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MeCP2 controls dendritic morphogenesis via miR-199a-mediated Qki downregulation

Abbreviated Title: MeCP2/miR-199a/Qki axis promotes dendritic growth

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Abstract

Rett syndrome (RTT) (OMIM: 312750) is a severe neurodevelopmental

disorder caused by mutations in the *MECP2* gene. Although decreased dendritic

morphogenesis has been observed in the brain of RTT patients and mouse models, the

molecular mechanisms underlying these dendritic anomalies remain unclear. We have

previously shown that MeCP2 facilitates specific microRNA (miRNA) processing by

associating with the miRNA microprocessor Drosha complex. In this study, we show that

MeCP2 positively regulates dendritic formation via *miR-199a*, a specific target of the

MeCP2-Drosha complex. Overexpression of MeCP2 and miR-199a promotes dendritic

development such as increases in dendrite length, branching number, and complexity. In

contrast, blocking miR-199a inhibited dendrite formation and abolished enhanced

dendritic development induced by MeCP2 expression. We also demonstrate that the

decreased dendrite outgrowth observed in MeCP2-deficient neurons could be rescued by

miR-199a expression. In addition, we found that miR-199a targets the 3' untranslated

region of *quaking (Qki)*, a negative regulator of dendritic development, and

downregulates its protein expression level. Furthermore, we report an increase in the Qki

protein expression level in *miR-199a-2*-deficient brains and show that Qki knockdown

restores the dendritic morphology of *miR-199a-2*-Knockout (KO) neurons. Taken

together, these results suggest that the MeCP2/miR-199a/Qki axis is critical for proper

dendritic development and its dysregulation contributes to the dendritic pathology in RTT.

Introduction

Dendritic morphogenesis is critical for establishing functional neural circuits during brain development (1, 2), with dendrite anomalies associated with neurodevelopmental and neuropsychiatric disorders (3-5). Rett syndrome (RTT) is a progressive neurodevelopmental disorder caused by loss-of-function mutations in the X-linked *methyl-CpG binding protein 2 (MECP2)* gene (6). RTT patients develop normally until 6-18 months of age, beyond which there is an onset of regression and various neurological symptoms including seizures, stereotypies, loss of motor skills, autistic features, and mental retardation (7, 8). Studies on postmortem brains have revealed dendritic atrophy in RTT patients (9-11). Several mouse models lacking *Mecp2* (MeCP2-KO) or carrying a mutation on *Mecp2* (12-16) mimic many RTT phenotypes including motor impairments, seizures, and stereotypies (12, 13, 16). Reduced dendritic growth was observed in these models (17, 18) and dendritic atrophy was reported in cultured MeCP2-deficient neurons (19, 20). Therefore, impaired dendritic development may contribute to RTT pathophysiology, with MeCP2 regulating dendritic formation. However, little is known about the mechanisms underlying these phenomena.

MeCP2 belongs to the methylated DNA binding protein family and functions

as a transcriptional repressor by recruiting co-repressors including histone deacetylases

(HDACs) (21, 22), in addition to being a transcriptional activator and a splicing regulator

(23, 24).

MicroRNAs (miRNAs) are small noncoding RNAs that regulate gene

expression at the post-transcriptional level and are involved in many biological processes

including cell proliferation, differentiation, and development (25, 26). Primary miRNAs

(pri-miRNAs) are transcribed from DNA, processed into precursor miRNAs (pre-

miRNAs) by the nucleus-associated Drosha complex, and transported to the cytoplasm

by Exportin-5, where these are processed into mature miRNAs by Dicer. Mature miRNAs

bind to the 3' untranslated region (3'UTR) of target mRNAs and regulate gene expression

by inhibiting their translation or degradation (27, 28). Increasing evidence suggests a key

regulatory role of miRNAs in dendritic development. For example, miR-137 represses

dendritic morphogenesis by inhibiting Mind bomb 1 expression (29) and miR-9 regulates

dendritic formation by targeting the *repressor element-1 silencing transcription factor*

(*REST*) gene (30). Recently, we have shown that miR-214 positively controls dendritic

morphogenesis by regulating the expression of the schizophrenia-associated protein

quaking (Qki) (31).

We have reported that MeCP2 facilitates specific pri-miRNA processing by

associating with the Drosha complex and identified miR-199a as a target of the MeCP2-

Drosha complex (32). We also showed that miR-199a-5p, a mature form of miR-199a,

regulates mTOR signaling downstream of MeCP2 by inhibiting the expression of mTOR

signal inhibitors, and dysregulation of the MeCP2/miR-199a-5p/mTOR signaling

contributes to RTT neuronal phenotypes such as reduction in excitatory synaptic

transmission and density and smaller neuronal soma size (32). Furthermore, we

demonstrated that genetic deletion of miR-199a-2, a genomic loci of miR-199a,

recapitulates RTT phenotypes in mice and leads to decreased brain mTOR activity (32).

Although these findings identify miR-199a as a critical downstream target of MeCP2 in

RTT pathology, its contribution to dendritic phenotype remains unexplored. Despite

reports on suppression of dendritic development by MeCP2-mediated disruption of the

Drosha-DGCR8 complex (33), this negative regulation of dendritic morphology by

MeCP2 is inconsistent with the dendritic phenotype in postmortem brains of RTT

individuals and animal models (9-11, 17, 18).

In this study, both miR-199a-5p and miR-199a-3p were found to contribute to

dendrite development downstream of MeCP2. Overexpression and inhibition of miR-

199a in primary cultures of hippocampal neurons promoted and impeded dendrite

development, respectively. Importantly, blocking miR-199a-3p strongly inhibited

dendritic morphogenesis and completely abolished MeCP2-induced enhancement of

dendritic growth, highlighting the crucial role of miR-199a-3p in dendritic development.

We also demonstrated that miR-199a overexpression rescued dendritic impairment in

MeCP2-KO neurons. Additionally, miR-199a-3p was observed to target *Qki* mRNA and

suppress Qki expression. Indeed, Qki expression was elevated in miR-199a-2-KO mice

brain and *Qki* knockdown improved impaired dendritic development in miR-199a-2-KO

neurons. These results suggest that the MeCP2/miR-199a/Qki pathway plays a critical

role in dendritic development and its dysregulation accounts for the dendritic phenotype

in RTT mouse models.

Results

Expression of MeCP2 correlates with mature-miR-199a production in developing

hippocampal neurons

Although previous studies have shown that MeCP2 positively regulates

neuronal maturation (34, 35), little is known about the mechanistic basis of its regulation

of dendritic development. Previously, we have reported that MeCP2 promotes specific

miRNA processing and identified miR-199a as a MeCP2 target accountable for the RTT

phenotypes in mouse models of the disease (32). For this reason, we focused on the role

of miR-199a for understanding MeCP2-regulated dendritic development of neurons. To

explore the impact of the MeCP2/miR-199a pathway on dendritic morphogenesis, we

examined the expression patterns of MeCP2 and miR-199a in cultures of developing

hippocampal neurons. MeCP2 expression level gradually increased during neuronal

development (Fig. 1A and B). The pri-miR-199a is expressed from 2 gene loci, *miR-199a-*

1 and *miR-199a-2*, and pre-miR-199a eventually generates 2 mature forms of miR-199a,

i.e., miR-199a-5p and miR-199a-3p, which are highly conserved across mammalian

species (Fig. 1C) (32). Expression levels of pri-miR-199a-1 and pri-miR-199a-2 in

cultured hippocampal neurons were found to increase at an early stage and then gradually

decrease at later stages of neuronal maturation (Fig. 1D and E), whereas that of the 2

types of mature miR-199a (miR-199a-5p and -3p) increased with neuronal dendrite

growth (Fig. 1F and G). These results imply that the MeCP2/miR-199a axis has a role in

the regulation of dendritic morphogenesis.

MeCP2 and miR-199a promote dendritic development in cultured hippocampal neurons

141   To investigate the influence of the MeCP2/miR-199a axis in the dendritic

development of neurons, we expressed MeCP2 or miR-199a in primary cultured

hippocampal neurons by infecting with lentiviral systems expressing GFP as control,

along with MeCP2 or miR-199a (Fig. 2A). Then, we evaluated dendritic morphology by

immunocytochemical assays utilizing antibodies against MAP2, a marker for neuronal

dendrites. In neurons expressing exogenous MeCP2, total dendrite length and branch

number were found to be significantly higher than that in control. Interestingly, we found

that miR-199a expression in neurons significantly increased dendrite length and branch

number to the same level as in MeCP2-expressing cells (Fig. 2B-D). We also performed

Sholl analysis by measuring the number of dendrites that cross concentric circles at

different radial distances from the cell body. The Sholl analysis revealed that miR-199a,

as well as MeCP2, enhances dendritic arborization compared to the control (Fig. 2E).

Together, these results suggest that the MeCP2/miR-199a pathway promotes dendritic

morphogenesis in hippocampal neurons.

Both mature forms of miR-199a are required for dendritic development in hippocampal

neurons

The pre-miR-199a generates 2 types of mature miRNAs, miR-199a-5p and -3p.

We, therefore, investigated which of these 2 mature forms contributes to dendritic

morphogenesis of neurons. We expressed the miRNA inhibitor (sponge) against either

miR-199a-5p (miR-199a5pi) or miR-199a-3p (miR-199a-3pi) in primary cultured

hippocampal neurons (Fig. 3A) and evaluated the resultant dendrite morphology.

Blocking of endogenous miR-199a-5p was found to lead to decreased total dendrite

length, but not branch number, compared with control samples, whereas miR-199a-3p

inhibition decreased both (Fig. 3B-D). Furthermore, expression of both miR-199a-5pi

and miR-199a-3pi reduced dendritic complexity, although the effect of miR-199a-5pi was

less pronounced compared with that of miR-199a-3pi (Fig. 3E). These results suggest that

both mature forms of miR-199a can regulate dendritic development, although miR-199a-

3p plays a more crucial role than miR-199a-5p.

miR-199a acts downstream of MeCP2 in the regulation of dendritic development

172   Since MeCP2 is known to promote the processing of pri-miR-199a, we next

examined whether miR-199a functions downstream of MeCP2 in dendrite formation. We

expressed the inhibitors for the respective mature miR-199a, along with MeCP2, in

cultured hippocampal neurons and evaluated the resultant dendritic morphology (Fig. 4A).

Inhibition of miR-199a-5p decreased the MeCP2-mediated enhancement of total dendrite

length, but not branch number, in neurons compared with control, whereas blocking miR-

199a-3p reduced both (Fig. 4B-D). Moreover, although the effect of miR-199a-3pi was

stronger than that of miR-199a-5pi, we found that expression of both the mature forms

inhibited MeCP2-induced increase in dendritic arborization (Fig. 4E). These results

suggest that both forms of mature miR-199a act as regulatory factors downstream of

MeCP2 in dendritic development, although miR-199a-3p is more stringently involved in

this regulation.

MeCP2 and miR-199a enhance dendritic development in vivo

We next examined the effects of MeCP2 and miR-199a in dendritic

morphogenesis *in vivo* in the mouse brain. For this, we expressed MeCP2 and miR-199a

in WT mouse brains by *in utero* electroporation at E14.5 and analyzed neuronal

morphology at P10. Almost all the GFP-positive transfected neurons at this stage were

located in layer IV (31). When we co-expressed MeCP2 and GFP in the WT mouse brain,

electroporated neurons showed enhanced dendrite length and branching number

compared with that of control (Fig. 5A-D). Furthermore, co-expression of miR-199a and

GFP in these WT neurons significantly promoted increase in dendrite length and

branching number, such that the latter were comparable to those in MeCP2-expressing

neurons (Fig. 5A-D). We also performed Sholl analysis and found that expression of

MeCP2 and miR-199a facilitates dendritic complexity and increases it to levels similar to

that in control conditions (Fig. 5E). Taken together, these data suggest that the

MeCP2/miR-199a axis positively regulates dendritic development *in vivo*.

Expression of miR-199a rescues dendritic impairment in MeCP2-deficient neurons in

vivo

202   We next asked whether miR-199a expression could reverse the impairment of

dendritic morphogenesis in MeCP2-deficient neurons. To this end, we expressed miR-

199a in the MeCP2-KO mouse brain by *in utero* electroporation at E14.5 and analyzed

neuronal morphology at P10. When we expressed only GFP in the MeCP2-KO mouse

brain, electroporated neurons showed decreased dendrite length and branching number

compared with that in WT. By contrast, the expression of miR-199a in MeCP2-KO

neurons significantly reversed the reduced dendrite length and branching number to the

level of WT neurons (Fig. 6A-D). Furthermore, Sholl analysis revealed that miR-199a

expression restored the dendritic complexity to levels observed in MeCP2-KO neurons

(Fig. 6E). Together with the *in vitro* results, these insights support the notion that miR-

199a functions as a downstream regulator of MeCP2 in dendritic development *in vivo*.

miR-199a-3p targets 3'UTR of Qki mRNA and suppresses its protein expression in

neurons

To understand the mechanisms by which miR-199a regulates dendritic

morphogenesis downstream of MeCP2, we attempted to identify genes targeted by each

mature form of miR-199a. We have previously demonstrated that miR-199a-5p positively

regulates mTOR signaling by targeting *Pde4d*, *Sirt1*, and *Hif1a* in hippocampal neurons

(32). Since mTOR signaling is known to promote morphological development of

dendrites (36), we reasoned that miR-199a-5p enhances dendritic arborization through

mTOR signaling. Additionally, since we have earlier shown that miR-199a-3p regulates

dendritic development to a greater extent compared with miR-199a-5p, we particularly

focused on identifying the genes targeted by miR-199a-3p. To this end, we first utilized

public　　databases　　such　　as　　miRDB　　(http://mirdb.org),　　TargetScan

(http://www.targetscan.org/vert_72/),　and　PicTar　(https://pictar.mdc-berlin.de),　and

identified 9 candidate target genes which were common in results from the three databases.

These included *amyloid beta (A4) precursor-like protein 2 (APLP2), Cbp/p300-*

interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 2 (Cited2), F-box

and WD-40 domain protein 11 (Fbxw11), far upstream element (FUSE) binding protein

1 (Fubp1), mitogen-activated protein kinase kinase kinase 4 (Map3k4), muscleblind-like

1 (Mbnl1), platelet-derived growth factor receptor (Pdgfr), protein phosphatase 2

regulatory subunit B' epsilon (Ppp2r5e), and quaking (Qki) (Fig. 7A). Since the 3'UTR

of *Qki* mRNA has the largest number of seed sites among these candidates and we have

recently shown that Qki negatively regulates dendritic development (31), we chose the

Qki gene as the most plausible target of miR-199a-3p. Next, to investigate whether miR-

199a-3p directly targets the *Qki* mRNA, we performed a luciferase reporter assay using

reporter constructs in which a luciferase reporter gene was placed under post-

transcriptional control of the native *Qki* 3'UTR or a *Qki* 3'UTR harboring mutations in

all three miR-199a-3p seed sites (Fig. 7B). These luciferase constructs, along with a miR-

199a-expressing plasmid, were co-transfected in hippocampal neurons, and luciferase

activity was measured. The latter showed significant decrease on miR-199a expression in

neurons containing the construct harboring all three native seed sequences. However, the

luciferase activities of reporter constructs with mutation in either of the three seed

sequences did not show such sensitivity to miR-199a expression (Fig. 7C). To determine

whether miR-199a-3p regulates Qki expression at the protein level, we expressed miR-

199a in hippocampal neurons and carried out immunoblotting assays using an anti-Qki

antibody. The *Qki* gene is known to generate three isoforms of the Qki protein (Qki-5,

Qki-6, and Qki-7) by alternative splicing (37). We found that the expression levels of all

three Qki isoforms were substantially decreased in miR-199a-expressing cells compared

with that in control cells (Fig. 7D and E). These findings suggest that miR-199a-3p

suppresses Qki protein expression in hippocampal neurons through interaction with the

3'UTR of *Qki* mRNA.

miR-199a-2-KO neurons show reduced dendritic formation and Qki knockdown

reinstates abnormal dendritic morphology in miR-199a-2-deficient neurons

We have previously generated miR-199a-2 deficient (miR-199a-2-KO) mice,

which recapitulates many RTT phenotypes such as reduction in body weight, brain size,

and neuronal soma size (32). We, therefore, attempted to confirm that miR-199a-3p

regulates Qki expression using the miR-199a-2-KO mouse model by estimating the

expression levels of miR-199a and Qki in miR-199a-2-KO neurons. Although miR-199a

262 is supposed to be expressed from 2 gene loci, expression levels of miR-199a-5p and miR-

263 199a-3p in the cortex of miR-199a-2 KO mice were significantly reduced compared with

264 those in WT mice (Fig. 8A). On the contrary, the expression of Qki protein was increased

265 in the same mice (Fig. 8B and C). Then, we investigated the dendritic morphology of

266 miR-199a-2 KO neurons and found that cultured hippocampal neurons from miR-199a-

267 2 KO mice exhibits decreased dendrite length and branching number compared with

268 neurons from WT mice. Further, we investigated whether downregulation of Qki

269 expression could reverse the dendritic phenotypes of miR-199a-2 KO neurons. For this,

270 we transfected miR-199a-2 KO neurons with lentivirus expressing shRNAs against *Qki*

271 and evaluated the resultant dendritic morphology. Interestingly, expression of shRNA

272 against *Qki* in miR-199a-2 KO neurons significantly restored the decrease in dendrite

273 length and branch number (Fig. 8D-F). Decreased dendritic complexity of miR-199a-2

274 KO neurons was also improved by Qki knockdown (Fig. 8G). From these results, we

275 concluded that miR-199a controls dendrite morphogenesis by targeting Qki.

Discussion

Although Rett syndrome is known to be caused by loss-of-function mutations

in the *MECP2* gene, the mechanisms by which dysfunction of *MECP2* leads to this

disease phenotype has remained largely elusive. A number of studies have reported

abnormal reductions in dendritic development in the postmortem brains of RTT

individuals and genetically engineered mouse models (9-11, 14, 17, 18, 34, 35).

Additionally, MeCP2-deficient neurons are known to exhibit abnormally decreased

dendritic development in *in vitro* culture systems (19, 20). Dendritic atrophy found in the

brain of RTT patients and mouse models of the disease have been related to dysfunctions

of the neural circuits (3). Therefore, these findings imply that defects in dendritic

morphology are a key feature in understanding RTT pathogenesis. However, the

molecular basis linking MeCP2 functions to the regulation of dendritic development is

still unclear. In this study, we have demonstrated the functional role of miR-199a in the

MeCP2-mediated regulation of dendritic morphogenesis. We first showed that the

expression of MeCP2 correlates with that of miR-199a in developing hippocampal

neurons. Transient up-regulation of pri-miR-199a expression was observed at early

developmental stages of these cultured hippocampal neurons, along with a reduction in

its expression at later stages. On the contrary, the expression levels of both MeCP2 and

mature miR-199a were upregulated during maturation of hippocampal neurons. These

observations led us to hypothesize that miR-199a has a critical role downstream of

MeCP2 in dendritic maturation and implied that the expression of mature miR-199a is

tightly regulated at the posttranscriptional level during neuronal maturation. Since we

have previously shown that miR-199a regulates multiple aspects of neurons, such as

neuronal growth and excitatory synaptic transmission and formation downstream of

MeCP2 (32), we postulated that miR-199a could also contribute to dendritic development

downstream of MeCP2. In support of our idea, we showed here that miR-199a positively

regulates morphological development of neuronal dendrites. Additionally, blocking of

mature miR-199a function was shown to abolish the MeCP2-mediated increase in

dendritic outgrowth. The expression of miR-199a in MeCP2-KO neurons was found to

improve the loss in dendritic morphology elicited by MeCP2 deficiency. In the present

study, we also investigated dendritic morphology of miR-199a-2 KO neurons and found

that dendrite formation was significantly decreased compared with WT, similar to that

observed in MeCP2 KO neurons. Since miR-199a-2 KO mice mimics various phenotypes

of MeCP2 KO mice (32), miR-199a KO neurons understandably recapitulated the

dendritic phenotype of MeCP2 KO neurons. Taken together, these findings clearly

indicate that miR-199a acts downstream of MeCP2 in the regulation of dendrite

morphogenesis and support the notion that miR-199a is a critical downstream target of

MeCP2 in RTT pathogenesis (32).

We also highlighted the differential effects of mature miR-199a, miR-199a-5p,

and mir-199a-3p on dendritic development. Whereas inhibition of miR-199a-5p reduced

only dendrite length, blocking miR-199a-3p induced a decrease in both dendrite length

and branch number. These results suggested that both mature forms of miR-199a regulate

dendrite formation through independent cascades by targeting different downstream

targets. We have earlier demonstrated that miR-199a-5p positively regulates neuronal

functions including neuronal growth, excitatory synaptic transmission, and

synaptogenesis by controlling mTOR signaling, and mTOR signaling has been reported

to enhance dendrite outgrowth (32, 36). Based on this, we postulated that miR-199a-5p

promotes dendritic formation, at least in part, by regulating mTOR signaling. On the

contrary, the target genes of miR-199a-3p in neurons have not been investigated so far.

Since *Qki* mRNA has three seed sequences of miR-199a-3p in its 3'UTR and we recently

reported the negative regulation of dendrite development by Qki (31), we hypothesized

that miR-199a-3p targets *Qki* gene in neurons. Our findings were in overall agreement

with the hypothesis and demonstrated that miR-199a-3p directly targets the 3'UTR of *Qki*

mRNA and suppresses Qki protein expression in neurons.

In the present study, the mechanisms underlying Qki-mediated regulation of

dendritic development remain to be determined. Qki is an RNA-binding protein that

belongs to the signal transduction and activation of RNA (STAR) protein family and is

reported to regulate RNA splicing, RNA export, RNA stability, and protein translation

(38, 39). A previous study has reported that Qki regulates the stability of actin-interacting

protein 1 (AIP1) mRNA during oligodendrocyte differentiation (40). Parallelly, a number

of studies have revealed that actin cytoskeleton dynamics plays an important role in the

regulation of dendritic morphogenesis (41, 42). Therefore, it is possible that Qki regulates

dendritic formation by targeting AIP1. Many other target mRNA candidates of Qki have

also been identified by SELEX (systematic evolution of ligands by exponential

enrichment) and bioinformatics based analysis (43). Intriguingly, *Tbr1*, *Wnt2a*, and

Rab5a have been identified as putative targets of Qki. A recent work has reported that

Tbr1 regulates laminar patterning of retinal ganglion cell dendrites (44). Another study

has shown that the Wnt/Dishevelled pathway controls development of dendritic

morphology and a component of the early endocytic pathway, Rab5a, enhances dendritic

morphogenesis of fly dendritic arborization neurons (45).

Genetic studies have implicated *Qki* in psychiatric disorders including

schizophrenia (46, 47), and mutations in *MECP2* have been reported in schizophrenia

patients (48). Given the dual lines of evidence on the contribution of altered dendritic

morphology in various neurological diseases (5, 49, 50) and MeCP2-mediated regulation

of Qki expression, it is possible that dysfunction of MeCP2/miR-199a/Qki axis

contributes to abnormal dendritic phenotypes associated with a variety of neurological

disorders.

Although an abnormal decrease in dendritic development is observed in the

postmortem brains of RTT patients and mouse models, the mechanisms underlying this

dendritic phenotype have not been understood to date. Our findings reveal a key role of

the MeCP2/miR-199a/Qki pathway in the regulation of dendritic morphogenesis and

provide a molecular basis linking MeCP2 dysfunction with impaired dendritic

development. We, therefore, propose that controlling this molecular pathway offers a new

avenue for the development of an effective therapeutic strategy for RTT.

Material and Methods

Animals – All aspects of animal care and treatment were carried out according

to the guidelines of the Experimental Animal Care and Use Committee of Kyushu

University and Nagoya University, respectively. Timed-pregnant ICR mice (Japan SLC,

Shizuoka, Japan) were used for the present research. The *Mecp2* mutant (*Mecp2^{tm1.1Jae}*)

used in this study was created by deleting exon 3 containing the methyl-DNA-binding

domain of MeCP2 (12) and was obtained from Jackson Laboratories. miR-199a-2-KO

mice (Acc. No.: CDB1185K; http://www2.clst.riken.jp/arg/micelist.html) were generated

as described previously (32). All mutant mice were maintained on a C57BL/6J (Jackson

Laboratories) or ICR (Japan SLC) background.

Cell culture – Hippocampal neuronal cultures were carried out as previously

described (51). Briefly, hippocampi were dissected from embryonic day 17.5 (E17.5)

mice and were digested in S-MEM (Gibco) containing 0.1% papain (Sigma), and

triturated with 60 mg/ml DNase I (Sigma) and 10% fetal bovine serum (FBS). Dissociated

cells were plated on poly-*L*-lysine (10 μg/ml, Sigma)-coated culture dishes at densities of

1×10^4 cells/cm^2 and 5×10^4 cells/cm^2 for immunocytochemical and immunoblotting

assays, respectively. Once neurons had adhered (after 3-4 h), the medium was replaced

with serum-free neuronal maintenance medium, comprising Neurobasal medium

(Invitrogen) supplemented with B-27 (Invitrogen) and 0.5 mM GlutaMAX (Invitrogen).

Cytosine β-D-arabinofuranoside hydrochloride (Sigma) was added 1 day after plating to

prevent the proliferation of undifferentiated and glial cells. The medium was changed

every 3 days. HEK 293T cells were cultured in DMEM containing 10% FBS and

gentamicin (Nacalai Tesque).

Construction of vectors – Lentiviral vectors used to express the short hairpin

RNA (shRNA) for *Qki* (pLLX-shQki) and FLAG-tagged Qki (pLEMPRA-Qki and

pLEMPRA-Qki-3'UTR) were generously provided by Drs. Z. Zhou and M. E. Greenberg.

pLLX and pLEMPRA are dual-promoter lentivirus vectors constructed by inserting the

U6 promoter-driven shRNA cassette 5' into the ubiquitin-C promoter in the FUIGW

plasmid (52, 53). The pLLX-primary-miR-199a plasmid was constructed by inserting a

PCR-amplified fragment from mouse genomic DNA into the HpaI and XhoI restriction

sites of pLLX. The shRNA for Qki and sponges against miR-199a-5p- or miR-199a-3p-

expressing lentivirus plasmids were constructed by inserting the following

oligonucleotides into the HpaI and XhoI sites of pLLX: sh-Qki-3-Fw, 5'-

TGGACTTACAGCTAAACAACTTTTCAAGAGAAAGTTGTTTAGCTGTAAGTCC

TTTTTTTGAAC-3', and sh-Qki-3-Rv, 5'-

tcgagttccaaaaaaGGACTTACAGCTAAACAACTTt-

ctcttgaaAAGTTGTTTAGCTGTAAGTCCa-3'; sponge-miR-199a-5p-Fw, 5'-

gacgttaacGAACAGGTACGTCTAACACTGGGGAACAGGTACGTCTAACACTGGG

GAACAGGTACGTCTAACACTGGGtttttttctcgaggtc, and sponge-miR-199a–5p-Rv; 5'-

gacctcgagaaaaaaCCCAGTGTTAGACGTACCTGTTCCCCAGTGTTAGACGTACCTG

TTCCCCAGTGTTAGACGTACCTGTTCgttaacgtc-3'; sponge-miR-199a-3p-Fw, and

5'-

gacgttaacTAACCAATGCATCGACTACTATTAACCAATGCATCGACTACTATTAAC

CAATGCATCGACTACTATtttttttctcgaggtc-3'; and sponge-miR-199a-3p-Rv and 5'-

gacctcgagaaaaaaATAGTAGTCGATGCATTGGTTAATAGTAGTCGATGCATTGGTTA

ATAGTAGTCGATGCATTGGTTAgttaacgtC-3'. *Qki*, cDNA fragments and its 3'UTR-

containing fragments were amplified by PCR using KOD polymerase (Toyobo) and

subsequently cloned into the EcoRI and AscI sites of pLEMPRA-MeCP2 (53). The

luciferase reporter plasmids pmirGLO-Qki-3'UTR-1ss-nat, -2ss-nat, and -3ss-nat were

constructed by inserting the genomic DNA fragments of Qki 3'UTR (1ss, +1361 to

+2374; 2ss, +3330 to +4337; and 3ss, +4134 to +5144) into the PmeI and XhoI sites of

pmir-GLO (Promega). Each of the mutated luciferase reporter constructs was obtained

by inserting the mutated 3'UTR of Qki with the seed regions of miR-199a-3p into the

PmeI and XhoI sites of pmir-GLO.

In utero electroporation – To investigate dendritic growth *in vivo*, *in utero*

electroporation was performed on WT or MeCP2-KO E14 mice (ICR background)

embryos, as described previously (54). Briefly, plasmid DNA (0.1 μg/μL in phosphate-

buffered saline (PBS) containing 0.1% Fast Green FCF dye) was injected (0.5-1 μl) into

the lateral ventricle of the embryonic brain from outside the uterus with a glass

micropipette (GD-1, Narishige). While holding the embryo in the uterus with forceps-

type electrodes (Nepa Gene), 50-ms electric pulses of 45 V each were delivered 5 times

at intervals of 950 ms using a CUY21 Single Cell Electroporator (Nepa Gene). Glass

micropipettes were prepared using a P-1000IVF micropipette puller (Sutter). Animals

were perfused with 4% paraformaldehyde at postnatal day 10 (P10). Collected brains

were postfixed with 4% paraformaldehyde overnight at 4°C and then equilibrated in 30%

sucrose. Brains were frozen at -80°C after embedding in Tissue-Tek optimal cutting

temperature (OCT) compound (Sakura Finetek) and serially sectioned at 40-μm thickness.

Immunocytochemical assays – Cells were fixed at the indicated day(s) *in vitro*

(DIV) with 4% paraformaldehyde in PBS, washed with PBS, permeabilized, and blocked

with blocking buffer (3% FBS and 0.1% Triton X-100 in PBS) at room temperature (RT).

The cells were then incubated with the primary antibody solution at RT for 3 h. The cells

were then washed with PBS and further incubated with the secondary antibody solution

at RT for 1 h. After further washing with PBS, these were mounted on glass slides.

Immunohistochemical assays – Brain sections were washed with PBS,

permeabilized, and blocked with blocking buffer at RT. These sections were then

incubated with the primary antibody solution overnight at 4°C, washed with PBS, and

further incubated with the secondary antibody solution at RT for 2 h. These sections were

mounted on glass slides, after washing with PBS. Fluorescence images were acquired

using a Zeiss LSM 700 confocal microscope with a 20× objective lens. Z series of 20

images were taken at 1-μm intervals at a resolution of 1,024 × 1,024 pixels.

Immunoblotting – Cells were lysed with a buffer containing 0.5% Nonidet P-

40, 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 1% protease inhibitor mixture (Nacalai

Tesque). Lysates were sonicated and centrifuged at 20,000xg for 15 min at 4°C. Total cell

lysates were subjected to SDS-PAGE and transferred to a PVDF transfer membrane (GE

Healthcare). The blots were blocked with 0.3% skim milk in TBST (50 mM Tris-HCl, pH

7.5, 150 mM NaCl, 0.1% Tween 20), incubated with a primary antibody, and washed

again with TBST. This was followed by incubation with a peroxidase-conjugated

secondary antibody. Immunoreactive bands were detected by enhanced

chemiluminescence using the ECL Prime Western Blotting Detection Reagent (GE

Healthcare).

Antibodies – For immunocytochemical and immunohistochemical assays, the

following primary antibodies were used: chicken anti-green fluorescent protein (GFP)

(1:1000; Aves Labs) and guinea pig anti-microtubule-associated protein 2 (MAP2)

(1:1000; Synaptic Systems) antibodies. The following secondary antibodies were used:

CF488A donkey anti-chicken IgY (IgG) (H + L) highly cross-adsorbed (1:500; Biotium)

and CF555 donkey anti-guinea pig IgG (H + L) highly cross-adsorbed (1:500; Biotium).

Nuclei were stained using bisbenzimide H 33258 fluorochrome trihydrochloride (Nacalai

Tesque).

For immunoblotting, the following primary antibodies were used: rabbit anti-

MeCP2 (1:500; Diagenode), rabbit anti-QKI (1:250; ProteinTech), rabbit anti-GAPDH

(1:1000; Cell Signaling Technology), and mouse anti-β-actin (1:1000; Cell Signaling

Technology). The following secondary antibodies were used: anti-mouse IgG, HRP-

linked whole antibody from sheep (1:5000; GE Healthcare) and anti-rabbit IgG, HRP-

linked whole antibody from donkey (1:5000; GE Healthcare).

Luciferase reporter assay – Hippocampal neurons were co-transfected with

control (pLLX) or miR-199a expression vectors and pmir-GLO-Qki-1ss-nat, pmir-GLO-

Qki-1ss-mut, pmir-GLO-Qki-2ss-nat, pmir-GLO-Qki-2ss-mut pmir-GLO-Qki-3ss-nat,

or pmir-GLO-Qki-3ss-mut using Lipofectamine 2000 (Life Technologies). After

transfection, the cells were incubated for 3 days, and lysed with a 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.

Morphological analysis of cultured hippocampal neurons – For analysis of

dendrite development *in vitro*, hippocampal neurons were subjected to immunostaining

with antibodies against MAP2 and GFP at 6 DIV. Dendrites were defined as MAP2-

positive neurites. Sholl analysis and quantification of dendritic length and branch number

were performed using the ImageJ software. For Sholl analysis, concentric circles with

radii increasing in 10 μm increments were defined from the center of the cell body. The

number of MAP2-positive dendrites crossing each circle was then counted.

For *in vivo* dendrite analysis, brain sections were subjected to

immunohistochemical labeling with the anti-GFP antibody. Total dendritic length and

branch number of GFP-positive neurons in layer IV were measured using ImageJ. For

Sholl analysis, concentric circles with radii increasing in 10 μm incrementsQ were

defined from the center of the cell body. The number of GFP-positive dendrites crossing

each circle was counted. The total number of crossings for each cell was calculated as an

objective measurement of total dendritic complexity.

Quantitative real time-polymerase chain reaction (qRT-PCR) assays – qRT-

PCR analysis was performed to measure the expression levels of primary and mature

miRNAs, as described previously (55). In brief, total RNA was extracted with TRIzol

(Invitrogen) and subjected to reverse transcription with the SuperScript VILO cDNA

Synthesis Kit (Invitrogen) according to the manufacturer's protocol. qRT-PCR was

performed with the StepOne Real-Time PCR System (Applied Biosystems). To detect

primary and mature miRNAs, a TaqMan MicroRNA Assay kit (Applied Biosystems) was

used in accordance with the manufacturer's protocol. Data analysis was performed using

the comparative Ct method. Results were normalized to the *Gapdh* or *U6* snRNA.

Statistical analysis – The results presented are the average of at least three

experiments, each performed in triplicates, with standard errors. Statistical analyses were

performed by one-way ANOVA, followed by Tukey's multiple comparison tests or by

Student's *t*-test, as appropriate, using Prism 5 (GraphPad software). Probabilities of $p <$

0.05 were considered significant.

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Legends to Figures:

Fig. 1. Expression patterns of MeCP2 and miR-199a in hippocampal neurons during

development

A, Expression levels of MeCP2 were measured by immunoblotting in cultures of

developing hippocampal neurons at 0, 1, 3, 6, and 10 days *in vitro* (DIV).

B, Quantification of MeCP2 expression in *A*.

C, Diagrammatic representation of the sequence of pre-miR-199a-1 and pre-miR-199a-2.

The nucleotide sequences of mature miR-199a-5p and miR-199a-3p are highly conserved

among several species. The regions highlighted in gray indicate the seed sequence.

D - G, Expression levels of primary miR-199a-1 (D), primary miR-199a-2 (E), mature

miR-199a-5p (F), and mature miR-199a-3p (G) were measured by qRT-PCR in cultures

of developing hippocampal neurons at 0, 1, 3, 6, and 10 DIV.

Fig. 2. MeCP2 and miR-199a promote dendritic development of cultured

hippocampal neurons

A, Diagrammatic representations of lentiviral vector constructs. GFP and MeCP2 are

expressed under the ubiquitin promoter. miR-199a is expressed as a short hairpin RNA

under the U6 RNA polymerase III promoter, and GFP is expressed under the ubiquitin

promoter.

B, Representative images of hippocampal neurons, labeled with anti-MAP2 (red) and

anti-GFP (green) antibodies at 6 days *in vitro* (DIV).

C and D, Quantification of total dendrite length (C) and dendrite branch number (D) in

B. Student's *t*-test, *, $p < 0.05$. n = 4 independent experiments; at least 50 neurons were

analyzed in each experiment.

E, quantification of dendrite complexity by Sholl analysis of neurons infected with

lentiviruses co-expressing MeCP2 and miR-199a at 6 DIV. Student's *t*-test, *, $p < 0.05$;

**, $p < 0.01$; ***, $p < 0.001$. Fifty neurons were analyzed at each condition.

Fig. 3. Both mature forms of miR-199a are required for dendritic development in

hippocampal neurons

A, diagrammatic representations of lentiviral vector constructs expressing sponges

against miR-199a-5p and miR-199a-3p. These sponges are expressed as a short hairpin

RNA under the U6 RNA polymerase III promoter, and GFP is expressed under the

ubiquitin promoter.

B, representative images of hippocampal neurons infected with lentivirus expressing

sponges against miR-199a-5p and miR-199a-3p. These neurons were labeled with anti-

MAP2 (red) and anti-GFP (green) antibodies at 6 days *in vitro* (DIV).

C and D, quantification of total dendrite length (C) and dendrite branch number (D) in *B*.

Student's *t*-test, ***, $p < 0.001$; n.s., not significant. n = 4 independent experiments; at

least 50 neurons were analyzed in each experiment.

E, quantification of dendrite complexity by Sholl analysis of neurons infected with

lentiviruses expressing sponges against miR-199a-5p and miR-199a-3p at 6 DIV.

Student's *t*-test, *, $p < 0.05$; **, $p < 0.01$. Fifty neurons were analyzed for each condition.

Fig. 4. Inhibition of miR-199a counteracts the effect of MeCP2 expression in

hippocampal neurons

A, diagrammatic representations of lentiviral vector constructs expressing MeCP2 and

sponges against miR-199a-5p or miR-199a-3p. These sponges are expressed as a short

hairpin under the U6 RNA polymerase III promoter, and GFP and MeCP2 are expressed

under the ubiquitin promoter.

B, representative images of hippocampal neurons infected with lentivirus expressing

MeCP2 and sponges against miR-199a-5p or miR-199a-3p. These neurons were labeled

with anti-MAP2 (red) and anti-GFP (green) antibodies at 6 DIV.

C and D, quantification of total dendrite length (C) and dendrite branch number (D) in *B*.

Student's *t*-test, *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. $n = 4$ independent experiments;

at least 50 neurons were analyzed in each experiment.

E, quantification of dendrite complexity by Sholl analysis of neurons infected with

lentiviruses expressing MeCP2 and sponges against miR-199a-5p or miR-199a-3p at 6

DIV. Student's *t*-test, *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Fifty neurons were analyzed

for each condition.

Fig. 5. MeCP2 and miR-199a promote dendritic development *in vivo*

A, representative image of a transfected brain section labeled with anti-GFP antibody. WT

mice embryos were transfected with plasmids expressing only GFP (control), or GFP

along with MeCP2 (MeCP2) or miR-199a (miR-199a) by *in utero electroporation* at E14

and sacrificed at P10. Scale bar, 50 μm.

B, representative tracing images of neurons transfected with the constructs described in

A. Scale bar, 50 μm.

C and D, quantification of total dendrite length (C) and dendrite branch number (D) in

B. One-way ANOVA, Tukey's post-hoc test, ***, $p < 0.001$. Number of cells analyzed:

control, 13 neurons from three brains; MeCP2, 14 neurons from three brains; miR-199a,

12 neurons from three brains.

E, quantification of dendrite complexity by Sholl analysis of neurons in *C*. One-way

ANOVA, Tukey's post-test, *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Fig. 6. Expression of miR-199a reverses dendritic impairment in MeCP2 knockout

neurons *in vivo*

A, Representative image of a transfected brain section labeled with anti-GFP antibody.

WT and MeCP2-KO mice embryos were transfected with plasmids expressing only GFP

(control), or GFP together with miR-199a (miR-199a) by *in utero electroporation* at E14

and sacrificed at P10. Scale bar, 50 μm.

B, Representative tracing images of neurons transfected with the constructs described in

A. Scale bar, 50 μm.

C and D, Quantification of total dendrite length (C) and dendrite branch number (D) in

B. One-way ANOVA, Tukey's post-test, *, $p < 0.05$; ***, $p < 0.001$. Number of cells

analyzed: WT + control, 19 neurons from three brains; MeCP2-KO + control, 17

neurons from three brains; MeCP2-KO + miR-199a, 16 neurons from three brains.

E, Quantification of dendrite complexity by Sholl analysis of neurons in *C*. One-way

ANOVA, Tukey's post-test, *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Fig. 7. miR-199a-3p targets *Qki* in hippocampal neurons

A, Flow chart showing the screening process for selection of downstream targets of miR-

199a-3p.

B, Diagrammatic representations of luciferase reporter constructs that were inserted in a

part of the Qki 3'UTR DNA fragment including each seed site, showing the miR-199a-

3p sequence, and each miR-199a-3p target sequence of native type (nat) and its mutated

sequence (mut). The 5' seed sequences of miR-199a-3p are marked by blue boxes.

C, Luciferase activity of *Qki* 3'UTR was suppressed by miR-199a overexpression at 3

days after transfection (left). Student's *t*-test, *, $p < 0.05$; n.s., not significant. n = 3

independent experiments.

D, Western blotting analysis showing that the levels of all three isoforms of Qki protein

were decreased in hippocampal neurons infected with lentivirus vector constructs

expressing miR-199a at 6 days *in vitro* (DIV).

E, Quantification of total Qki protein level in *B*. Student's *t*-test, *, $p < 0.05$. n = 3

independent experiments.

Fig. 8. miR-199a-deficient neurons show reduced dendrite formation and Qki

knockdown restores abnormal dendritic phenotype

A, Expression levels of mature forms of miR-199a-5p and miR-199a-3p in miR-199a-2-

KO cortex at E17 by qRT-PCR assays.

B, Representative immunoblots of Qki, generated by immunoblotting with anti-Qki

antibody in miR-199a-2-KO mice cortex for estimation of Qki protein levels.

C, Quantification of Qki protein levels in *B*. Student's *t*-test, *, $p < 0.05$. n = 3 independent

experiments.

D, Representative images of cultured hippocampal neurons from miR-199a-2-KO mice

as indicated, infected with lentivirus vector constructs expressing shRNA against *Qki* and

labeled with anti-GFP (green) and anti-MAP2 (red) antibodies at 6 days *in vitro* (DIV).

E and F, Quantification of total dendrite length (E) and dendrite branch number (F) in *D*.

Student's *t*-test, *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. n = 4 independent experiments;

at least 50 neurons were analyzed in each experiment. Scale bar, 50 μm.

G, Quantification of dendrite complexity by Sholl analysis of neurons from miR-199a-2-

KO mice infected with lentivirus vector constructs expressing shRNA against *Qki*.

Student's *t*-test, *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Fifty neurons were analyzed for

each condition.

A

| E14 electroporation → P10 analysis | | |
| Wildtype + Control | MeCP2-KO + Control | MeCP2-KO + miR-199a |

B

Wildtype + Control

MeCP2-KO + Control

MeCP2-KO + miR-199a

C

Relative total dendritic length

■ Wildtype + Control □ MeCP2-KO + Control ▨ MeCP2-KO + miR-199a

D

Relative dendritic branch number

E

Number of intersections

Distance from soma (μm)

● WT + Control
○ MeCP2-KO + Control
◉ MeCP2-KO + miR-199a

A

TargetScan 309

miRDB 216

PicTor 329

Aplp2, Cited2, Fbxw11, Fubp1, Map3k4, Mbnl1, Pdgfr, Ppp2r5e, Qki

The largest seed number

Qki

B

miR-199a-3p   AUUGGUUACACGUC UGAUGACA
1ss nat   ····CAGUUGAUUUUAAA ACUACUGU G····
1ss mut   ····CAGUUGAUUUUAAA UGAUGACU G····

miR-199a-3p   AUUGGUUACACGUC UGAUGACA
3ss nat   ····GUCUUAAAAAUUAU ACUACUGU U····
3ss mut   ····GUCUUAAAAAUUAU UGAUGACU U····

miR-199a-3p   AUUGGUUACACGUC UGAUGACA
2ss nat   ····CAUUAUUUUAUGAU ACUACUGU ····
2ss mut   ····CAUUAUUUUAUGAU UGAUGACU ····

PGK promoter   *luciferase*   *qki* 3'UTR fragment (1000 bp)

C

Relative luciferase activity

■ Control ■ miR-199a

* n.s.
1ss nat 1ss mut

* n.s.
2ss nat 2ss mut

* n.s.
3ss nat 3ss mut

D

E17 hippocampal neuron 6DIV

Control   miR-199a

Qki-5
Qki-7
Qki-6

β-actin

E

Relative expression of Qki

■ Control ■ miR-199a

QKI-5 QKI-6 QKI-7 Total QKI