression.

We observed that the phosphorylation of rpS6 is reduced in both MeCP2− and MeCP2+ neurons in the brains of Mecp2 heterozygous female. This finding suggests that rpS6 phosphorylation in MeCP2 expressing cells is deregulated by the presence of neighboring Mecp2 null neurons. This might be caused by the aberrant secretion from mutant neurons or glia cells of soluble factor(s) with neurotoxic effects. It might be hypothesized as well that the functional impairment of mutant neurons inhibits neuronal activity in adjoining cells therefore reducing rpS6 phosphorylation. Both possibilities are not alternative and can be simultaneously occurring at a different grade. Dysfunctions in non-cell autonomous pathways have been already proposed to contribute to RTT progression. In fact, Mecp2 mutant astrocytes as well as microglia were found to induce multiple defects in WT neurons including the dendritic patterning, synapse formation and microtubule stability (45–48). However, our study indicated that p-rpS6 immunolabeling expression in glial cells is under the detection level of high-resolution confocal microscopy, thus suggesting that glia may not significantly contribute to the reduction in rpS6 phosphorylation in the brain of MeCP2 KO mice. The
elucidation of non-cell autonomous molecular mechanisms acting in neuronal networks mosaic for the MeCP2 mutation will be critical for our understanding of the disease pathogenesis in humans, which indeed affects primarily heterozygous girls.

Herein, we discovered a reduction in AKT/mTOR signaling associated with protein synthesis impairment in MeCP2 mutant brains. To our knowledge, this is the first time that a cellular molecular cascade with pleiotropic functions in controlling cell homeostasis is found altered in RTT. These combined molecular defects might be responsible at least in part for the decreased number of excitatory hippocampal synapses described in mice lacking MeCP2 (13,15,34–36). Nevertheless, RTT presents opposite molecular defects when compared with similar neurodevelopmental disorders, such as Fragile X, tuberous sclerosis and Phelan–Dunn syndrome, where, alternatively, mTOR signaling and protein synthesis result aberrantly up-regulated (49). Thus, RTT present peculiar molecular features where the hypoaeration of the mTOR signaling and limited protein synthesis is associated with reduced synaptic connectivity as already hypothesized by Kelleher and Bear (17). Despite the different molecular basis of RTT with respect to the other mentioned diseases, the final output is not so dissimilar, as in both cases, it causes a loss in neuronal network performance. In this scenario, the overall clinical neurological symptoms described as cognitive deficits, autism and impaired language and communication might arise from a positive as well as negative unbalance of the same molecular processes.

A large body of evidence indicates that the AKT/mTOR signaling pathway is a key modulator of the translation process, and its deficiency is frequently associated with defects in protein synthesis control (41,42,50). Consistent with this evidence, we identified a notable reduction in the overall translation rate in MeCP2 mutant brains. This impairment was evident by analyzing the polysomal fraction of ribosomal profiling, whereas less significant in an ex vivo metabolic labeling system. This may be due to the different sensitivity/noise levels associated with the two different approaches. Alternatively, a deficit in protein turnover in the mutant brains might influence the final result of the metabolic labeling, while not affecting in any means the ribosomal profiling...

Figure 7. Activation of rpS6 is reduced in the brain of symptomatic MeCP2+/− female mice. (A) Immunohistochemical detection of p-rpS6 (Ser235/236), p-rpS6 (Ser240/244) and total rpS6 in the S1 cortex and in the CA1 area of the hippocampus in MeCP2+/− female mice and in WT controls at 10 months of age. Insets show higher magnification views of the immunolabeling pattern in layer V neurons of the S1 cortex. (B and C) Histograms show quantitative analysis of the labeling intensity of p-rpS6 and total rpS6 expression in layers I–VI of the S1 cortex as well as in the CA1 pyramidal cell layer of the hippocampus. The expression of p-rpS6 (Ser235/236) and p-rpS6 (Ser240/244) is significantly decreased in MeCP2+/− mice compared with WT controls in each cortical layer (B) as well as in the CA1 area of the hippocampus (C) compared with control animals. Total rpS6 immunostaining intensity is unchanged between genotypes in the two areas examined. Data are expressed as the mean ± SEM. **P < 0.01, ***P < 0.001 (scale bars, 50 μm).
which represents a snapshot of the initiation of translation. Future studies will investigate whether protein turnover is impaired in MeCP2 mutant brains.

Intriguingly, protein synthesis defects are occurring very early in RTT. Even if, at present, we cannot indicate whether this translational defect stands as a cause or an early effect of the disease, it is likely that it contributes to the overall progression of the disease. In this study, we did not address whether the rate of local protein synthesis is similarly affected. However, the reduction in mTOR activity is such pervasive in Mecp2 mutant brains that is likely to impact on both global and local synthesis processes in comparable extent.

To determine whether translational repression in Mecp2 mutants is affecting different transcripts with a similar
magnitude, we evaluated the effective amount of mRNAs on polysomes for non-directly related genes with a pivotal role in neuronal activity like CAMKII and PSD-95. Interestingly, we found that the polysomal incorporation of the two transcripts was significantly lower in MeCP2 null mice with respect to WT. A similar trend was observed for Rack1, hnRNP A2/B1 and GADPH mRNAs. These three genes code for molecules that are not involved in neuronal activity and plasticity. Although restricted to the analysis of some genes, these results indicate that the translation impairment is not an event restricted to only few gene transcripts that impact to their protein production rate in similar levels.

On this basis, we favor the idea that a widespread limited protein production in MeCP2 null neurons might restrain over time their metabolic activity and, ultimately, their excitability and plasticity.

Given that MeCP2 is a chromatin-associated transcriptional regulator, a large effort has been put in identifying the altered gene expression occurring in Mecp2 mutant brains. However, a relative small amount of genes have been identified to be aberrantly expressed that do not account for fully explaining the development of the neuropathological signs (1,27,28). Why this is the case it remains still elusive. It has been proposed that the disease might be rooted in a genome-wide, but subtle, deregulation of global gene expression levels (51). Alternatively, the pathological gene alterations are strictly neuronal cell type specific and arduous to be identified in the expression profile studies conducted so far on entire brain subregions (52). Nevertheless, we described a new level of complexity of the disease represented by the widespread dysregulation of the protein synthesis process.

Considering our results, gene functional impairment in RTT might result from the sum of deregulated gene expression followed by a limited capability to produce the functional proteins.

Given the alterations that we discovered in two closely related processes like AKT/mTOR signaling and the protein synthesis rate, we suggest that a defect in this cellular pathway is responsible for the altered translational control in Mecp2 mutant neurons. In contrast, we have not found any evidence for a direct role of MeCP2 in the ribosomal machinery controlling translational processes. This is also consistent with the subcellular localization of MeCP2 almost exclusively confined in the nucleus of differentiated neurons (11,44,53). Further, MeCP2 is excluded from nucleoli in the nuclear compartment where ribosomal genesis is mainly taking place (54,55) (S. Ricciardi and V. Broccoli, unpublished results). However, we cannot exclude that MeCP2 might regulate translation initiation by associating with components of the pre-initiation complex (56). This might be also consistent with the ability of MeCP2 to bind RNA and regulate its processing (57). This possibility warrants future investigations to be better delineated.

In conclusion, we identified the AKT/mTOR signaling as a molecular pathway down-regulated in MeCP2 null neurons. Consistent with this finding, rpS6 phosphorylation is reduced in large areas of the Mecep2 male null or female heterozygous brains providing an interesting biomarker for following the dynamics of the pathology. Finally, the general process of protein synthesis was found evidently impaired surprisingly even at early pre-symptomatic stage, making likely its contribution to the progression of the disease. Although our results revealed the translational control as an additional process altered by the pathological process; nevertheless, they provide a novel context where therapeutic interventions can be developed to successfully restrain or ameliorate the development of RTT.

MATERIALS AND METHODS

Animals

The experiments performed in this study were conducted in accordance with European Community Council Directive 86/609/EEC for care and use of experimental animals with protocols approved by the Italian Minister for Scientific Research. To obtain the litters of WT animals and MeCP2 mutants used for this study, heterozygous Mecp2<sup>−/−<sup> mice with exon 3 deletion in MeCP2 (8) were crossed to C57BL6 for one generation, followed by breeding among offspring and were maintained on a mixed background. Age-matched litters were used in all experimental conditions to avoid possible consequences of genetic background unrelated to the MeCP2 mutation (37).

Immunohistochemistry and immunofluorescence

Animals were anesthetized with an intraperitoneal injection of chloral hydrate and transcardially perfused with ice cold 4% paraformaldehyde in 0.1 M phosphate buffer (PB; pH 7.4). After perfusion, the brains were dissected and kept in the same fixative solution overnight at 4°C. After several washes in 0.1 M PB, the brains were then cryoprotected by immersion in 10, 20 and 30% sucrose solutions and subsequently cut in 30-μm sections with a cryostat. Cryosections were collected in phosphate-buffered saline (PBS, 0.01 M, pH 7.4) and processed for free-floating immunohistochemistry as described (58). After a blocking step in a PBS solution containing 0.05% Triton X-100 and 10% normal goat serum (NGS), sections were then incubated overnight at room temperature with the following primary antibodies: rabbit anti-phospho-rpS6 (Ser235/236) (1:100); rabbit anti-phospho-rpS6 (Ser240/244) (1:200); rabbit anti-phospho-rpS6 (Ser429/431) (1:100) from Cell Signaling Technology (Danvers, MA, USA). Antibodies were diluted in PBS with 3% NGS and 0.05 Triton X-100. Sections were then washed in PBS (4 × 10 min), incubated for 1 h with goat anti-rabbit biotinylated secondary antibodies (1:250; Vector Labs, Burlingame, CA, USA) diluted in 3% NGS and 0.05% Triton X-100 in PBS and transferred to a solution containing a biotin–avidin complex (1:100, Vector Labs) diluted in PBS with 3% NGS and 0.05 Triton X-100. Sections were then washed in PBS (4 × 10 min) and mounted on gelatin-coated glass slides and observed with a light microscope (Eclipse 80i, Nikon, Japan) equipped with a CCD camera (AxioCam HRc, Zeiss, Germany).

Double immunofluorescence was performed with simultaneous addition of the primary antibodies as described (59). Briefly, cryosections were blocked in 10% normal donkey serum with 0.05% Triton X-100 in PBS for 1 h, then incubated at room temperature overnight with the following primary...
antibodies: rabbit anti-phospho-rpS6 (Ser235/236) (1:100), rabbit anti-phospho-rpS6 (Ser240/244) (1:200), goat anti-MeCP2 (1:250, Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse anti-NeU-N (1:100, Chemicon, Billerica, MA, USA) and mouse anti-GFAP (1:100, Cell Signaling Technology). After PBS washing, sections were incubated with secondary fluorescent antibodies (donkey anti-rabbit Alexa 488, Molecular Probes; donkey anti-goat Alexa 594, Invitrogen; donkey anti-mouse Alexa 594, Molecular Probes) for 1 h and, after several PBS rinses, incubated with DAPI (1:500, Invitrogen), mounted on gelatin-coated glass slides and observed with a confocal microscope (Zeiss LSM-5 Pascal, Germany).

**Immunohistochemical data analysis**

Total labeling of peroxidase in immunohistochemistry experiments was analyzed quantitatively by measuring OD on micrographs (10×) using a public-domain dedicated software (ImageJ, USA) by an operator blinded to the genotype. Measurements of OD in the cortex were obtained from 100 × 50 µm measuring boxes that were randomly placed in each cortical layer. OD values in the hippocampus were obtained from the CA1 pyramidal cell layer. Histograms illustrate the average OD obtained from three repeated measures in 4–5 sections per experimental animal at all ages examined (male WT n = 3; male MeCP2 KO n = 3; female WT n = 5; female MeCP2+/− n = 5). The mean OD of the corpus callosum was subtracted as background staining.

Double-fluorescence micrograph of the S1 cortex samples was acquired with a laser scanning confocal microscope, using the multitrack mode to avoid fluorescence crosstalk (pinhole: 1.0 airy unit). For the quantitative analysis of phospho-rpS6 fluorescence labeling in cortical neurons, Z-series stacks of four consecutive confocal sections (512 × 512 pixels) spaced by 2 µm were acquired at 20×. Images from both channels were overlaid and background labeling was subtracted. Fluorescence levels were analyzed on confocal images by measuring mean pixel intensity in either MeCP2-negative or MeCP2-positive neurons using ImageJ software. Histograms represent the mean fluorescence intensity calculated in at least 50 cortical neurons per animal (female WT n = 4; female MeCP2+/− n = 4). All data are presented as the mean ± SEM. Statistical analysis was done by Student’s t-test as well as one- or two-way ANOVA using GraphPad Prism software (La Jolla, CA, USA). For presentation, digital micrographs were processed with the software ImageJ. Files were imported into Adobe Photoshop (Adobe Systems, San Jose, CA, USA), where images were cropped.

**Polysomal profiles**

Polysomal profiles were performed as described previously (60), with some modifications. Brain tissues were extracted from the skull and homogenized in 50 mM Tris–HCl (pH 7.4), 100 mM NaCl, 30 mM MgCl₂, 0.1% NP40, 100 µg/ml of cycloheximide, 40 U/ml of RNasin® and protease inhibitor cocktail (Sigma-Aldrich). Whole-brain extracts were clarified at 4°C for 10 min at 15 000g. The equivalent of 10 absorbance units at 254 nm were layered on a 15–50% sucrose gradient in 50 mM Tris–acetate, pH 7.5, 50 mM NH₄Cl, 12 mM MgCl₂ and 1 mM DTT and centrifuged at 4°C in a SW41Ti Beckman rotor for 3.5 h at 39 000 rpm. The absorbance at 254 nm was recorded by BioLogic LP software (Bio-Rad) and fractions were collected. In order to disrupt 80S ribosomes and polysomes, total extracts were treated with 30 mM EDTA for 10 min at 4°C. EDTA-treated and -untreated samples were then loaded on a sucrose gradient. Fractions were precipitated with 10% TCA (trichloroacetic acid) according to the standard protocol, separated on SDS–PAGE and analyzed by western blot. RNA was isolated from each fraction by phenol:chloroform:isoamylalcohol (25:24:1) (Sigma-Aldrich) and mRNAs were analyzed by qRT-PCR. For quantitative measurements, polysomal profiles were scanned and the area of ribosomal peaks assessed with ImageJ (NIH) software.

**Metabolic labeling**

Six 400-µm sections were prepared from whole brain per animal (average age P40) using a tissue slicer. The slices were maintained in carbogenated, at 32.5°C ACSF (120 mM NaCl, 2.5 mM KCl, 1.2 mM MgCl₂, 2.5 mM CaCl₂, 26.2 mM NaHCO₃, 1 mM NaH₂PO₄, 11 mM Glucose) for 1.5 h. Following a 1.5 h recovery, slices were transferred to 15 mm diameter netwells (Corning) containing 5 ml of carbogenated ACSF. For each animal, three slices were incubated for 30 min in carbogenated ACSF with 100 µg/ml of cycloheximide. Slices were then incubated in carbogenated ACSF containing 11 µCi/ml of Promix ³²S-labeled methionine (Amersham) for 1 h. Netwells were then transferred to 12-well dish containing ice-cold dissection buffer to stop protein synthesis and remove the excess of ³²S-labeled methionine. Slices were subsequently removed and homogenized in ice-cold lysis buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 1% NP40 and a mix of proteases inhibitors from Sigma-Aldrich). Extracts of 10 µl were TCA-precipitated on glass micro fibre filters (Whatman) and counted. Obtained values were normalized by sample protein content, quantified using the bicinchoninic acid protein assay (Pierce). Final data were expressed as the ratio between the incorporated counts per minute (CPM) of non-treated slices and the CPM of cycloheximide-treated slices.

**Western blotting**

Brain tissues were extracted from the skull and homogenized in lysis buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 1% NP40, 0.1% SDS and a mix of phosphatases and proteases inhibitors from Sigma-Aldrich) at 4°C. Lysates were clarified by centrifugation for 15 min at 18 000g, and protein concentration of the supernatant was determined using bovine serum albumin (BSA) as a standard (Bradford reagent assay, Sigma-Aldrich). Total lysates were boiled in SDS sample buffer, separated by SDS–PAGE and blotted to nitrocellulose membrane (Amersham). Filters were blocked in tris-buffered saline Tween-20 (0.1%) (TBST) (10 mM Tris–HCl, pH 8.0, 150 mM NaCl and 0.05% Tween-20) plus 5% BSA (Sigma-Aldrich) and incubated with primary antibodies for 16 h at 4°C. The following primary antibodies were used: rabbit polyclonal anti-MeCP2 (1:1000, Sigma-Aldrich),
Quantitative RT-PCR
An equal volume of RNA from each eleven-numbered fraction was reverse transcribed with random hexamer primers by Transcriptor High Fidelity cDNA Synthesis Kit (Roche), according to the manufacturer’s instructions. Real-time PCR was performed with specific primers and SsoFast™ EvaGreen® Supermix (Bio-Rad) in a C1000™ Thermal Cycler (Bio-Rad). The amount of template and the number of amplification cycles were preliminarily optimized for each PCR to avoid conditions of saturation. For mRNA quantification, we constructed a standard curve with a serial dilution of RNA. For the experimental sample, the mRNA level was calculated using its standard curve obtained for that mRNA.

Supplementary Material
Supplementary Material is available at HMG online.

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Conflict of Interest statement
None declared.

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References


