1 **miR-9 and miR-124 synergistically affect regulation of dendritic branching via the**

2 **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.

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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.

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Results

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

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

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

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

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

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

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

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

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

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

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

neurites morphology and promote dendritic branching.

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

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

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

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

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

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

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

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.

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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

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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.

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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

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

RNA extraction and quantitative real-time PCR

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

Luciferase assay

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

Cell transfection and transduction

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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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

564 **Additional Information**

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

566

567

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.

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	