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A brain-specific microRNA regulates dendritic spine development

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MicroRNAs are small, non-coding RNAs that control the translation of target messenger RNAs, thereby regulating critical aspects of plant and animal development. In the mammalian nervous system, the spatiotemporal control of mRNA translation has an important role in synaptic development and plasticity. Although a number of microRNAs have been isolated from the mammalian brain, neither the specific microRNAs that regulate synapse function nor their target mRNAs have been identified. Here we show that a brain-specific microRNA, miR-134, is localized to the synaptodendritic compartment of rat hippocampal neurons and negatively regulates the size of dendritic spines—postsynaptic sites of excitatory synaptic transmission. This effect is mediated by miR-134 inhibition of the translation of an mRNA encoding a protein kinase, Limk1, that controls spine development. Exposure of neurons to extracellular stimuli such as brain-derived neurotrophic factor relieves miR-134 inhibition of *Limk1* translation and in this way may contribute to synaptic development, maturation and/or plasticity.

Highly orchestrated programmes of gene expression act to shape the developing nervous system. This tight regulation is mediated by a variety of transcriptional and post-transcriptional events that control the expression of individual gene products[1,2]. The discovery of small, non-coding RNAs has greatly expanded our understanding of the cellular mechanisms that regulate gene expression at the post-transcriptional level. MicroRNAs (miRNAs) act by binding to target mRNAs and initiating either cleavage or a reduction in the translational efficiency of the target mRNA, depending on the degree of sequence complementarity[3–5]. Biochemical and genetic studies have revealed important functions for specific miRNAs in a variety of cellular processes, including differentiation, apoptosis and metabolism[6–10].

A number of miRNAs have been isolated from the vertebrate nervous system[11–13], and a recent study has demonstrated a crucial role for the miRNA pathway in early zebrafish brain development[14]. Expression analysis also supports a role for miRNAs in later stages of neuronal maturation and synapse development[12,15,16]. A potential role for miRNAs in synaptic function is particularly intriguing given the evidence that selected mRNAs in neurons are transported to sites of synaptic contact that are quite distant from the cell body[17–19]. Within dendrites, and at synapses, the translation of these mRNAs may be inhibited until neurons are exposed to appropriate extracellular stimuli such as a neurotrophic factor (for example, brain-derived neurotrophic factor (BDNF)) or neurotransmitter release at the synapse. Local translation of these previously dormant mRNAs has been hypothesized to have a key role in synaptic development and plasticity[20–22]. Whether miRNAs might inhibit the translation of synaptically localized mRNAs in neurons until their translation is activated by neurotrophic factors or neuronal activity remains to be investigated.

miR-134 expression during synapse development

To identify miRNAs that might function in dendritic and/or synaptic development, we investigated the expression and localization of candidate miRNAs that had been previously isolated from mouse brain[13]. Both northern blotting and an RNase protection assay (RPA) revealed that the expression of microRNA-134 (miR-134) is restricted to the brain, similar to the expression pattern of the previously characterized miR-124a (Fig. 1a and Supplementary Fig. 1a, b). Unlike miR-124a, however, miR-134 levels in the hippocampus gradually increase with development, reaching maximum levels at postnatal day 13 (P13), the time at which synaptic maturation occurs (Fig. 1b). A similar developmental expression profile was also observed in dissociated hippocampal neurons that were allowed to mature over time in culture (Fig. 1c). Moreover, membrane depolarization of cortical neurons induced a significant increase in the level of the miR-134 precursor (Supplementary Fig. 1c). Taken together, these results suggested a potential role for miR-134 in dendritic and/or synaptic development.

We used an *in situ* hybridization (ISH) protocol to examine the subcellular localization of the miR-134 RNA within cultured hippocampal neurons. Unlike the mismatch control probe, hybridization with the miR-134-specific probe revealed the presence of miR-134 within dendrites, where it is present in a punctate pattern (Fig. 1d and Supplementary Fig. 1d). Quantification of the two signal intensities (miR-134-specific versus the mismatch probe) along the length of multiple dendrites confirmed significantly higher levels of miR-134-specific signal within dendrites as compared to that obtained with the mismatch control (Supplementary Fig. 1e) or the U6 small nuclear (sn)RNA (data not shown). A substantial fraction of the dendritic miR-134 was found to partially co-localize with synapsin immunostaining, indicating that miR-134 is present near synaptic sites on dendrites (Fig. 1d, lower panel and inset at higher magnification). The presence of miR-134 in synaptic compartments was also corroborated by subcellular fractionation experiments; miR-134 was enriched in synaptoneurosome preparations (Fig. 1e), which represent membrane preparations highly enriched for synaptic terminals[23]. The presence of miR-134 within dendrites near synapses suggested a possible functional role for this miRNA at post-synaptic sites.

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miR-134 regulates dendritic spine morphology

To investigate a possible function of miR-134 at the synapse, we examined the effects of modulating miR-134 activity on dendritic spine development. Dendritic spines are actin-rich protrusions from the dendritic shaft and represent the major sites of excitatory synaptic contact[24,25]. The size of dendritic spines is a good correlate of the strength of excitatory synapses[26–28]. To achieve miR-134 overexpression, we designed a vector that permits efficient expression of exogenous miR-134 (Supplementary Fig. 2a). Alternatively, miR-134 function in neurons was suppressed by introducing a 2′-O-methylated antisense oligonucleotide that interferes with endogenous miR-134 activity in a sequence-specific manner[29,30]. The efficacy of these approaches was confirmed in neurons using a previously described miRNA sensor assay (Supplementary Fig. 2b)[31].

An analysis of dendritic spines in cultured hippocampal neurons (cultured for a total of 18 days, transfected at day 8: 8 + 10 days *in vitro* (DIV)) overexpressing miR-134 showed a significantly decreased spine volume as compared to the spines of neurons transfected with empty vector or overexpressing the unrelated let-7c miRNA (Fig. 2a (bottom panel), b (bottom panel), e and Supplementary Fig. 3a–c).

Further analysis revealed that this decrease in spine volume was mainly a consequence of a reduction in spine width ($-16.9 \pm 5.8\%$, $n = 3$, $P = 0.02$) as opposed to a change in spine length ($-3.5 \pm 7.1\%$, $n = 3$, $P = 0.23$, Fig. 2f). A similar reduction in dendritic spine size was observed when synthetic miR-134 was introduced into neurons at a later stage (15 DIV) and for shorter times (72 h, Supplementary Fig. 3d). Because hippocampal neurons at 15 DIV have already developed the vast majority of their spines, these findings suggest that miR-134 may perturb the morphology of pre-existing spines.

In contrast to the effect of miR-134 overexpression, sequence-specific inhibition of endogenous miR-134 function using a 2′-O-methylated antisense oligonucleotide[29,30] (2′-O-Me-134) led to small but statistically significant increases in spine volume and width ($7.6 \pm 3.7\%$, $n = 3$, $P = 0.03$) when compared to neurons transfected with an unrelated 2′-O-Me-control oligonucleotide (Fig. 2c (bottom panel), d (bottom panel), e, f). No significant effects on spine length were observed in the presence of 2′-O-Me-134 ($2.6 \pm 5.6\%$, $n = 3$, $P = 0.25$). Neither miR-134 overexpression nor the use of 2′-O-methylated oligonucleotides had any measurable effect on spine density or overall dendritic complexity (Supplementary Fig. 3e, f). We conclude that miR-134 acts as a negative regulator of dendritic spine volume in hippocampal neurons, raising the possibility that miR-134 may be involved in the regulation of synapse development and/or function.

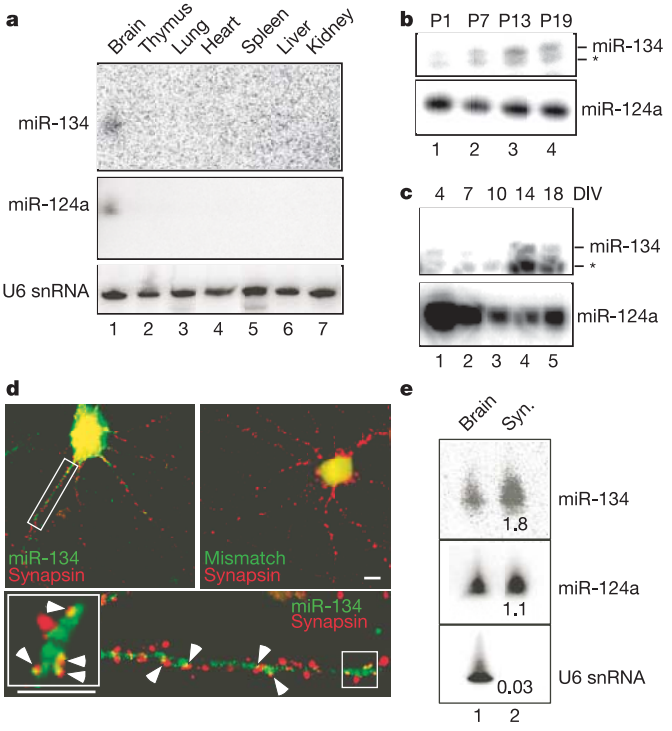

Figure 1 | miR-134 is specifically expressed in the brain and localized to neuronal dendrites. a, Northern blot of adult tissues was probed for the indicated miRNAs or U6 snRNA. **b**, RNase protection assay (RPA) to detect the indicated miRNAs in postnatal (P1–P19) hippocampus. **c**, RPA to detect the indicated miRNAs in hippocampal neurons cultured for 4–18 DIV. Asterisk indicates an unknown protected fragment. **d**, Co-staining of the presynaptic marker protein synapsin (red) together with miR-134 ISH (green) in 14 DIV hippocampal neurons (upper left). The boxed area in the upper-left panel is shown at greater magnification in the bottom panel, which also has a higher-magnification inset. Arrows point to synapses that partially overlap with miR-134-positive puncta. Scale bars, 10 μm. **e**, Northern blot of P15 whole brain or synaptoneurosomes (syn.) probed for indicated miRNAs or U6 snRNA. Fold enrichment in synaptoneurosomes is depicted.

miR-134 inhibits translation of *Limk1* mRNA

To gain insight into the mechanisms by which miR-134 regulates dendritic spine morphology, we sought to identify miR-134 target mRNAs. Towards this end, we scanned the 3′ untranslated regions (UTRs) of mRNAs for potential miR-134 binding sites. For this analysis, we focused on a set of 48 genes that we recently identified in a screen for mRNAs for which translation is enhanced in neurons upon treatment with BDNF[32]. As BDNF promotes dendritic spine growth[33] and regulates synaptic function, at least in part, by activating dendritic protein synthesis[21], we reasoned that mRNAs for which translation is regulated by BDNF might also represent miR-134 targets. Three of the BDNF-regulated mRNAs (discs large homologue 2 (*DLG2*), *Neurod2* and Lim-domain-containing protein kinase 1 (*Limk1*)) were found to contain conserved 3′ UTR sequence elements that were partially complementary to mouse miR-134 (Fig. 3a and data not shown). Among these potential miR-134 target mRNAs, *Limk1* was of particular interest. Limk1 regulates actin filament dynamics through inhibition of ADF/cofilin[34], and *Limk1* knockout mice show abnormalities in dendritic spine structure similar to those observed upon miR-134 overexpression[35].

Using an electrophoretic mobility shift assay, we demonstrated that *Limk1* mRNA and miR-134 interact *in vitro* (Supplementary Fig. 4). We next determined whether the *Limk1* mRNA co-localized with miR-134 within dendrites of live neurons. Fluorescently labelled miR-134 was introduced into hippocampal neurons by micro-injection together with a fluorescent *Limk1* 3′ UTR that contains the miR-134 binding site. Both miR-134 and the *Limk1* 3′ UTR, in contrast to the non-dendritic *Gapdh* and histone H3 mRNAs, were found to be present within dendrites in a granular pattern (Fig. 3b, upper two panels and data not shown). Moreover, miR-134- and *Limk1* 3′ UTR-positive granules were co-localized within dendrites (Fig. 3b, lower panel). Furthermore, efficient co-localization of miR-134 and *Limk1* mRNA required the presence of an intact miR-134 binding site within the *Limk1* 3′ UTR (Fig. 3b, bar graph). The dendritic localization of endogenous *Limk1* mRNA was further confirmed by ISH in cultured neurons and by subcellular fractionation (Supplementary Fig. 5a, b).

In mammalian cells, miRNAs are thought to regulate the expression of target mRNAs predominantly through the inhibition of productive translation[3]. We therefore hypothesized that miR-134 binding to the *Limk1* mRNA might act to inhibit *Limk1* translation. In support of this idea, miR-134 overexpression in both 293T cells and primary neurons was found to decrease specifically the activity of a luciferase reporter gene fused to the wild-type *Limk1* 3′ UTR, whereas expression of the unrelated let-7c miRNA had no significant

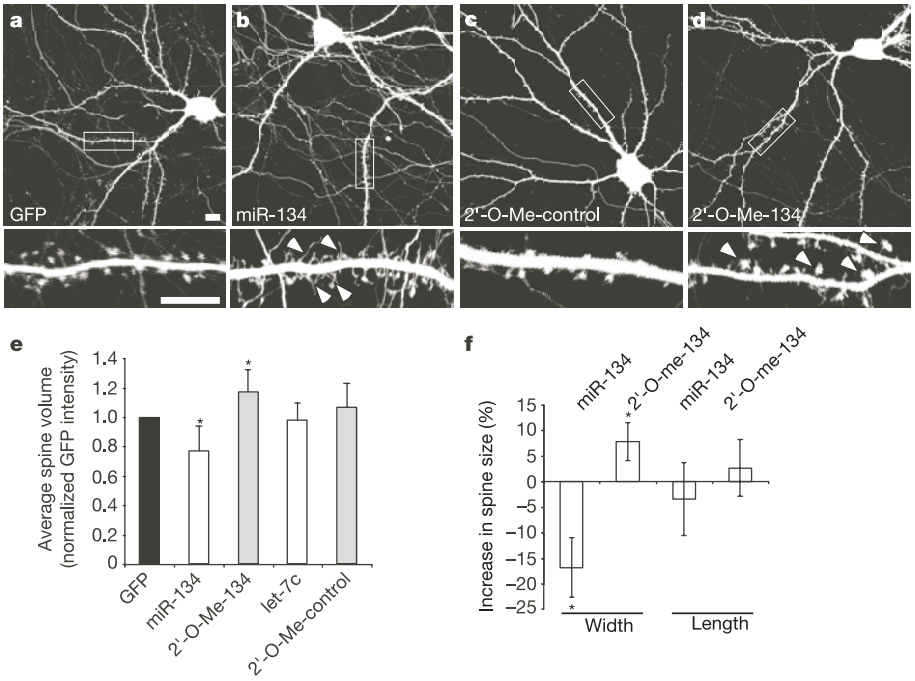

Figure 2 | miR-134 negatively regulates dendritic spine size in hippocampal neurons. a–d, Representative neurons (18 DIV) transfected with control vector (**a**), miR-134 expression vector (**b**), 2′-O-Me control (**c**) or 2′-O-Me-134 oligonucleotide (**d**). Bottom panels (insets of boxed areas) illustrate higher frequency of thinner spines in miR-134-expressing cells (arrows in **b**) and enlarged spines in 2′-O-Me-134-transfected neurons (arrows in **d**). Scale bars, 10 μm. **e**, Normalized average volume of spines ($n > 600$) from neurons ($n = 15$) transfected as in **a–d**. Data are presented as average spine volume ±s.d. from three independent experiments. Asterisk, $P < 0.05$ (paired Student's *t*-test). **f**, Per cent changes in the width and length of spines ($n > 600$) in neurons ($n = 15$) expressing miR-134 or 2′-O-Me-134 compared to GFP. Data are presented as mean change in spine length/width ±s.d from three independent experiments. Asterisk, $P < 0.05$ (paired Student's *t*-test).

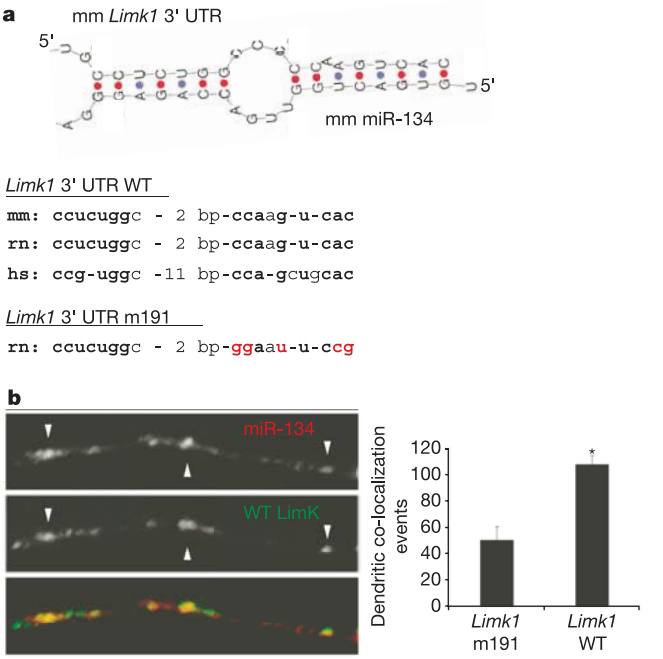

Figure 3 | *Limk1* mRNA is a putative miR-134 target. a, Upper panel: predicted duplex formation between mouse *Limk1* 3′ UTR (top) and miR-134 (bottom). Middle panel: Sequence conservation of the miR-134 binding site within the *Limk1* 3′ UTR of mouse (mm), rat (rn) and human (hs). Lower panel: sequence of the m191 *Limk1* 3′ UTR, containing mutations in the miR-134 binding site (red). **b**, Localization of microinjected miR-134 (red) and *Limk1* RNA (green) in hippocampal neurons. Arrows indicate miR-134 and *Limk1* co-localization in granule-like structures. Bar graph: quantification of dendritic co-localization events between microinjected miR-134 and either *Limk1* m191 or *Limk1* wild-type (WT) RNA. Data represent the mean of $n = 12$ cells per condition counted in triplicate ±s.d. Asterisk, $P < 0.05$.

effect on the expression of this reporter construct (Fig. 4a and Supplementary Fig. 6a, b). The steady-state levels of the reporter gene mRNA were unaffected by miR-134 overexpression, suggesting that the observed effect of miR-134 on luciferase expression does not reflect a change in the stability of the luciferase mRNA (Supplementary Fig. 6c). The effect of miR-134 on translation of the luciferase mRNA is dependent on the presence of the miR-134 cognate binding site within the 3′ UTR, as expression of a luciferase reporter containing the mutant m191 *Limk1* 3′ UTR (that is, with a mutated miR-134 binding site) was unaffected by the presence of exogenous miR-134 (Fig. 4a, white bars). In contrast to the effect on *Limk1* mRNA translation, mutation of the miR-134 binding site did not affect dendritic targeting of a *Gfp–Limk1* reporter RNA (Supplementary Fig. 5c).

The inhibition of endogenous miR-134 in neurons by 2′-O-Me-134 led to a statistically significant increase in the expression of the luciferase reporter fused to the wild-type *Limk1* 3′ UTR (Fig. 4b, black bars), but had no significant effect on expression of the m191 mutant reporter that is incapable of binding miR-134 (Fig. 4b, white bars). By contrast, an antisense oligonucleotide directed against let-7c (2′-O-Me-let-7c) had no effect on *Limk1* reporter gene activity. Peptide-mediated delivery of miR-134 into neurons led to a dose-dependent decrease in the level of endogenous Limk1 protein, whereas delivery of its inhibitor 2′-O-Me-134 led to an increase in protein level (Fig. 4c, d), suggesting that miR-134 inhibits translation of the endogenous *Limk1* mRNA. Taken together, these data suggest that endogenous miR-134 inhibits *Limk1* mRNA translation in neurons by binding to a single site present in the *Limk1* 3′ UTR.

Although these studies provide evidence that miR-134 acts to repress *Limk1* mRNA translation, they do not distinguish whether the inhibition occurs within the cell body and/or dendrites. To address this issue, we generated a GFP-based protein synthesis reporter (*myr-d1Gfp*) with limited diffusion and a shortened half-life (1 h). Results from a previous study using a similar construct

demonstrated that GFP expressed from the reporter gene allows for the study of local protein synthesis within intact dendrites[36]. The *myr-d1GFP* reporter was fused to either wild-type or m191 mutant *Limk1* 3′ UTR and introduced into hippocampal neurons. GFP expression was monitored by confocal microscopy, and the intensity of the GFP signal was determined in the dendrites of many neurons at varying distances from the cell body (Fig. 4e). This analysis revealed that the average expression of the wild-type *Limk1* reporter was significantly reduced (by 18–28%) along the entire length of the dendrites compared to that of the m191 reporter (Fig. 4f). Given the dendritic localization of endogenous *Limk1* mRNA and miR-134, these findings suggest that miR-134 partially inhibits *Limk1* mRNA translation locally within dendrites.

miR-134 regulates spine size through Limk1

Because both overexpression of miR-134 and disruption of Limk1 function lead to decreased spine size[35], we next investigated whether miR-134-mediated repression of *Limk1* mRNA translation might be an explanation for the observed reduction in dendritic spine size upon miR-134 overexpression. Towards this end, we expressed miR-134 in hippocampal neurons together with constructs expressing either a wild-type *Limk1* mRNA or mutant m191 *Limk1* mRNA, and monitored dendritic spine size (Fig. 5a). We reasoned that if the effect of miR-134 on spine morphology occurs through suppression of endogenous *Limk1* mRNA translation, ectopically expressed *Limk1* mRNA that is incapable of interacting with miR-134 (m191) should be able to rescue the spine defect. In contrast, co-expression of the wild-type *Limk1* mRNA, which is still subject to miR-134-mediated translational inhibition, might be expected to prove less effective in the rescue of the dendritic spine phenotype caused by miR-134 overexpression. Consistent with this idea, we found that the m191 mutant *Limk1* mRNA efficiently rescued both the spine volume and width decrease imposed by miR-134 overexpression, whereas the wild-type *Limk1* mRNA was not as effective at rescuing the decrease in spine volume and width (Fig. 5a, upper and lower left panels). Both *Limk1* constructs had no effect on dendritic spine length (Fig. 5a, lower right). In addition, the observed difference between the effect of the wild-type and m191 mutant *Limk1* mRNA on spine width was not due to intrinsic differences in the ability of the two mRNAs to be translated, because in the absence of miR-134, Limk1 protein levels were equivalent in 293Tcells transfected with the wild-type and mutant *Limk1* constructs (Fig. 5b). Immunohistochemistry revealed that in neurons, overexpressed Limk1 protein was targeted to synaptic sites within spines (Fig. 5c), consistent with the possibility that an increased level of Limk1 protein within spines might be responsible for the rescue of the spine morphology phenotype. Taken together, these results suggest that Limk1 is a downstream effector of miR-134 in the control of dendritic spine development.

miR-134 functions in BDNF-stimulated Limk1 synthesis

Dendritic mRNAs are transported to the synapto-dendritic compartment within RNA granules. During their transport and once they have arrived at synaptic sites, the translation of dendritic mRNAs may be suppressed until extracellular factors such as those released upon synaptic stimulation activate the translation of these dormant mRNAs[17,19]. We asked whether the suppression of *Limk1* translation by miR-134 is relieved by extracellular stimuli such as BDNF. We first assessed whether the translation of *Limk1* mRNA is regulated by BDNF. Towards this end, synaptoneurosomes prepared from P15 rat brain were incubated with ^{35}S-methionine to label newly synthesized

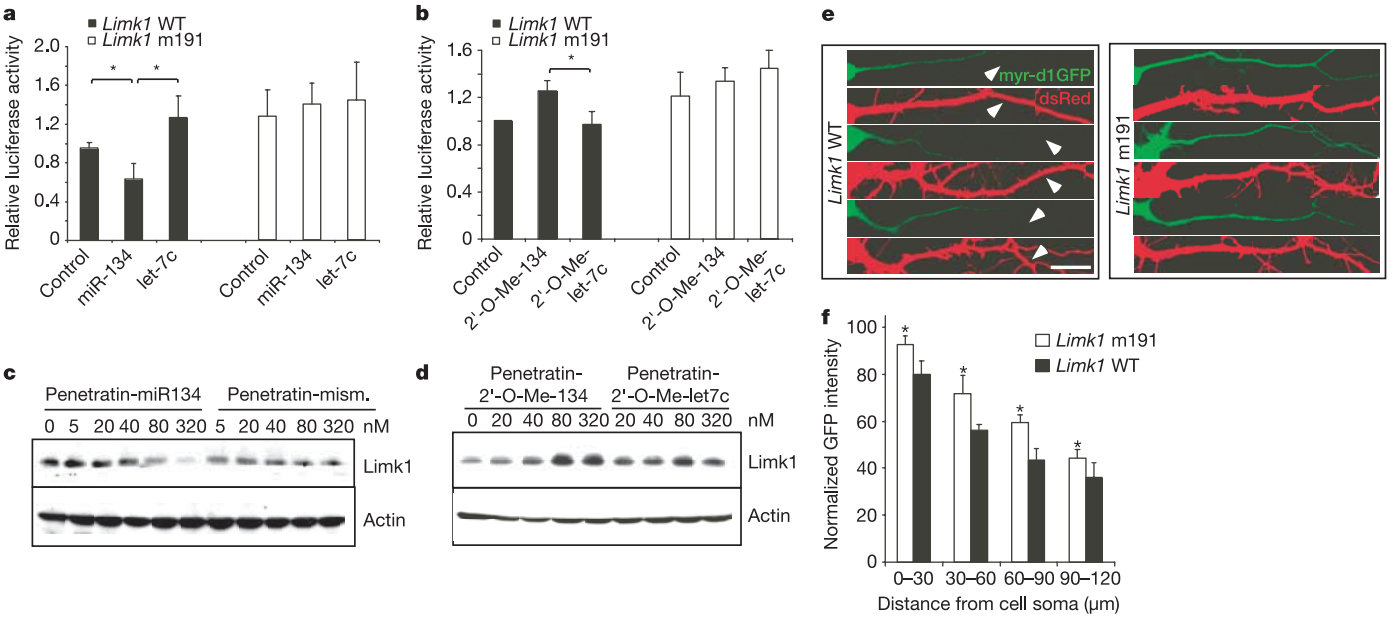

Figure 4 | miR-134 inhibits *Limk1* mRNA translation in neurons. **a**, Luciferase activity of wild type (black bars) or m191 (white bars) *Limk1* 3′ UTR reporter genes in the absence (control) or presence of the indicated miRNAs (10 μM). Data represent the mean from three independent experiments ±s.d. Asterisk, $P < 0.05$ (paired Student's *t*-test). **b**, Luciferase activity of reporter genes described in **a** in the presence of the indicated antisense 2′-O-Me oligonucleotides (20 μM). Data represent the mean from three independent experiments ±s.d. *Limk1* wild-type control = 1; asterisk, $P < 0.05$ (paired Student's *t*-test). **c**, Western blot analysis of endogenous Limk1 (upper panel) and actin (lower panel) expression in lysates from cortical neurons (12 + 2 DIV) transduced with penetratin-coupled miR-134 or mismatch (mism.) control. **d**, Western blot analysis as in **c**, except that penetratin-coupled antisense 2′-O-Me oligonucleotides were used. **e**, Local translation assay in hippocampal neurons (12 + 2 DIV) using destabilized, membrane-anchored *myr-d1GFP* reporter genes (green) harbouring either the wild type (left panel) or m191 (right panel) *Limk1* 3′ UTR. Co-transfected dsRed was used to track dendrites. Three representative dendrites are shown per experimental condition. Arrows point to dendritic regions of *myr-d1GFP-Limk1* wild-type UTR transfected neurons where little GFP signal is detectable. Scale bar, 20 μm. **f**, Average normalized GFP intensity in dendritic segments ($n > 60$) depicted in **e**. Data are from three independent experiments and presented as mean ±s.d. at 30-μm dendritic intervals. The average GFP intensity of the *Limk1* m191 reporter at the most proximal part of the dendrite was set to 100. Asterisk, $P < 0.05$ (paired Student's *t*-test).

proteins, and the amount of newly synthesized Limk1 protein was monitored by radio-immunoprecipitation. BDNF treatment significantly increased synthesis of Limk1 protein within isolated synaptoneurosomes as indicated by an increase in ^{35}S-methionine-labelled protein in Limk1 immunoprecipitates. This increase was sensitive to treatment with rapamycin, an inhibitor of the mTOR kinase pathway, which we and others have shown to mediate BDNF signalling to the translational machinery (Fig. 6a)[32,37].

We next asked whether the ability of BDNF to induce *Limk1* mRNA translation reflects the ability of BDNF to relieve miR-134-dependent repression of *Limk1* translation. Towards this end, we examined the effect of BDNF treatment on the translation of a *Limk1* 3′ UTR luciferase reporter mRNA in neurons at a time when endogenous miR-134 is highly expressed (14 DIV). When cells were transfected with luciferase mRNA fused to the wild-type *Limk1* 3′ UTR, BDNF led to a statistically significant induction of translation of the reporter mRNA (Fig. 6b). Expression of the m191 luciferase reporter mRNA was derepressed relative to the wild-type reporter in the absence of BDNF treatment, presumably due to the failure of endogenous miR-134 to bind to the m191 reporter gene (Fig. 6b). BDNF treatment did not lead to a further increase in the expression of the m191 reporter gene. To investigate the effect of miR-134 on BDNF-induced *Limk1* translation more directly, we introduced synthetic miR-134 into neurons that express little endogenous miR-134 (4 DIV, Fig. 1c). We found that miR-134 partially interferes with BDNF induction of the wild-type, but not the m191 mutant, reporter mRNA (Fig. 6c). These findings suggest that miR-134 represses *Limk1* mRNA translation and that BDNF treatment relieves this repression. However, the observation that there is still residual BDNF induction of reporter mRNA translation

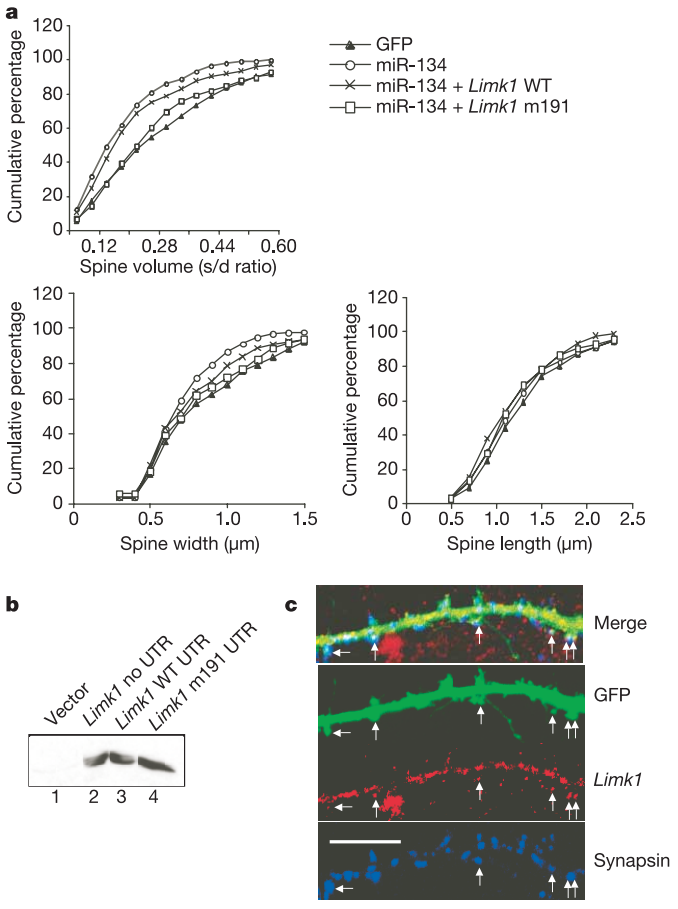

Figure 5 | Limk1 expression rescues miR-134-mediated reduction in spine size. **a**, Cumulative percentage plots of spine volume, width and length in hippocampal neurons (18 DIV) transfected with miR-134 alone or together with the indicated Limk1 expression constructs ($n > 500$ spines per condition from two independent experiments, five neurons per experiment). Spines of miR-134-transfected neurons have a significantly decreased volume compared to GFP ($P < 0.001$) or *Limk1* m191 3′ UTR ($P < 0.001$)—but not *Limk1* wild-type 3′ UTR ($P = 0.229$)—transfected neurons, and are significantly thinner than those of GFP ($P < 0.001$) or *Limk1* m191 3′ UTR ($P = 0.006$)—but not *Limk1* wild-type 3′ UTR ($P = 0.189$)—transfected neurons. Statistical significance was assessed by Kolmogorov–Smirnov test. **b**, Anti-Limk1 western blot of 293T whole-cell lysates transfected with vector alone or the indicated Limk1 expression constructs. **c**, Immunocytochemistry of GFP (green), Limk1 expressed from the *Limk1* m191 3′ UTR construct (red) and synapsin (blue) in 18 DIV hippocampal neurons. Arrows point to the co-localization of Limk1 and synapsin in GFP-positive dendritic spine heads. Scale bar, 10 μm.

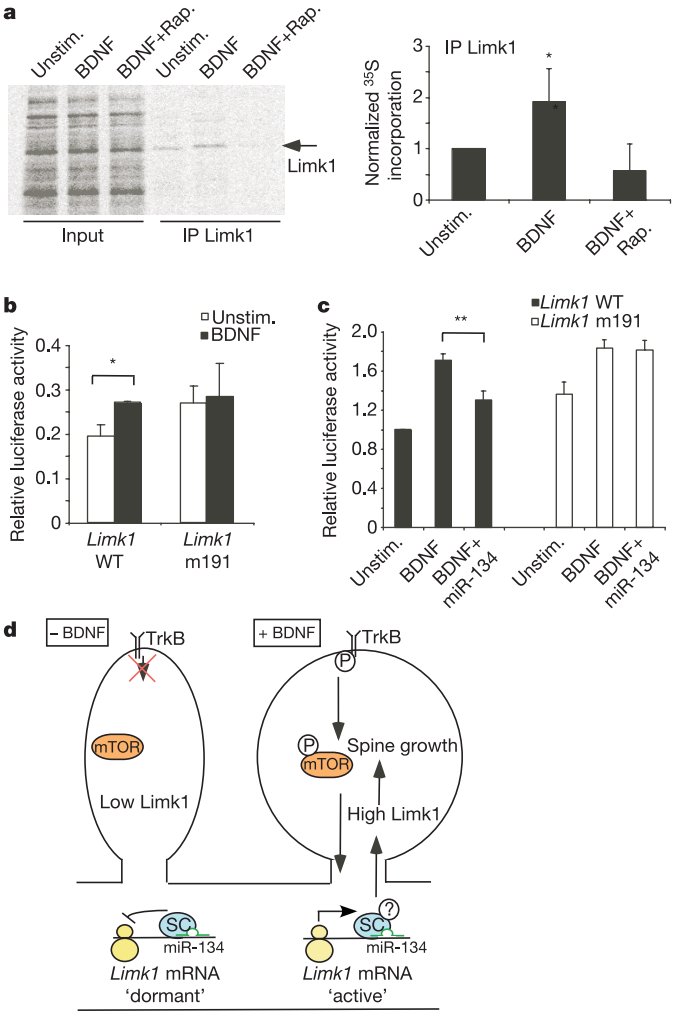

Figure 6 | miR-134 is involved in BDNF-induced *Limk1* mRNA translation. **a**, Left panel: immunoprecipitation (IP) of Limk1 from P15 synaptoneurosomes incubated with ^{35}S in the presence or absence of BDNF/rapamycin (rap). Right panel: average of the Limk1 immunoprecipitation signal intensities from three independent experiments ±s.d. Unstim. = 1. Asterisk, $P < 0.05$. **b**, Relative luciferase activity in 14 DIV cortical neurons transfected with *Limk* wild type (black bars) or *Limk1* m191 (white bars) reporter mRNAs. Neurons were either unstimulated or treated with 100 ng ml^{-1} BDNF for 4 h. Data represent the average of three independent experiments ±s.d. Asterisk, $P < 0.05$. **c**, Relative luciferase activity in 4 DIV cortical neurons transfected with *Limk* wild type (black bars) or *Limk1* m191 (white bars) reporter mRNAs treated as in **b** together with miR-134 where indicated. Data represent the average of three independent experiments ±s.d. Asterisk, $P < 0.005$. **d**, Model for the role of miR-134 (green) in the regulation of Limk1 synthesis and spine growth. For details, see text.

when miR-134 cannot bind to the *Limk1* 3′ UTR suggests an involvement of additional miR-134-independent mechanism(s) in BDNF-induced *Limk1* translation.

Discussion

We have identified a dendritically localized miRNA that regulates the expression of the synaptic Limk1 protein, thereby controlling dendritic spine size. We hypothesize that the association of *Limk1* mRNA with miR-134 keeps the *Limk1* mRNA in a dormant state while it is being transported within dendrites to synaptic sites (Fig. 6d). In the absence of synaptic activity, miR-134 may recruit a silencing complex that has a key role in repressing *Limk1* mRNA translation. This then limits the synthesis of new Limk1 protein and restricts the growth of dendritic spines. Upon synaptic stimulation, the release of BDNF may trigger activation of the TrkB/mTOR signalling pathway, which inactivates the miR-134-associated silencing complex by an as-yet-unknown mechanism, leading to enhanced Limk1 protein synthesis and spine growth. Our preliminary finding that miR-134 moves to the polysome-associated mRNA pool upon BDNF stimulation (G.S. and M.E.G., unpublished observations) suggests that miR-134 itself may not dissociate from the *Limk1* mRNA upon exposure of neurons to BDNF. Instead, we speculate that BDNF alters the activity of other translational regulators within the miR-134-containing complex. In addition to miR-134, other neuronal miRNAs have been predicted to bind the *Limk1* 3′ UTR[38]. Therefore, the combinatorial action of multiple miRNAs on the *Limk1* 3′ UTR might explain our observation that miR-134 only partially inhibits *Limk1* mRNA translation (Fig. 4).

A recent bioinformatics approach predicted several additional neuronal mRNAs that may also represent miR-134 targets[39]. Given that BDNF has important roles at multiple steps of synaptic development[40,41], it is possible that miR-134 regulates distinct sets of target genes involved in the formation, maturation or plasticity of synapses.

We propose that miRNA regulation of the translation of a variety of neuronal mRNAs will be found to contribute in an important way to synaptic function[42]. It is tempting to speculate that miRNAs act locally at individual synapses, thereby contributing to synapse-specific modifications that occur during synaptic plasticity. A future challenge will be to identify the full complement of dendritic miRNAs as well as their target mRNAs, and to determine their role in synaptic development.

METHODS

DNA constructs. The rat *Limk1* 3′ UTR (1,171 base pairs) was amplified by polymerase chain reaction (PCR) from rat brain cDNA (P15). Mutation of the miR-134 binding site (m191) was achieved using the Quick Change site directed mutagenesis kit (Stratagene). PCR products were cloned into pGL3 basic (Promega), pBSK (Stratagene) or *myr-d1GFP* (gift of B. Sabatini) for constructs used in luciferase assay, *in vitro* transcription, or local reporter assay, respectively. For Limk1 expression constructs, the *Limk1* cDNA (gift of K. Mizuno) was cloned into pcDNA3 (Promega) together with rat *Limk1* 3′ UTR (wild type or m191). For the miR-134 expression construct, a genomic sequence spanning 150 base pairs 3′and 5′ of the miR-134 sequence (Supplementary Fig. S1c) was PCR-amplified and cloned into pcDNA3. See Supplementary Information for further details.

Cell culture, transfection and stimulation. Cultures of dissociated primary cortical and hippocampal neurons were prepared as described[32]. Hippocampal neurons were maintained in Neurobasal plus B27 supplement; cortical neurons in Basal Medium Eagle plus 5% FBS. Neuronal transfections were performed with LipofectAmine 2000 (Invitrogen). For BDNF stimulation, neurons were starved overnight in the presence of UO126 (1 μM) and then treated with BDNF (Preprotech, 100 ng ml^{-1}) for 4 h before cell harvest.

Northern blotting and RNase protection assays. RNA was isolated from synaptoneurosomes or cultured neurons by phenol/chloroform extraction using RNA Stat-60 (Tel-Test). For northern blots, 30 μg of total RNA was resolved on 15% urea/polyacrylamide gels and transferred to Hybond N$^+$ membrane (Amersham). See Supplementary Information for further details. RNase protection assays were performed with the mirVana miRNA detection kit (Ambion) as per the manufacturers' recommendations.

***In situ* hybridization.** *In situ* hybridization of endogenous mRNAs and GFP reporter mRNAs was as described[32]. For the detection of small RNAs, a digoxigenin tail was added to antisense-locked nucleic acid (LNA) oligonucleotides (Exiqon) with the DIG tailing kit (Roche). Tailed LNA oligonucleotides were purified and used for overnight hybridization at 42 °C. All other steps were the same as for mRNAs.

Microinjection. Mature hippocampal neurons[43] were microinjected using an AIS2 microinjection system (Cellbiology Trading) attached to a Zeiss Axiovert 200M. Annealed 3′-end labelled (Alexa-546) sense and unmodified antisense strands of miR-134 (IBA) were used at 100 ng μl^{-1}. *Limk1* RNA was labelled by *in vitro* transcription in the presence of Alexa-488-5′ UTP (Molecular Probes) and used at 200 ng μl^{-1}. Microinjection needles with a tip size between 0.2 and 0.3 μm were used (P-87, Sutter Instruments) with a holding pressure of 40 hPa and an injection pressure of 80 hPa. Cells were imaged 20 min after injection, and randomly selected images were analysed by three independent observers in a blind manner.

Peptide-mediated delivery. Double-stranded small RNA or 2′-O-methylated DNA oligonucleotides containing a 5′ thiol group (80 μm, IDT) were reduced with TCEP (80 μm, Sigma) at room temperature for 15 min. Penetratin (80 μm, Qbiogene) was added and the mixture was incubated at 65 °C followed by 1 h at 37 °C. Coupled oligonucleotides were heated at 65 °C for 15 min before adding to cells at the indicated concentrations for 4 h. Neurons were harvested for western analysis 48 h after transduction.

Image analysis. For spine analysis, neurons were transfected at 8 DIV or 15 DIV with indicated expression plasmids in combination with EGFP and processed for confocal microscopy at 18 DIV. See Supplementary Information for further details on spine analysis.

For Sholl analysis, a series of concentric circles of 10-μm increments was manually drawn around the cell body, and the number of dendritic intersections at each individual circle was counted. At least ten individual neurons were measured for each experimental condition. To quantify dendritic GFP levels in the local reporter assay, random dendrites were selected based on dsRed staining and plot profiles of the GFP intensity of the same dendrites were derived using ImageJ (NIH). The obtained values were background corrected and normalized to the respective signal in the red channel. At least 20 dendrites per experimental condition of a total of three independent experiments were measured.

Quantitative real-time PCR. Quantitative real-time PCR was performed on a Taq-Man (Perkin Elmer Life Sciences) using the SYBR-green-containing PCR kit (PE Applied Biosystems) as described[32].

Preparation of synaptoneurosomes and radio-immunoprecipitation. Synaptoneurosomes were prepared from P15 long-Evans rat pups (Charles River) as described[32]. For radio-immunoprecipitation, a mouse monoclonal anti-Limk1 antibody (Pharmingen) was used.

Immunocytochemistry. Hippocampal neurons (18 DIV) were immunostained as described[32], using a mouse monoclonal anti-Limk1 (Pharmingen) or a rabbit anti-synapsin (Chemicon) antibody as primary antibody.

Luciferase assay. Cortical neurons were transfected at 4 DIV or 12 DIV, and luciferase assays were performed 2 days later with the Dual-Luciferase Reporter Assay System (Promega).

Received 15 August; accepted 25 October 2005.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

Acknowledgements We thank D. Bartel for providing the *lin-41* reporter constructs and northern blot protocols, K. Mizuno for the rat *Limk1* cDNA, G. Corfas for initial help with ISH, Y. Lin and A. West for reagents, J. Bikoff, E. Griffith, E. Hong, S. Paradis and B. Sabatini for critically reading the manuscript, and all the members of the Greenberg laboratory for support and discussion. This work was supported by grants from the NINDS and NICHD (M.E.G.), HFSP (G.S. and M.K.), the Charles Hood Foundation (G.S.), the Hertie-Foundation (M.K.), the Schram-Stiftung (M.K.) and a Boehringer Ingelheim Fonds fellowship (F.T.). M.E.G. acknowledges the generous support of the F. M. Kirby Foundation to the Neurobiology Program of Children's Hospital.

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