miR-199a Links MeCP2 with mTOR Signaling and Its Dysregulation Leads to Rett Syndrome Phenotypes

Highlights
- MeCP2 facilitates the processing of miR-199a as a component of the Drosha complex
- miR-199a ameliorates RTT neuronal phenotypes and its inhibition blocks MeCP2 function
- miR-199a positively controls mTOR signaling by targeting mTOR signaling inhibitors
- Genetic deletion of miR-199a-2 recapitulates numerous RTT phenotypes

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In Brief
Tsujimura et al. find that MeCP2 facilitates processing of miR-199a, which, in turn, leads to upregulation of mTOR signaling. Genetic deletion of miR-199a-2 recapitulates RTT phenotypes, suggesting that the MeCP2/miR-199a/mTOR axis may contribute to RTT pathophysiology.
miR-199a Links MeCP2 with mTOR Signaling and Its Dysregulation Leads to Rett Syndrome Phenotypes

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SUMMARY

Rett syndrome (RTT) is a neurodevelopmental disorder caused by MECP2 mutations. Although emerging evidence suggests that MeCP2 deficiency is associated with dysregulation of mechanistic target of rapamycin (mTOR), which functions as a hub for various signaling pathways, the mechanism underlying this association and the molecular pathophysiology of RTT remain elusive. We show here that MeCP2 promotes the posttranscriptional processing of particular microRNAs (miRNAs) as a component of the microprocessor Drosha complex. Among the MeCP2-regulated miRNAs, we found that miR-199a positively controls mTOR signaling by targeting inhibitors for mTOR signaling, miR-199a and its targets have opposite effects on mTOR activity, ameliorating and inducing RTT neuronal phenotypes, respectively. Furthermore, genetic deletion of miR-199a-2 led to a reduction of mTOR activity in the brain and recapitulated numerous RTT phenotypes in mice. Together, these findings establish miR-199a as a critical downstream target of MeCP2 in RTT pathogenesis by linking MeCP2 with mTOR signaling.

INTRODUCTION

Rett syndrome (RTT) is a severe progressive neurodevelopmental disorder that affects approximately one in 10,000 females. Afflicted individuals appear to develop normally for the first 6–18 months but then regress, with the onset of various neurological symptoms including impaired motor function, mental retardation, seizure, autistic features, and stereotyped behaviors (Bienvenu and Chelly, 2006). RTT is predominantly caused by mutations in the X-linked gene encoding methyl-CpG binding protein 2 (MeCP2) (Amir et al., 1999). Mutations in MECP2 are also associated with other neurodevelopmental diseases including psychiatric disorders, cognitive disorders, and some cases of autism (Cohen et al., 2002; Klauck et al., 2002; Lam et al., 2000; Orrico et al., 2000).

MeCP2 was originally identified as a methylated-DNA-binding transcriptional repressor (Jones et al., 1998; Nan et al., 1997). MeCP2 induces repression of gene expression by recruiting a corepressor complex (Harikrishnan et al., 2005; Jones et al., 1998). However, MeCP2 is becoming recognized as a pleiotropic protein, which can also mediate transcriptional activation and mRNA splicing (Chahrour et al., 2008; Youngh et al., 2005). Although MeCP2-deficient neurons clearly underlie the RTT phenotype, other studies have shown that the loss of MeCP2 in glia negatively influences normal neuronal functions in a non-cell-autonomous fashion in vitro and in vivo (Ballas et al., 2009; Liyo et al., 2011; Maeszawa and Jin, 2010).
Several mouse models of RTT have been reported (Chen et al., 2001; Guy et al., 2001; Shahbazian et al., 2002). These models recapitulate many characteristic features of RTT, including the delayed-onset neurological phenotype and early mortality. Conditional deletion of MeCP2 in the brain causes symptoms that are indistinguishable from those observed in conventional MeCP2-knockout (KO) mice. Moreover, re-expression of MeCP2 in the MeCP2-deficient brain is sufficient to prevent the onset of the neurological phenotype (Guy et al., 2007; Luikenhuis et al., 2004).

In addition to genetic studies, several lines of evidence further support the effects of MeCP2 dysfunction on neuronal properties in RTT patients and model mice. Although both exhibit profound neurological abnormalities, the major neuropathological changes in the CNS are characterized by smaller neuronal soma and an overall decrease of brain size (Armstrong, 2005; Bauman et al., 1995; Chahrouh and Zoghbi, 2007; Chen et al., 2001; Kaufmann et al., 2000). Subtle alterations of neuronal density, dendritc arborization, and spine formation in some specific brain regions and stages are also found in both patients and model mice in which MeCP2 is mutated (Armstrong, 2005; Belichenko et al., 1994; Chapleau et al., 2009; Landi et al., 2011). It has been proposed that synaptic alterations constitute a major substrate of the disease symptoms (Boggio et al., 2010; Zoghbi, 2003).

Neurophysiological studies of RTT patients and model mice have revealed alterations in excitatory synaptic functions (Asaka et al., 2006; Dani et al., 2005; Glaze, 2005). In particular, synaptic properties of MeCP2-deficient neurons are well studied in in vitro culture systems. Primary cultured hippocampal neurons from MeCP2-KO mice show a decrease in the frequency of spontaneous excitatory synaptic transmission (Nelson et al., 2006), and loss and overexpression of MeCP2 have opposite effects, respectively decreasing and increasing the number of excitatory synapses in individual neurons (Chao et al., 2007). The molecular mechanisms underlying these morphological and physiological alterations are unknown.

The mechanistic target of rapamycin (mTOR) protein kinase acts as a critical sensor/integrator of diverse environmental signals that are converted to neuronal activity and synaptic inputs. mTOR function is mediated through two distinct complexes, mTOR complex 1 (mTORC1) and mTORC2. mTORC1 phosphorylates a range of substrates to control major cell processes such as mRNA translation, lipid synthesis, and autophagy. mTORC2 is implicated in the activation of Akt and in the regulation of the mTOR signaling pathway, we performed comprehensive proteomic screening of mouse brain cells to identify protein partners of MeCP2 in highly enriched populations of the four major CNS cell types: neural stem cells (NSCs), neurons, astrocytes, and oligodendrocytes (Figure S1A). We then categorized the MeCP2-interacting candidate molecules in each cell type according to the Gene Ontology (GO) biological processes in which they are involved (Figure 1A). Because MeCP2 is a well-known transcriptional repressor/activator, it was expected that transcription-related molecules would appear among the MeCP2 interactors; surprisingly, however, we also observed numerous members of other functional categories such as nucleosome assembly, translation, and RNA processing (Figure 1A). Strikingly, five components of the miRNA microprocessor Drosha complex (Gregory et al., 2004)—the DEAD-box RNA helicases DX5 and DX17, and heterogeneous nuclear ribonucleoproteins G/M/U (hnRNPG/M/U)—were common to at least three or all four of the CNS cell types (Figures 1B and 1C). These same Drosha complex components were also detected, by LC-MS/MS, in endogenous MeCP2 complexes obtained from postnatal day (P)1 mouse brains (Figure 1D). These findings prompted us to assume that MeCP2 plays hitherto-unknown roles in miRNA processing.

The miRNA microprocessor Drosha complex consists of Drosha, DGDeGeorge syndrome critical region gene 8 (DGC8R), and multiple classes of RNA-associated proteins, including DX5 and DX17, that are required for the processing of some, but not all, miRNAs (Gregory et al., 2004). The appearance of Drosha complex proteins among MeCP2 co-immunoprecipitates prompted us to examine whether MeCP2 might function unexpectedly as a facilitator of miRNA processing.

In this study, we found that MeCP2 associates with the microRNA microprocessor Drosha complex and identified miR-199a as an mTOR-regulating downstream target of the MeCP2-Drosha complex. We further demonstrated that genetic deletion of miR-199a-2 led to a reduction of mTOR activity in the brain and recapitulated numerous RTT phenotypes in mice. These findings therefore suggest that MeCP2/miR-199a/mTOR axis contribute to RTT pathophysiology.

RESULTS

MeCP2 Associates with miRNA Microprocessor Drosha Complex

To obtain a clue to elucidate the molecular mechanism that underlies RTT pathogenesis and links MeCP2 to the mTOR signaling pathway, we performed comprehensive proteomic screening of mouse brain cells to identify protein partners of MeCP2 in highly enriched populations of the four major CNS cell types: neural stem cells (NSCs), neurons, astrocytes, and oligodendrocytes (Figure S1A). We then categorized the MeCP2-interacting candidate molecules in each cell type according to the Gene Ontology (GO) biological processes in which they are involved (Figure 1A). Because MeCP2 is a well-known transcriptional repressor/activator, it was expected that transcription-related molecules would appear among the MeCP2 interactors; surprisingly, however, we also observed numerous members of other functional categories such as nucleosome assembly, translation, and RNA processing (Figure 1A). Strikingly, five components of the miRNA microprocessor Drosha complex (Gregory et al., 2004)—the DEAD-box RNA helicases DX5 and DX17, and heterogeneous nuclear ribonucleoproteins G/M/U (hnRNPG/M/U)—were common to at least three or all four of the CNS cell types (Figures 1B and 1C). These same Drosha complex components were also detected, by LC-MS/MS, in endogenous MeCP2 complexes obtained from postnatal day (P)1 mouse brains (Figure 1D). These findings prompted us to assume that MeCP2 plays hitherto-unknown roles in miRNA processing.

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We first investigated molecular interactions between MeCP2 and components of the Drosha complex. Exogenous MeCP2 associated with DDX5 and Drosha in human HEK293T cells (Figure 1E). Using a reciprocal co-immunoprecipitation assay, we also confirmed that endogenous MeCP2 associated with core components of the Drosha complex including Drosha, DGCR8, and DDX5 in a mouse brain lysate (Figure 1F). Biochemical analysis also revealed that exogenous and endogenous MeCP2 complex contains the components of Drosha complex (Figures S1B–S1D). These results indicate that MeCP2 associates with...
the core components of the Drosha complex, both in vitro and in vivo.

Recent work has reported that MeCP2 interferes with the association of Drosha with DGCR8 in the Drosha complex (Cheng et al., 2014). However, we did not find any significant change in the association between Drosha and DGCR8 regardless of MeCP2 expression level (Figures S1E–S1H).

**MeCP2 Regulates Processing of a Specific miRNA**

To identify target miRNAs of the MeCP2-Drosha complex, we performed deep RNA sequencing using a small non-coding RNA fraction extracted from neurons and NSCs of wild-type (WT) and KO mice. No drastic and/or overall alterations of mature miRNA expression levels were observed in either cell type (Figures 2A and 2B), suggesting that MeCP2 does not affect core Drosha-DGCR8 microprocessor function. We then searched for miRNAs whose level decreased over 1.5-fold in MeCP2-KO cells compared with WT. Sixty and 75 miRNAs were reduced in MeCP2-KO neurons and NSCs, respectively (Figure 2C). Since MeCP2 associates with the Drosha complex in both cell types, we focused on miRNAs that decreased in both cell types and found nine that met this criterion.

We then performed functional screening for mTOR signal-regulating miRNAs that act downstream of MeCP2. We expressed the nine miRNAs in hippocampal neurons and evaluated neuronal soma size, since mTOR signaling is known to regulate cellular soma size, and found that only miR-199a increased the neuronal soma size to a similar extent as MeCP2 expression did (Figure 2D). We also found that miR-199a expression and blockade (see below for details) potentiated and attenuated, respectively, the phosphorylation levels in neurons of S6 ribosomal protein, which signify the activation of mTOR signaling, indicating that miR-199a positively regulates mTOR signaling activity (Figures S2A and S2B). Therefore, we decided to focus on miR-199a in the following experiments.

miR-199a is expressed from two genomic loci, miR-199a-1 and miR-199a-2, and pri-miR-199a-2 is synthesized with pri-miR-214 as a single transcript (Lee et al., 2009). Two types of mature form, miR-199a-5p and -3p, are generated from both miR-199a-1 and miR-199a-2 (Figure S2C), but no function of miR-199a in the nervous system has yet been reported. To determine whether MeCP2 specifically regulates posttranscriptional processing of miR-199a, we first measured the expression levels of primary and mature forms of miR-199a and miR-214, by qRT-PCR, in hippocampal neurons from WT and MeCP2-KO mice. We found that the mature forms miR-199a-5p and miR-199a-3p were downregulated in MeCP2-KO neurons (Figure 2E), the same held true in MeCP2-KO NSCs (Figure S2D), as reported previously (Sulwach et al., 2010). In contrast, pri-miR-199a-1 and -2 levels showed no significant change in MeCP2-KO neurons compared with those in WT neurons (Figure 2F), suggesting that the reduced mature-form levels were not caused by decreased levels of transcription. In addition, expression levels of the mature forms of miR-214 and miR-137, the latter previously identified as a transcriptional repression target of MeCP2 in NSCs (Sulwach et al., 2010), were unchanged (Figures 2E and 2F; Figures S2D and S2E). Conversely, overexpression of MeCP2 in WT neurons led to significant increases of mature miR-199a-5p and -3p but not of mature miR-214 (Figure 2G). Expression of primary miR-199a and miR-214 was either unchanged or slightly reduced in response to MeCP2 overexpression in WT neurons (Figure 2H). To further determine whether MeCP2 regulates the expression of miR-199a, we also re-expressed MeCP2 in MeCP2-KO neurons. As expected, this restored the level of mature-miR-199a expression to that in WT neurons (Figure 2I). No change in the mature miR-137 expression level was observed (Figure S2E). Together, these results suggest that MeCP2 modulates specifically the posttranscriptional processing of pri-miR-199a, rather than its transcription. Dysregulation of miR-199a expression at posttranscriptional level was also observed in the frontal cortex of RTT patients (Table S1; Figures S2F and S2G), suggesting that the reduction of miR-199a expression may associate with RTT pathophysiology.

We then examined the in vivo association of pri-miR-199a with the MeCP2 complex by RNA immunoprecipitation assay and found that both pri-miR-199a-1 and pri-miR-199a-2 clearly associated with the complex (Figure 2J). We also performed an in vitro pri-miRNA processing assay by incubating radiolabeled pri-miR-199a-2 substrate with anti-FLAG antibody immunoprecipitates from Neuro2A cells transfected with empty vector (mock), or with FLAG-tagged MeCP2- or FLAG-tagged Drosha-expressing constructs. pri-miR-199a-2 processing activity in the MeCP2 complex immunoprecipitates was comparable to that in Drosha complex immunoprecipitates, and higher than
Figure 3. miR-199a Overcomes Neuronal Abnormalities Caused by the Loss of MeCP2 and Acts Downstream of MeCP2
(A) Representative traces of individual mEPSC frequency recordings. P0–P2 WT and MeCP2-KO hippocampal neurons were infected with lentiviruses expressing GFP alone (control) or GFP together with the precursor form of miR-199a and cultured for 10–14 days.
(B) Quantification of mEPSC frequency and amplitude in (A). The number of neurons analyzed is shown under each bar.
(C) Representative images of cultured hippocampal neurons as indicated, stained with antibodies against VGLUT1 (green), PSD-95 (red), and MAP2 (blue). Scale bar, 5 μm.
(D) Normalized densities of colocalized VGLUT1 and PSD-95 signal in the hippocampal cultures in (C) were quantified (n = 10 neurons per group).
(E) Quantification of neuronal soma size relative to control WT neurons. n = 3 independent experiments; in each experiment at least 100 neurons were assessed.
(F) Representative traces of individual mEPSC frequency recordings of WT hippocampal neurons co-infected with control or MeCP2-expressing lentiviruses and those expressing sponge miRNA inhibitors against either miR-199a-5p or miR-199a-3p.
(G) Quantification of mEPSC frequency and amplitude in (F). The number of neurons analyzed is shown under the bars.
(H) Representative images of cultured hippocampal neurons as indicated, stained with antibodies against VGLUT1 (green), PSD-95 (red), and MAP2 (blue). Scale bar, 5 μm.

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that in immunoprecipitates from mock-transfected cells (Figures S2H and S2I). Moreover, Drosha complex from MeCP2-knockdown cells showed lower processing activity against pri-miR-199a than that from control cells (Figures S2J and S2K). In contrast, processing activity by the Drosha immunoprecipitates against pri-miR-214 was similar regardless of MeCP2 expression levels. Taken together, these results indicate that MeCP2 specifically contributes to the posttranscriptional processing of pri-miR-199a.

**miR-199a Acts Downstream of MeCP2 In Vitro and In Vivo**

MeCP2 target molecules that can rescue major RTT neuronal phenotypes such as decreased excitatory synaptic transmission, synaptic density, and soma size have not been identified. Therefore, we investigated whether miR-199a, identified here as a MeCP2 target, restores RTT neuronal properties of MeCP2-deficient neurons.

MeCP2-KO neurons displayed a significantly lower miniature excitatory postsynaptic current (mEPSC) frequency, lower synaptic density, and smaller soma size than WT neurons, as reported previously (Chao et al., 2007; Nelson et al., 2006) (Figures 3A–3E). These impairments were overcome by the re-introduction of MeCP2 (Figures S3A–S3E). Likewise, expression of precursor form miR-199a in MeCP2-KO neurons completely restored RTT neuronal phenotypes such as decreased mEPSC frequency, excitatory synaptic density, and soma size, indicating that miR-199a can substitute for the function of MeCP2 in neurons (Figures S3A–3E; Figures S3F and S3G). No significant differences in mEPSC amplitude were observed under any conditions (Figures 3A–3E).

To determine whether miR-199a indeed acts downstream of MeCP2, we inhibited miR-199a function in MeCP2-overexpressing WT hippocampal neurons using miRNA inhibitors against miR-199a-5p or miR-199a-3p. The increase of mEPSC frequency and synaptic density as well as the enlargement of neuronal soma size induced by MeCP2 overexpression were abolished when the cells were treated with miR-199a-5p inhibitor but not with control or miR-199a-3p inhibitor (Figures 3F–3J). Although miR-134 was reported to act downstream of MeCP2 (Cheng et al., 2014), the expression of miR-134 in neurons did not influence the effects of MeCP2 overexpression in our experimental setting (Figures S3H–S3L).

To test whether blocking endogenous activities of miR-199a recapitulates the RTT neuronal phenotype, we treated WT hippocampal neurons with miRNA inhibitors against miR-199a-5p or miR-199a-3p. In contrast to scrambled control and miR-199a-3p inhibitor, treatment with an inhibitor of miR-199a-5p resulted in a significant reduction of mEPSC frequency, synaptic density, and soma size (Figures S3M–S3Q). We also observed that overexpression of miR-199a in WT hippocampal neurons phenocopied MeCP2 overexpression (Figures S3R–S3V). We further demonstrated that inhibition of endogenous miR-214 and expression of miR-214 could not abolish the effect of MeCP2 expression (mEPSC) (Figures S3W and S3X) and could not restore the RTT neuronal phenotypes (synaptic density and soma size) (Figures S3Y and S3Z), respectively. When we performed in utero electroporation to mouse embryonic brains, the effects of MeCP2 and miR-199a on soma size observed in vitro could be reproduced in vivo (data not shown). These results strongly indicate that miR-199a, specifically miR-199a-5p, is a direct MeCP2 target that critically contributes to MeCP2 functions (Figure 3K).

**miR-199a Targets Inhibitory Factors of the mTOR Signaling Pathway**

To identify target genes downstream of miR-199a that are physiologically relevant to RTT pathogenesis, we searched for mTOR signal-inhibiting genes that have miR-199a-5p target sequences in their 3′ UTRs using public databases including TargetScan 5.1, miRDB, Pictor, and miRanda. This search yielded three candidate genes: Pde4d, Sirt1, and Hif1a (Ghosh et al., 2010; Kim et al., 2010; Wouters and Koritzinsky, 2008) (Figure 4A). To examine whether these genes are actual targets of miR-199a, we conducted luciferase assays using reporter constructs harboring 3′ UTR of each gene, within which were native or mutated sequence complementary to the miR-199a seed sequence, in hippocampal neurons. The luciferase activities of native Pde4d, Sirt1, and Hif1a-3′ UTR constructs were all reduced significantly by miR-199a expression, whereas those of mutated Pde4d, Sirt1, and Hif1a-3′ UTR constructs were unaffected (Figure 4B). We then examined protein levels of PDE4D, SIRT1, and HIF1α in MeCP2-KO brain and found that they were indeed upregulated compared to those in WT (Figures 4C and 4D), suggesting that miR-199a targets Pde4d, Sirt1, and Hif1α downstream of MeCP2 and downregulates their cognate protein levels.

Having suggested that MeCP2 and miR-199a are epistatic to and negatively regulate PDE4D, SIRT1, and HIF1α, we tested whether expression of these mTOR signal negative regulators reverses the effect of MeCP2 expression in neurons. Because HIF1α is degraded through hydroxylation of specific proline residues in a normal atmosphere, we used in this experiment a constitutively active form of HIF1α (HIF1α-CA) whose hydroxylation-targeted proline residues are substituted by alanine (Jaakkola et al., 2001). The expression of Pde4d, Sirt1, and Hif1α (without their cognate 3′ UTR) abolished the enhanced mEPSC frequency, the increase in synapse number, and the enlarged soma size induced by MeCP2 overexpression (Figures 4E–4S). Interestingly, Pde4d, Sirt1, and Hif1α-CA with their native 3′ UTR sequences failed to abolish these effects of MeCP2 overexpression, while constructs with a 3′ UTR harboring a mutation in the miR-199a-target sequence significantly attenuated the effects of MeCP2 (Figures 4E–4S), indicating that miR-199a plays a critical role in controlling the expression of these mTOR signal inhibitors.
Figure 4. Three Negative Regulators of mTOR Signaling Are Functional Downstream Targets of miR-199a

(A) Luciferase reporter assays of the predicted miR-199a target genes. Schematics of luciferase assay reporter constructs are shown in (A). The miR-199a-5p seed sequence, miR-199a-5p target (upper, Nat), and its mutated (lower, Mut) sequences of each gene’s 3' UTR are indicated (blue boxes). The mir-199a-5p seed and its target sequences in Pde4d, Sirt1, and Hif1α 3' UTRs are highlighted in light blue. Reduction of luciferase activity of Pde4d, Sirt1, and Hif1α 3' UTRs with native miR-199a-5p sequences was observed (B). Data are represented as mean ± SEM. *p < 0.05. n = 3 independent experiments.

(C) Western blot analysis showing that protein levels of PDE4D, SIRT1, and HIF1α are upregulated in MeCP2-KO brain (P1 cortex).

(D) Quantification of protein levels of PDE4D, SIRT1, and HIF1α. n = 3 brains from each genotype.

(E–S) PDE4D, SIRT1, and HIF1α act downstream of MeCP2/miR-199a.

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We next asked whether the inhibition of these miR-199a downstream targets ameliorated RTT neuronal phenotypes. When we pharmacologically inhibited PDE4D with rolipram and SIRT1 with nicotinamide (NIC) and EX527, the defects in mEPSC, synaptic density, and soma size of MeCP2-KO neurons were all overcome (Figures S4A–S4D). Knockdown of Pde4d also remarkably restored these RTT neuronal phenotypes (Figures S4P–S4T). In addition, treatment with rolipram, NIC, and EX527 significantly recovered the neuronal impairments induced by the inhibition of miR-199a-5p (Figures S4U–S4D). Moreover, when PDE4D, SIRT1, and HIF1α-CA were expressed in WT hippocampal neurons, similar neuronal impairments to those caused by MeCP2 deficiency and miR-199a inhibition were observed (Figures S4E–S4J). Taken together, these data support our model that Pde4d, Sirt1, and Hif1α are functional downstream targets of miR-199a in RTT neuropathology.

mTOR Underlies MeCP2-Related RTT Neuronal Phenotypes

Although the mTOR signaling pathway is dysregulated in MeCP2-deficient mice and cells (Li et al., 2013; Ricciardi et al., 2011), it has not been ascertained that mTOR signaling indeed functions downstream of MeCP2. Therefore, we first investigated whether activation of mTOR signaling ameliorates RTT neuronal phenotypes. We found that expression of Rheb, an upstream activator of mTOR, in MeCP2-KO hippocampal neurons clearly restored the neuronal impairments observed in MeCP2-KO neurons affecting mEPSC, synaptic density, and soma size (Figures 5A–5E). Moreover, activation of mTOR signaling by Rheb expression reversed the defects in neurons induced by miR-199a-5p inhibition and by PDE4D, SIRT1, and HIF1α-CA expression (Figures 5F–5J; Figures S5A–S5O). In support of these results, inhibition of mTOR by rapamycin strikingly abrogated the effects induced by MeCP2 and miR-199a expression, and by the pharmacological inhibition of miR-199a targets (PDE4D with rolipram and SIRT1 with EX527) (Figures 5K–5T; Figures S5P–S5Y). Furthermore, activation and inhibition of mTOR signaling in WT hippocampal neurons recapitulated the gain and loss of MeCP2 phenotypes, respectively (Figures S5Z–S5I). Collectively, these findings demonstrate that mTOR signaling underlies MeCP2-related RTT phenotypes in neurons (Figures 5U and 5V).

Loss of miR-199a-2 Recapitulates RTT Phenotypes In Vivo

miR-199a-1 KO mice had already been produced and do not display any gross physical or behavioral abnormalities (information from The Jackson Laboratory: http://jaxmice.jax.org/strain/017512.html) (Park et al., 2012), and it has been reported that the Dnm3os KO mice, which lack both miR-199a-2 and miR-214, show similar skeletal abnormality and growth retardation to Rett patients and MeCP2 KO mice (O’Connor et al., 2009; Watanabe et al., 2008). In contrast, miR-214 single KO mice develop normally (Aurora et al., 2012), clearly indicating that the phenotypes observed in Dnm3os KO mice are attributable to the lack of miR-199a-2. Based on these observations, we further investigated the functions of miR-199a by establishing miR-199a-2 KO mice using conventional method (Figures S6A–S6C). To our surprise, miR-199a-2 KO mice displayed many RTT features that were similar to MeCP2 KO mice (Chen et al., 2001; Guy et al., 2001). We observed a marked reduction in size and body weight of the miR-199a-2 KO mice compared to WT littermates, while miR-199a-2+/− heterozygotes are almost indistinguishable from WT (Figures 6A, 6B, and 6D). Brain size was also smaller in miR-199a-2 KO mice (Figure 6C). Although most miR-199a-2 KO mice appeared normal for the first ~3 postnatal weeks, but they then developed abnormal behavior, such as a stiff, uncoordinated gait, body trembling, pilar erection, irregular breathing, and hypopigmentation (Figure 6E), and began to die at around 6 weeks (Guy et al., 2007). Immunohistochemical analysis revealed that miR-199a-2 KO mice had normal brain architecture (Figures 6F and 6G), whereas their hippocampal and cortical neurons exhibited a smaller cell body and nucleus (Figures 6H–6K) regardless of MeCP2 expression (Figure 6D). In addition, we found that these neurons in miR-199a-2 KO mice were more densely packed than those in WT, consistent with findings in RTT patients and MeCP2 KO mice (Figures 6H–6K) (Bauman et al., 1995; Chen et al., 2001; Guy et al., 2001). Taken together, these results indicate that miR-199a is a critical downstream target of MeCP2 for RTT phenotypes in vivo.

Loss of miR-199a-2 Results in Decreased mTOR Activity in the Brain

To ask whether miR-199a regulates mTOR signaling in the brain, we assessed mTOR activity in the WT and miR-199a-2 KO brain. Concordant with our hypothesis that miR-199a positively regulates mTOR activity, immunohistochemical analysis showed a striking reduction of pS6-positive neurons in the cortex and hippocampus of miR-199a-2 KO mice (Figure 7). Collectively, these data suggest that miR-199a activates mTOR activity in vivo and that the MeCP2/miR-199a/mTOR axis makes a substantial contribution to RTT pathophysiology.
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DISCUSSION

In this study, we demonstrated that MeCP2 facilitates specific miRNA processing as a component of Drosha complex and identified miR-199a as a direct MeCP2 target. We also revealed that exogenous miR-199a could overcome several impairments in MeCP2-KO neurons, and that inhibition of endogenous miR-199a abolished the effect of MeCP2 overexpression. Taken together, these results provide strong evidence that miR-199a is a direct MeCP2 target underlying the RTT phenotype. It has been reported recently that the interaction between Drosha and DGCR8 was attenuated in the brain lysate of MeCP2-overexpressing transgenic mice due to the competition of MeCP2 with Drosha for binding to DGCR8, suggesting MeCP2 has a negative role in miRNA processing. However, in our experimental setting using embryonic hippocampal neurons, overexpression of MeCP2 did not interfere with association of Drosha with DGCR8 and enhanced the specific miRNA processing (Figures S5C and SSD; Figure 2G). Although currently we do not have specific explanation of how MeCP2 exerts these opposite functions in miRNA processing, it could be, e.g., because of that posttranslational modification status of MeCP2 is different among different brain regions and neuronal subtypes, since phosphorylation of MeCP2 is known to alter MeCP2 functions (Tao et al., 2009). To verify the molecular mechanism is also an important challenge for future studies.

In the present study, we demonstrated that MeCP2 associates with pri-miR-199a-1 and pri-miR-199a-2. Nevertheless, how MeCP2 recognizes its target miRNAs remains elusive. Since pri-miR-199a-1 and pri-miR-199a-2 have exactly the same sequence in their mature miRNA regions but are completely different on the outsides, it is tempting to speculate that mature miRNA sequence is responsible for defining the specificity of MeCP2. In this relation, it has been indicated that transcription factor Smad1 directly binds to the cognate sequence within the mature sequence of target primary miRNA (Davis et al., 2010). However, precise consensus sequence of MeCP2 binding has not so far been determined, and we could not identify any common sequence among mature sequences in the miRNA suggested as MeCP2 targets in this study; we cannot rule out a possibility that other factors, such as conformation and methylation state of the outside regions, can play a critical role on regulating the MeCP2 binding specificity.

Although it has been assumed that a reduction of mTOR signal activity is involved in RTT pathogenesis, plausible molecular link between MeCP2 and the mTOR signaling pathway have been scant. Our findings clearly reveal a role for MeCP2 in the regulation of the mTOR signaling pathway as well as a previously unknown miRNA-based mechanism by which MeCP2 controls neuronal functions, which could clearly explain RTT pathophysiology (Figures 5U and 5V). Most importantly, we have demonstrated that genetic deletion of miR-199a-2 attenuates mTOR activity in the brain and recapitulates numerous features of RTT patients and MeCP2 KO mice, supporting the idea that the MeCP2/miR-199a/mTOR axis is deeply involved in RTT phenotypes. Based on these findings, we propose that dysregulation of the MeCP2/miR-199a/mTOR axis contributes to the pathogenesis of neurodevelopmental disorders, and that an avenue for the development of an effective therapeutic strategy against RTT is to control molecules involved in this pathway.

EXPERIMENTAL PROCEDURES

See Supplemental Experimental Procedures for additional details.

RNA immunoprecipitation

Co-immunoprecipitation of RNAs with anti-MeCP2 antibody and their subsequent isolation were performed using a RibocCluster Profiller RIP-Assay kit (MBL International Corporation) according to the manufacturer’s protocol. Whole-cell lysates were sonicated and used for the RNA immunoprecipitation assay. Rabbit immunoglobulin G (lgG) supplied by the manufacturer was used as a control and the immunoprecipitated RNA was analyzed by qRT-PCR using a TaqMan pri-microRNA assay kit (Applied Biosystems) with specific primers.

Luciferase assay

Hippocampal neurons were co-transfected with control (pLLX) or miR-199a expression vector and pmirGLO-Pde4a, Sirt1, Hif1α-3’ UTR-Nat or...
Figure 6. Loss of miR-199a-2 Recapitulates RTT Phenotypes
(A and B) Comparison of body size of WT, heterozygous (Het), and miR-199a-2 KO littermates.
(C) Comparison of brain size of WT, Het, and miR-199a-2 KO littermates.
(D) miR-199a-2 KO mice body weight is less than that of WT and Het littermates (measured from postnatal 3–6 weeks). Data are represented as mean ± SEM. n = 3–5.
(E) Plots of the phenotypic scores of WT, Het, and miR-199a-2 KO littermates. Data are represented as mean ± SEM. n = 3–6.
(F) Representative immunohistological images of WT, Het, and miR-199a-2 KO hippocampi. Brain sections at 6 weeks were stained with anti-NeuN (green) antibody and Hoechst 33258 (blue). Hippocampus size of miR-199a-2 KO is smaller than that of WT, but abnormal structural changes were not observed.
(G) Representative immunohistological images of WT and miR-199a-2 KO cortices. Brain sections at 6 weeks were stained with anti-NeuN (green) antibody and Hoechst 33258 (blue). Abnormal structural changes are not detected in miR-199a-2 KO brain.
(H) Representative immunohistological images of cortical layer II/III regions of WT and miR-199a-2 KO mice. Brain sections at 6 weeks were stained with anti-NeuN (green) and Hoechst 33258 (blue). Decreased soma size of neurons and increased neuronal packing density were observed in miR-199a-2 KO mice.
(I) Quantification of neuronal soma size in (H). *p < 0.05 compared to WT; n = 9 sections (n = 3 brains of each genotype).
(J) Representative immunohistological images of hippocampus CA3 regions of WT and miR-199a-2 KO mice. Brain sections at 6 weeks were stained with anti-NeuN (green) and Hoechst 33258 (blue). Decreased soma size of neurons and increased neuronal packing density are observed in miR-199a-2 KO mice.
(K) Quantification of neuronal soma size in (J). *p < 0.05 compared to WT; n = 9 sections (n = 3 brains from each genotype).
pmirGLO-Pde4d, Sirt1, Hif1α-3' UTR-Mut using Lipofectamine 2000. After transfection, the cells were incubated for 6 days and lysed with reporter lysis buffer. Luciferase activity of the lysates was measured with the Dual-Glo Luciferase Assay System (Promega) according to the manufacturer’s protocol. Firefly luciferase activities were determined by three independent transfections and normalized by comparison with the Renilla luciferase activities of the internal control.

**Scoring of Symptoms**
Neurological symptoms of mice were scored as previously described (Guy et al., 2007).

**Statistical Analysis**
The results presented are the average of at least three experiments, each performed in triplicate, with SEs. Statistical analyses were performed by one-way ANOVA, followed by Tukey’s or Bonferroni multiple comparison tests, or by Student’s t test as appropriate, using Prism 5 (GraphPad Software). Probabilities of p < 0.05 were considered significant.

**ACCESSION NUMBERS**
The DNA DATA Bank of Japan (DDBJ) Sequence Read Archive (DRA) data-bank accession number for all deep sequencing data reported in this paper is DRA: DRA002731, which includes data sets of neurons and NSCs.

**SUPPLEMENTAL INFORMATION**
Supplemental Information includes Supplemental Experimental Procedures, six figures, and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2015.08.028.

**AUTHOR CONTRIBUTIONS**
K.T. conceived the original ideas, designed the project, and wrote the paper. K.T. performed the majority of the experiments with participation from K.I. and H.N. Y.N. contributed to experiments about SIRT1. S.T. and Y.E.
contributed to electrophysiology experiments. Y.F. and M.F. contributed to mass spectrometry. M.I. contributed to analysis using human RTT patient samples. T.I. and M.U. contributed to bioinformatic analyses. Y.Y. and T.A. contributed generation of miR-199a-2 KO mice. K.N. supervised the whole project and wrote the paper.

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