miR-9 and miR-124 synergistically affect regulation of dendritic branching via the AKT/GSK3β pathway by targeting Rap2a

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Abstract

A single microRNA (miRNA) can regulate expression of multiple proteins, and expression of an individual protein may be controlled by numerous miRNAs. This regulatory pattern strongly suggests that synergistic effects of miRNAs play critical roles in regulating biological processes. miR-9 and miR-124, two of the most abundant miRNAs in the mammalian nervous system, have important functions in neuronal development. In this study, we identified the small GTP-binding protein Rap2a as a common target of both miR-9 and miR-124. miR-9 and miR-124 together, but neither miRNA alone, strongly suppressed Rap2a, thereby promoting neuronal differentiation of neural stem cells (NSCs) and dendritic branching of differentiated neurons. Rap2a also diminished the dendritic complexity of mature neurons by decreasing the levels of pAKT and pGSK3β. Our results reveal a novel pathway in which miR-9 and miR-124 synergistically repress expression of Rap2a to sustain homeostatic dendritic complexity during neuronal development and maturation.

Introduction

The Ras superfamily consists of highly conserved small GTP-binding proteins that function as genetic switches to control cell proliferation, differentiation, adhesion, and survival. Some members of the Ras superfamily are key regulators of neuronal development and synaptic plasticity[1-3]. The Rap GTP-binding proteins, a subfamily of the Ras superfamily, mediate various biological functions in the hematopoietic, immune, and nervous systems[4, 5]. The Rap family has five members: Rap1a, Rap1b, Rap2a, Rap2b, and Rap2c[4]. In the nervous system, the Rap proteins are involved in neuronal polarity, synaptogenesis, and synaptic plasticity. In particular, Rap1b plays important roles in establishment of neuronal polarity[6-10], and Rap2a causes spine loss and dendritic shortening[11].

As posttranscriptional regulators of gene expression expressed in all tissues, miRNAs are involved in control of almost all physiological and pathologic processes, including differentiation, proliferation, apoptosis, development, inflammation, and cancer. MiRNAs also play important roles in the central nervous system, where they are involved in neuronal development and biological functions. MiR-134 controls spine development by targeting the mRNA encoding the protein kinase Limk1, thereby regulating memory and plasticity[12]. MiR-132 promotes dendritic morphogenesis in hippocampal neurons and controls the circadian clock in mice[13-15]. MiR-138, which is enriched in the brain, negatively regulates the size of dendritic spines[16].

MiR-9 and miR-124, two highly conserved miRNAs that are most abundantly expressed in the mammalian nervous system, both play critical roles in controlling neuron fate and

59 synaptic morphology. miR-9 negatively regulates proliferation of neural stem cells (NSCs)
60 and promotes their neuronal differentiation[17, 18]. MiR-9 controls axonal extension and
61 branching by regulating Map1b in neurogenesis[19]. MiR-124 is upregulated during neuronal
62 differentiation, suggesting that it plays an important role in this process. MiR-124 represses
63 translation of a large number of non-neuronal transcripts, indicating that it plays a role in
64 maintaining neuronal characteristics[20]. Knockdown of miR-124 results in a ~30% decrease in
65 the total number of postmitotic neurons and an increase in the total number of dividing cells[21].
66 Furthermore, miR-124 and miR-9 regulate neural lineage differentiation in embryonic stem
67 cells *in vitro*[22].
68 Synergism between miR-9/9* and miR-124 mediates the conversion of human
69 fibroblasts to neurons, but separate expression of these miRNAs has no effect[23-25]. MiR-9*
70 and miR-124 reduce proliferation of neural progenitors by repressing the Brg/Brm-associated
71 factor BAF53a, which in turn represses its neuron-specific homolog BAF53b[26, 27], a critical
72 factor in dendritic development. Although miR-9 and miR-124 have some distinct targets,
73 their synergistic effects on neuronal development are still not clear and merit further
74 investigation. In this study, we identified Rap2a as a common target gene of miR-9 and
75 miR-124. Moreover, we found that repression of Rap2a by miR-9 and miR-124 affects the
76 activation of AKT and GSK3β, which control neuronal differentiation and dendritic branching.
77 Our findings reveal a novel pathway that governs dendritic branching via the synergistic
78 effects of miR-9 and miR-124.
79
80

81 **Results**

82

83 *MiR-9 and miR-124 synergistically promote dendritic branching of differentiated neurons,*

84 *and Rap2a is predicted to be a common target of both miRNAs*

85 Previous studies demonstrated that miR-9 and miR-124 play crucial roles in determining

86 neuron fate. In addition, both of these miRNAs start to be expressed at almost the same time,

87 and their levels gradually increase over the course of neuronal development[22, 28, 29]. These

88 observations suggest that miR-9 and miR-124 have synergistic effects on neural development.

89 Therefore, we transfected NSCs *in vitro* with lentiviruses that overexpress miR-9, miR-124,

90 or both (Fig. 1A and Supplementary Fig. S1B). Surprisingly, MAP2-positive neurons derived

91 from NSCs co-overexpressing of miR-9 and miR-124 for 7 days had many more dendritic

92 branches than those transfected with control virus or virus expressing miR-9 or miR-124

93 alone (Fig.1A). These results suggest that miR-9 and miR-124 can synergistically regulate

94 neurites morphology and promote dendritic branching.

95 To screen for target genes of miR-9 and miR-124, we used the online prediction tools

96 TargetScan and PicTar[30-32]. Several Ras superfamily members were predicted to be the targets

97 of miR-9 or miR-124 (Table 1). Among them, Rhog was previously verified as a target of

98 miR-124 and shown to control axonal and dendritic branching[33, 34]. This observation

99 suggested that miR-9 and miR-124 regulate dendritic branching through the Ras superfamily

100 members. Both algorithms strongly predicted that Rap2a is a common target of miR-9 and

101 miR-124 (Table 1). Sequence analysis revealed that the 3′ UTR of Rap2a contains regions

102 complementary to the seed regions of miR-9 and miR-124 (Fig. 1B), i.e., that the Rap2a

103 mRNA has putative miR-9 and miR-124 binding sites in its 3′ UTR (Fig.1B).

104 To determine the expression patterns of miR-9, miR-124, and Rap2a, we measured the
105 levels of miR-9 and miR-124 in NSCs, the undifferentiated multipotent neural progenitor cell
106 line C17.2, and mature neurons. The levels of miR-9 and miR-124 were considerable higher
107 in postmitotic neurons than in NSCs or C17.2 cells (Fig.1C, D). On the contrary, the level of
108 Rap2a was much lower in postmitotic neurons than in NSC and C17.2 cells (Fig.1E, F).
109 Mature neurons contained a higher level of Tuj1 and lower level of nestin than NSC and
110 C17.2 cells (Fig. 1G, H). The inverted expression patterns of miR-9/-124 and Rap2a
111 supported our hypothesis that Rap2a is a common target of both of these miRNAs.

112

113 *Confirmation of Rap2a as a common target of miR-9 and miR-124*

114 To determine whether miR-9 and miR-124 directly repress the Rap2a protein level, we
115 constructed pCAG-miRNA expression plasmids (pCAG-miREPs) pCAG-miR-9,
116 pCAG-miR-124, and pCAG-miR-9-124, in which pri-miR-9, pri-miR-124, or both the
117 pri-miR-9 and pri-miR-124 sequences were placed under the control of the CAG promoter
118 (Supplementary Fig. S1A). All of these plasmids efficiently expressed high levels of the
119 corresponding miRNAs (data not shown).

120 We also constructed four reporter plasmids containing the luciferase cDNA sequence
121 fused to the Rap2a 3'UTR with intact miR-9 and miR-124 binding sites (Rap2a 3'UTR), a
122 mutated miR-9 binding site (named as ΔmiR-9), a mutated miR-124 binding site (named as
123 ΔmiR-124), or mutations in both the miR-9 and miR-124 binding sites (ΔmiR-9-124) (Fig.
124 2A). After co-transfection of individual reporter plasmids containing the pCAG-miREPs into

125 HEK293 cells harboring the Rap2a 3'UTR reporter, we found that either pCAG-miR-9 or
126 pCAG-124 efficiently suppressed the activity of luciferase relative to pCAG-Ctrl (Fig. 2B).
127 Moreover, pCAG-miR-9-124 suppressed luciferase activity to a greater extent than
128 pCAG-miR-9 or pCAG-124 plasmid (Fig. 2B). However, neither pCAG-miR-9 nor
129 pCAG-miR-124 suppressed luciferase activity in cells carrying a reporter in which its binding
130 site was mutated (i.e., ΔmiR-9 and ΔmiR-124, respectively) (Fig. 2C, D), whereas both
131 suppressed the reporter with the reciprocal mutation in the binding site for the other miRNA
132 (Fig.2C, D). None of the pCAG-miREPs could suppress the activity of luciferase in
133 ΔmiR-9-124 (Fig. 2E). These results indicate that mutation of the sequences complementary
134 to miRNA seed regions in the Rap2a 3'UTR can efficiently abolish the suppressive activity of
135 miR-9 and miR-124. Moreover, miR-9 and miR-124 synergistically suppressed the Rap2a
136 3'UTR together, both miRNAs exerted a greater than additive effect on expression.

137 In addition, we also constructed LV-miREPs in lentivirus: LV-Ctrl, LV-miR-9,
138 LV-miR-124, and LV-miR-9-124 (Supplementary Fig, S1B). LV-miR-9-124 repressed the
139 protein level of Rap2a in NSCs significantly more effectively than either LV-miR-9
140 (32%±4% vs 74%±3%, P=0.0046) or miR-124 (32%±4% vs 69%±2%, P=0.0015) (Fig.
141 2F, G). Since the target sites of miR-9 and miR-124 in Rap2a 3' UTR sequence were
142 conserved among the species (Supplementary Fig. S2), we transfected pCAG-miREPs
143 into HEK293 and C17.2 cells to further confirm that miR-9 and miR-124 can directly repress
144 Rap2a protein expression. Either pCAG-miR-9 or pCAG-miR-124 repressed the expression
145 of Rap2a in both HEK293 and C17.2 cells (Fig. 2H, I). The Rap2a protein level was more
146 reduced by pCAG-miR-9-124 than by either pCAG-miR-9 or pCAG-miR-124 alone (Fig. 2H,

147 I). The synergistic suppressive effect of miR-9 and miR-124 on Rap2a was abolished by
148 miR-9 and miR-124 sponges (miRNA sponges), which contain eight tandem binding sites for
149 either miR-9 or miR-124, respectively (Fig. 2J, K and Supplementary Fig. S3A-C). Together,
150 we demonstrated that Rap2a is a common target of miR-9 and miR-124, and that miR-9 and
151 miR-124 exert a synergistic effect on the suppression of Rap2a in cells.
152
153 *MiR-9 and miR-124 synergistically promote neuronal differentiation and dendritic complexity*
154 *of NSCs by directly repressing Rap2a*
155 To examine the synergistic effects of neuronal differentiation and the dendritic complexity of
156 differentiated neurons, we transfected LV-miR-9-124 into NSCs. In this experiment, low and
157 high titers of lentivirus of LV-miR-9-124 were used to infect NSCs (Fig. 3A). After 7 days of
158 culture, LV-miR-9-124 promoted more differentiation of NSCs into MAP2-positive neurons
159 than the control virus (Fig. 3B). More cells were MAP2-positive when a higher viral titer was
160 used (33% ± 3.2% vs 21% ± 2.7%, P=0.0078) (Fig. 3B). After treatment with LV-Rap2V12
161 (Supplementary Fig, S1C), a constitutively active form of Rap2a, in combination with
162 LV-miR-9-124, the number of MAP2-positive cells significantly decreased relative to that in
163 cells treated with LV-miR-9-124 alone (33% ± 3.2% vs 20% ± 3%, P=0.006) (Fig. 3B). We
164 also detected another postmitotic neuron marker NeuN in differentiated neurons after
165 LV-miR-9-12 transfected into NSCs. The numbers of NeuN-positive cells was consistent with
166 MAP2-positive cells in neuronal differentiation (Supplementary Fig. S4).
167 We also analyzed the dendritic complexity of differentiated neurons following
168 transfection with LV-miR-EPs. The complexity of dendritic branching was analyzed in terms

169 of in morphology, number of dendritic intersections (NDIs), and the total number of dendritic
170 end tips (TNDEPs) (Fig. 3C-E). MAP2-positive neurons derived from NSCs had more
171 dendritic branches, NDIs, and TNDEPs in the LV-miR-9-miR-124 (hi) group than in the
172 LV-Ctrl and LV-miR-9-124 (lo) group (Fig. 3C-E). Rap2V12 decreased the dendritic
173 complexity of neurons transfected with LV-miR-9-124 (hi) (Fig. 3C-E). These findings
174 suggest that miR-9 and miR-124, in a concentration-dependent manner, synergistically
175 regulate the neuronal differentiation of NSCs and dendritic complexity of differentiated
176 neurons. Furthermore, increasing the activity of Rap2a can diminish the synergistic effects of
177 miR-9 and miR-124 on neuronal differentiation and dendritic branching.

178 Next, we investigated the influence of culture time on the synergistic effects of miR-9
179 and miR-124 in NSCs. Both 3 and 7 days after transfection with LV-miR-9-124 [miR-9-124
180 (3d) and miR-9-124 (7d), respectively], NSC cultures contained more MAP2-positive cells
181 than controls (Fig. 3F, G). In addition, dendritic complexity of MAP2-positive cells increased
182 over time following miR-9-124 transfection (Fig. 3H, I, J). However, LV-Rap2V12 also
183 significantly decreased (P=0.008) the number of MAP2-positive cells three days after
184 LV-miR-9-124 transfection (Fig. 3F, G). These results suggest that miR-9 and miR-124
185 synergistically regulate the neuronal differentiation of NSCs and dendritic complexity of
186 differentiated neurons in a time-dependent manner. However, elevated Rap2a activity could
187 also diminish the synergistic effects of miR-9 and miR-124 on the dendritic complexity of
188 MAP2-positive differentiated neurons. Thus, our results demonstrate that miR-9 and miR-124
189 promote neuronal differentiation of NSCs and increase dendritic branching by inhibiting
190 Rap2a protein.

191

192 *Rap2a Reduce dendritic complexity of mature neurons*

193 To further examine the importance of Rap2a inhibited by miR-9 and miR-124 in mature
194 neurons, we transfected postmitotic neurons isolated from cortex with lentivirus expressing
195 LV-Ctrl, LV-Rap2N17 (a dominant-negative mutant of Rap2a protein) or LV-Rap2V12
196 (Supplementary Fig, S1C), respectively. Seven days after transfected, the postmitotic neurons
197 transfected with LV-Rap2N17 maintained dendritic branch morphology similar to that of
198 LV-Ctrl-transfected neurons (Fig. 4A, left panel and middle panel). Dendritic analysis
199 revealed that neither NDIs nor TNDEPs differed between LC-Ctrl- and
200 LV-Rap2N17-transfected neurons (Fig. 4B, C). In LV-Rap2V12-transfected cells (Fig. 4A,
201 right panel), the number of neuronal dendritic branches was strikingly reduced relative to
202 those in LV-Ctrl- and LV-Rap2N17-transfected cells (Fig. 4A, B, and C). These results
203 suggested that inhibition of Rap2a is indispensable for dendritic branching and complexity of
204 mature neurons.

205

206 *AKT-GSK3β signal pathway is involved in the regulation of dendritic complexity of mature*
207 *neurons by Rap2a*

208 To identify the signaling pathway(s) involved in the regulation of dendritic complexity by
209 Rap2a, we overexpressed miR-9-124, Rap2N17, and Rap2V12 in neurons. LV-Rap2V12
210 transfection considerable decreased the level of pAKT in mature neurons relative to LV-Ctrl,
211 LV-miR-9-124, and LV-Rap2N17 transfection (Fig. 5A, B). Thus, Rap2a, but not miR-9 or
212 miR-124, can change the level of pAKT, as mature neurons maintained high levels of miR-9

213 and miR-124 and a low level of Rap2a (Fig. 1C-F). This result also suggests that the AKT

214 signaling pathway is involved in the regulation of dendritic complexity of mature neurons by

215 Rap2a.

216 Glycogen synthase kinase 3 beta (GSK3β) acts downstream of Akt, and its activity is
217 inhibited via phosphorylation of its serine 9 residue (Ser9) by pAKT, leading to control of
218 neurogenesis, neuronal polarization, and axonal outgrowth[35]. To further detect the influence
219 of Rap2a on the activity of AKT and GSK3β, we forced mature neurons to overexpress Rap2a.
220 Compared to the LV-Rap2N17 control, overexpression of Rap2V12 resulted in greater
221 reductions in the levels of pAKT and pGSK-3β (Fig5C, D). This inhibition pattern was also
222 apparent in LV-Rap2V12-transfected neurons cultivated for longer periods (Fig. 5E, F).
223 Because miR-9 and miR-124 synergistically inhibited Rap2a translation, and NSCs contained
224 low levels of miR-9 and miR-124 and high level of Rap2a (Fig. 1E, F), we wondered whether
225 miR-9 and miR-124 could synergistically alter the levels of pAKT and pGSK-3β in NSCs.
226 Neither miR-9 nor miR-124 could change the levels of pAKT or pGSK-3β in NSCs following
227 transfection with LV-miR-EPs (Fig. 5G-H); only LV-miR-9-124 transfection could
228 significantly increase the levels of pAKT (P=0.0009) and pGSK-3β (P=0.0008) in NSCs (Fig.
229 5G-H). These results further demonstrate that Rap2a, the common target of miR-9 and
230 miR-124, exerts its physical roles in NSCs and neurons by regulating the activity of AKT and
231 GSK3β.

232

233 **Discussion**

234

235 Relationships between miRNAs and targets can be both one-to-many and many-to-one, i.e.,
236 one miRNA can repress many proteins, and one protein can be regulated by many miRNAs.
237 For example, miR-155 can target the bone morphogenetic protein (BMP)-responsive
238 transcriptional factors SMAD2 and SMAD5, nuclear factor κB (NF-κB) inhibitor κB-Ras1,
239 and MyD88 to modulate macrophage responses, lymphomagenesis, hematopoiesis, and
240 inflammation[36-39]. On the other hand, miR-15 and miR-16 control apoptosis by targeting
241 BCL-2 mRNA[40]. MiR-224 and miR-203 downregulate NPAS4 (Neuronal Per-ARNT-SIM
242 homology domain 4) expression through its 3′UTR[41]. This characteristic of miRNAs and their
243 targets has drawn increasing attention to the synergistic effects of miRNAs. For instance,
244 miR-499 and miR-133 synergistically promote cardiac differentiation[42]. Likewise, the
245 combined action of miR-106b, miR-93, and miR-25 effectively repress expression of PTEN
246 transcripts in prostate cancer[43].

247 In this study, we observed that co-overexpression of miR-9 and miR-124 in NSCs
248 promoted neuronal differentiation and dendritic branching, whereas neither miRNA had an
249 effect, strongly suggesting that miR-9 and miR-124 exert synergistic effects on neuronal
250 differentiation and dendritic tree complexity. Recent studies report that genetic switches
251 responsible for control of neuronal gene expression are targets of both miR-9 and miR-124.
252 MiR-9 targets repressor-element-1-silencing transcription factor (REST), and miR-9* targets
253 CoREST[44]. MiR-124 also targets CoREST to regulate intrinsic temporal changes in RGC
254 growth cone sensitivity and radial migration of projection neurons[45, 46]. Although these studies

255 proposed that miR-9 and miR-124 play crucial roles in neuron fate, they did not clearly
256 elucidate the synergistic effects. Here, we showed that miR-9 and miR-124 play synergistic
257 roles in neuron fate, and that Rap2a is their common target.

258 Previous work shows that Rap2a controls dendritic spine morphology and synaptic
259 plasticity[47-49], and our results were consistent with those observations. We confirmed that
260 Rap2a represses dendritic branching and neuronal differentiation, and found that miR-9 and
261 miR-124 promote neuronal differentiation and dendritic tree complexity by inhibiting Rap2a.
262 In fact, some Ras superfamily members interact with miR-9 and miR-124. For example,
263 miR-9 is suppressed by the Ras/PI3K/AKT axis, resulting in glioblastoma tumorigenicity[50].
264 Overexpression of miR-124 in differentiating mouse P19 cells promotes neurite outgrowth by
265 regulating the members of Rho GTPase[51]. MiR-124 controls axonal and dendritic
266 development by targeting the small GTPase RhoG. Our results showed that another member
267 of the Ras superfamily is regulated by miR-9 and miR-124. In addition, overexpression of
268 Rap2V12 could not completely offset the synergistic effects of miR-9 and miR-124, leading
269 us to speculate that miR-9 and miR-124 may regulate neuron fate via another mechanism.

270 The multifunctional serine/threonine kinase GSK3β plays a variety of roles in
271 activity-dependent regulation of dendritic development and maintenance[52, 53]. Phosphorylation
272 of GSK3β on Tyr216 leads to activation, whereas phosphorylation of Ser9 by AKT results in
273 inactivation[35, 54]. We found that levels of pAKT (phosphorylation of Ser473) and pGSK3β
274 (phosphorylation of Ser9) were dramatically downregulated by overexpression of Rap2a in
275 mature neurons (Fig. 5A, B). Thus, the AKT/GSK3β signaling pathway is regulated by Rap2a,
276 and miR-9 and miR-124 can control AKT/GSK3β signaling pathway by targeting Rap2a. It is

277 reported that in B cells Rap2V12 reduces Akt activity via PI3K inhibition[55]. Our results
278 proved that Rap2V12 can also repress Akt activity to inhibit neuronal differentiation
279 and dendritic branching in nervous system. Although Rap2a is involved in the JNK and
280 ERK signaling pathways[56, 57], we did not detect obvious changes in the levels of pERK or
281 pJNK upon overexpression of miR-9 and miR-124 in NSCs (data not shown). As homologous
282 proteins of Rap2a, Rap2b was reported to closely correlate with cancer[58]. The biological
283 function of Rap2c was still unclear. The roles of both Rap2b and Rap2c have not yet been
284 reported in nervous system. Considering the vital function of Ras superfamily in nervous
285 system, Rap2b and Rap2c may have some novel roles in differentiation of NSCs, which still
286 need to investigate further.

287 Our results reveal the mechanism by which miR-9 and miR-124 synergistically promote
288 neuronal differentiation and dendritic branching (Fig. 6). Rap2a decreases phosphorylation
289 levels of AKT, thereby inactivating it. MiR-9 and miR-124 repress Rap2a by binding to
290 specific sites in the Rap2a 3′ UTR, thereby releasing the inhibition of AKT, ultimately
291 resulting in inactivation of GSK3β by phosphorylation on Ser9. Inactivation of GSK3β boosts
292 neuronal differentiation and dendritic branching. In short, the results suggest that the
293 synergistic effects of miR-9 and miR-124 control AKT/GSK3β signaling to regulate neuronal
294 differentiation and dendritic complexity by inhibiting Rap2a.

295 The results of this study reveal a previously unknown interaction between miR-9,
296 miR-124 and Rap2a, and emphasize the synergistic effects of miR-9 and miR-124 on
297 neuronal differentiation and dendritic complexity.

298

299 **Materials and methods**

300

301 *DNA Constructs and lentivirus preparation*

302 Expression vectors for miR-9 and miR-124 were constructed as described previously[59].
303 Briefly, the two primary miRNA transcripts (pri-miR-9 and pri-miR-124; specifically, ~500
304 base pairs around *mmu-miR-9-3* and *mmu-miR-124-1*) were amplified, and either or both of
305 them were cloned downstream of the CAG promoter of pCAG to yield pCAG-miR-9,
306 pCAG-miR-124, and pCAG-miR-9-124 (Supplementary Fig. 1A), or downstream of the EF1
307 promoter of pCDH-EF1-MCS (System Biosciences, San Diego, CA USA) to yield LV-miR-9,
308 LV-miR-124, and LV-miR-9-124 (Supplementary Fig. 1B).

309 Vectors for luciferase reporter experiments were established as reported[60]. Bases
310 2310-3059 of the Rap2a 3′ UTR were amplified by RT-PCR from mouse brain mRNA and
311 inserted downstream of the stop codon of luciferase in vector pGL3 (Promega, Madison, WI,
312 USA). The binding sites in the Rap2a 3′ UTR for miR-9, miR-124, or both (i.e., sequences
313 complementary to bases 2-6 in the miRNA seed regions) were mutated, and the resultant
314 mutant UTRs were inserted downstream of the stop codon of luciferase in pGL3 to yield
315 pGL3-Rap2a, pGL3-Rap2aΔmiR-9, pGL3-Rap2aΔmiR-124, and pGL3-Rap2aΔmiR-9-124
316 (Fig. 2A).

317 The *Rap2a* cDNA was amplified from mouse brain using the primer pair
318 5′-ATGCGCGAGTACAAAGTGG-3′ and 5′-CTATTGTATGTTACAGGCAGAA-3′. To
319 generate dominant-negative Rap2a (Rap2N17) or constitutively active Rap2 (Rap2V12)[57], a
320 mutant containing a Ser-to-Asn substitution at position 17 (Rap2N17) or Gly-to-Val

321 substitution at position 12 (Rap2V12) was cloned downstream of the EF1 promoter in vector
322 pCDH-EF1-MCS to yield LV-Rap2N17 and LV-Rap2V12 (Supplementary Fig. 1C).
323 For the miRNA sponge expression vector, eight tandem miR-124 binding sites (Sangon
324 Biotech, Shanghai, China) were ligated into pGL3 (Promega). Likewise, eight tandem mouse
325 miR-9 binding sites were amplified from pBabe-puro-miR-9 sponge (Addgene) and ligated
326 into pGL3 (Supplementary Fig. 2A).

327

328 *Cell culture*

329 Human embryonic kidney HEK293 cells were grown in Dulbecco's Modified Eagle Medium
330 (DMEM) (Gibco, Karlsruhe, Germany) supplemented with 10% fetal bovine serum (Gibco)
331 and 10 mM L-glutamine (Gibco). The multipotent neural progenitor cell line C17.2 was
332 maintained in DMEM supplemented with 10% fetal bovine serum, 5% horse serum (Gibco),
333 and 10 mM L-glutamine. NSCs and neurons were separately established from cortex of
334 embryonic day (E) 14-E16 C57BL/6 mice. Briefly, cortex was microdissected and stripped of
335 meninges, and then tissues were mechanically dissociated into single-cell suspensions. For
336 NSCs, cells were grown in DMEM/F-12 (Gibco) supplemented with 10 mM L-glutamine, 1%
337 N2 supplement (Gibco), 20 μL/mL B-27 supplement minus vitamin A (Gibco), 100 μg/mL
338 penicillin/streptomycin (Gibco), 20 ng/mL epidermal growth factor (EGF), and 20 ng/mL
339 fibroblast growth factor bFGF (PeproTech, London, UK). For neurons, cells were seed in
340 poly-L-lysine-coated plates and grown in serum-free Neurobasal medium (Gibco)
341 supplemented with 10 mM L-glutamine, 100 μg/mL penicillin/streptomycin, and 20 μL/mL
342 B-27 supplement. Cells were maintained in a humidified incubator with 5% CO_2 at 37°C.

343

344 *RNA extraction and quantitative real-time PCR*

345 For quantitative real-time PCR of miRNA, RNA was extracted with TRIzol (Invitrogen,
346 Carlsbad, CA, USA) and reverse-transcribed with miRNA-specific primers using the miScript
347 Reverse Transcription Kit (Qiagen, Hilden, Germany). Quantitative RT-PCR of mature
348 miRNA was performed using a miRNA-specific primer on a CFX96 Real-Time PCR
349 Detection System (Bio-Rad Laboratories, Hercules, CA, USA). U6 was amplified as a
350 normalization control. Quantitative RT-PCR of miRNAs was performed using the following
351 primers: miR-9, 5'- GGTCTTTGGTTATCTAGCTGTATGA -3' ; miR-124,
352 5'-TTTCCTATGCATATACTTCTTT-3'.

353

354 *Luciferase assay*

355 HEK293 cells were seeded in 24-well plates and transfected the next day with 0.4 μg of
356 miRNA expression vector, 0.4 μg of firefly luciferase reporter vector, and 0.08 μg of the
357 control vector pRL-TK (Promega, Madison, USA), which contains *Renilla* luciferase.
358 Transfections were performed using Lipofectamine 2000 (Invitrogen). Each treatment was
359 performed in triplicate in three independent experiments, and the activities of firefly and
360 *Renilla* luciferase were measured consecutively using dual-luciferase assays (Promega) 24 h
361 after transfection.

362

363 *Cell transfection and transduction*

364 HEK293 cells and C17.2 cells were seeded in 24-well plates and transfected the next day with

365 miRNA expression vectors with or without miRNA sponges, Transfections were performed
366 using Lipofectamine 2000. The cells were then incubated for 48 h.
367 For virus transduction, NSCs were digested into single-cell suspensions, and then seeded
368 in poly-L-lysine-coated 24-well plates at 1×10^5cells/cm^2. The next day, low (5μL, titer:
369 1×10^8 TU/mL) or high amounts (10μL, titer: 1×10^8 TU/mL) of viral supernatants were added
370 to the cells. The medium containing virus was removed and discarded 24 h after transduction
371 and replaced with fresh growth medium of NSCs. Neurons derived from cortex of E14-E16
372 C57BL/6 mice were plates at 1×10^5cells/cm^2 and cultured for 3 days. On the fourth day, low
373 (5μL, titer: 1×10^8 TU/mL) or high amounts (10μL, titer: 1×10^8 TU/mL) of viral supernatant
374 were added to the cells. The medium containing virus was removed and discarded 24 h after
375 transduction and replaced with fresh growth medium of neurons. The cells were incubated for
376 3 or 7 days, and then harvested or immunostained.
377
378 *Immunocytochemistry*
379 Cells were fixed in 4% paraformaldehyde for 30 min, and then blocked for 1 h with 1%
380 bovine serum albumin containing 0.3 % Triton X-100. Blocked cells were incubated
381 overnight at 4°C with Rabbit polyclonal antibody to MAP2 (Millipore) and Rabbit polyclonal
382 antibody to NeuN antibody (Millipore), and then for 2 h at room temperature with the relative
383 secondary antibodies (DyLight 488-conjugated AffiniPure Donkey anti-rabbit IgG, Jackson
384 ImmunoResearch Laboratories, West Rove, PA, USA). Images were acquired using an IX71
385 inverted microscope (Olympus, Japan).
386

387 *Western blotting*

388 Cells were lysed in lysis buffer (pH 8.0; 50 mM Tris-HCl containing 150 mM NaCl, 5 mM
389 ethylenediaminetetraacetic acid, 1 mM dithiothreitol, 0.5% deoxysodium cholate, 0.1% SDS,
390 20 μg/mL protease inhibitors aprotinin, 1 mM sodium orthovanadate, 1 mM mercaptoethanol,
391 and 5 mM sodium fluoride), incubated on ice for 30 min, and centrifuged. Protein
392 concentrations in supernatants were determined by Bradford analysis.

393 Proteins were separated on 10% or 15% (for Rap2a) SDS-PAGE gels at a constant 100
394 mV voltage and transferred to Polyvinylidene Difluoride (PVDF) membranes at 300 mV for 1
395 h. PVDF membranes were blocked in 5% nonfat milk for 1 h; incubated overnight at 4°C with
396 primary antibodies against Rap2a (Proteintech, Wuhan, China), nestin (Sigma-Aldrich, St.
397 Louis, MO, USA), Tuj1 (Sigma-Aldrich), p-AKT (Ser473) (Cell Signaling Technology,
398 Boston, MA), p-GSK3β (Ser9) (Cell Signaling Technology), or β-actin (Sigma-Aldrich); and
399 then incubated for 2 h at room temperature with the relative secondary antibodies conjugated
400 with horseradish peroxidase (Abcam). Immunoreactive bands were visualized using an
401 enhanced chemiluminescence kit on a Bio-Rad Image Lab system.

402

403 *Statistical analysis*

404 All statistical analyses of experimental data were performed using GraphPad Prism 5.0
405 (GraphPad) and are presented as group mean ± SEM. All experiments were repeated at least
406 three times. Comparison of the two groups was performed using independent two-tailed
407 Student's t tests, and P values <0.05 were considered significant.

408

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553

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557

558 **Author Contributions**

559 Q. X., L. L. and Y. W. performed experiments with assistance from F. F. L., C. Y. Y. and G.
560 L. B.; C. F., and K. Z. analyzed the data; Q. X., G. J., and J. W. conceived the research and
561 wrote the manuscript; B. S. and A. G. Y. contributed to manuscript editing; and all authors
562 read the paper.
563

Additional Information

Conflict of interest: The authors declared no conflict of interest.

568 **Figure Legends**

569 Figure 1. Experimental suggestion of Rap2a as a common target of miR9 and miR-124.

570 (A) Dendritic morphology of neurons differentiated from NSCs transfected with LV-Ctrl,

571 LV-miR-9, LV-miR-124, or LV-miR-9-124 for 7 days. Scale bar, 100 μm. (B) Schematic

572 representation of the putative base-pairing interactions of miR-9 and miR-124 with the 3′

573 UTR of Rap2a. qPCR analysis of miR-9 (C) and miR-124 (D) expression in NSCs, C17.2

574 cells, and mature neurons. Western blot analysis (E) and quantitation by densitometry (F) for

575 Rap2a, Tuj1 (J), and nestin (H) in mature neurons, NSCs, and C17.2 cells; signals were

576 normalized to β-actin. (**, $P < 0.01$; *** $P < 0.001$).

577

578

579 Figure 2. Confirmation of Rap2a as the common target of miR-9 and miR-124.

580 (A) Schematic representation of the four reporter plasmids. pGL3-Rap2a 3'UTR (Rap2a

581 3'UTR): Rap2a 3′ UTR (2310-3059 bp) containing miR-9 and miR-124 binding sites was

582 cloned downstream of luciferase. Underlined bases are sequences complementary to the seed

583 regions of miR-9 and miR-124. pGL3-Rap2a 3'UTR/miR-9 (ΔmiR-9): pGL-Rap2a 3'UTR

584 with a mutation in the miR-9 binding site. pGL3-Rap2a 3'UTR/miR-124 (ΔmiR-124):

585 pGL-Rap2a 3'UTR with a mutation in the miR-124 binding site. pGL3-Rap2a

586 3'UTR/miR-9-124 (ΔmiR-9-124): pGL-Rap2a 3'UTR with mutations in both the miR-9 and

587 miR-124 binding sites. The boxed bases indicate mutations in sequences complementary to

588 the seed regions of miR-9 and miR-124. (B-E) Luciferase activity in HEK293 cells

589 co-transfected with Rap2a 3′ UTR (B), ΔmiR-9 (C), ΔmiR-124 (D), or ΔmiR-9-124 (E)

590 reporter plasmid with four miR-EPs. *Firefly* luciferase data were normalized to *renilla*

591 luciferase data. (F and G) Western blot analysis (F) and quantitation by densitometry (G) for
592 Rap2a in NSCs transfected with four miR-EPs. (H and I) Western blot analysis (H) and
593 quantitation by densitometry (I) for Rap2a in HEK293 and C17.2 cells transfected with four
594 miR-EPs. (J and K) Western blot analysis (J) and quantitation by densitometry (K) for Rap2a
595 in HEK293 and C17.2 cells transfected with miR-9-124 and miRNA sponge. Signals were
596 normalized to β-actin. (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).
597
598 Figure 3. miR-9 and miR-124 synergistically regulate neuronal differentiation and dendritic
599 branching of NSCs by repressing Rap2a.
600 (A and B) Representative profiles (A) and the percentage (B) of MAP2-positive differentiated
601 neurons after transfection of NSCs with miR-9-124 at different viral titers and rescue by
602 Rap2V12. Scale bar, 200 μm. (C) Typical dendritic morphology of differentiated neurons after
603 transfection of NSCs with miR-9-124 at different viral titers and rescue by Rap2V12. Scale
604 bar, 100 μm. (D and E) Sholl analysis of NDIs (D) and TNDETs (E) of dendritic complexity
605 in differentiated neurons in (C) (n=30 neurons). (F and G) Representative profiles (F) and
606 percentage (G) of MAP2-positive differentiated neurons after transfection of NSCs with
607 miR-9-124 for different culture times and rescue by Rap2V12. Scale bar, 200 μm. (H) Typical
608 dendritic morphology of differentiated neurons after transfection of NSCs with miR-9-124 for
609 different culture times and rescue by Rap2V12. Scale bar, 100 μm. (I–J) Sholl analysis of
610 NDIs (I) and TNDETs (J) of dendritic complexity of differentiated neurons in (H) (n=30
611 neurons). (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).
612
613 Figure 4. Rap2a repressed dendritic branching in mature neurons.

614 (A) Typical dendritic morphology of mature neurons after transfection with LV-Ctrl,
615 Rap2N17 or Rap2V12 for seven days. Scale bar, 100 μm; (B and C) Sholl analysis in NDIs (B)
616 and TNDETs (C) of dendritic complexity in mature neurons in (A). (n=30 neurons, ***,
617 P < 0.001).
618
619 Figure 5. Loss of Rap2a leads to enhanced AKT-GSK3β signaling pathway.
620 (A and B) Western blot analysis (A) and quantitation by densitometry (B) for pAKT (Ser473)
621 and total AKT of mature neurons after transfection with LV-Ctrl, Rap2N17, Rap2V12, or
622 miR-9-124. (C and D) Western blot analysis (C) and quantitation by densitometry (D) for
623 Rap2a, pAKT (Ser473), total AKT, pGSK3β (Ser9) and total GSK3β in mature neurons
624 transfected with Rap2V12 at different viral titers. (E and F) Western blot analysis (E) and
625 quantitation by densitometry (F) for Rap2a, pAKT (Ser473), total AKT, pGSK3β (Ser9) and
626 total GSK3β in mature neurons after Rap2V12 transfection for different culture times. (G–H)
627 Western blot analysis (G) and quantitation by densitometry (H) for pAKT (Ser473) total AKT,
628 pGSK3β (Ser9) and total GSK3β in NSCs after transfection with LV-Ctrl, miR-9, miR-124,
629 and miR-9-124 transfection. All signals were normalized to β-actin. (*, P < 0.05; **, P < 0.01;
630 ***, P < 0.001).
631
632 Figure 6. Schematic of miR-9/-124–mediated regulation of neuronal differentiation and
633 dendritic branching by inhibition of Rap2a.
634
635
636

637 Table 1 Members of the Ras superfamily were predicted as conserved targets of miR-9 and

638 miR-124 by the online prediction tools TargetScan and PicTar.

639

miR-124	miR-9
Rap2a	Rap2a
Rab34, Rab38	Rab43
Rhog	Rhoq
Raph1	RAS p21 protein activator 2
Rreb1	
Ras repressor protein 1	
Ras-GTPase-activating protein SH3-domain binding protein 1	