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Original Article

Tubby-like protein 1 (Tulp1) is a target of microRNA-134 and is down-regulated in experimental epilepsy

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Abstract: MicroRNAs are important determinants of gene expression via post-transcriptional control of the protein levels of their mRNA targets. MicroRNA-134 (miR-134) has emerged as an important brain-specific microRNA which has been implicated in the control of dendritic spine morphology, neuronal differentiation and apoptosis. Here we show that Tubby-like protein 1 (Tulp1) is a target of miR-134. Tulp1 protein showed a similar cellular distribution pattern in the hippocampus to miR-134 and displayed an inverse expression pattern in the mouse retina. Bioinformatics analyses identified a conserved miR-134 binding site in the 3' untranslated region of both mouse and human *Tulp1* and luciferase reporter assays confirmed miR-134 targets Tulp1 in vitro. Induction of prolonged seizures in mice resulted in upregulation of miR-134 and downregulation of protein levels of Tulp1 which were reversed in animals injected with locked nucleic acid-modified antagomirs targeting miR-134. Finally, knockdown of Tulp1 in human neurons caused an increase in vulnerability to excitotoxicity. These data identify Tulp1/TULP1 as a novel target of miR-134, which may contribute to underlying pathomechanisms in epilepsy.

Keywords: Epilepsy, hippocampal sclerosis, non-coding RNA, status epilepticus

Introduction

MicroRNAs (miRNA) are an important class of small (~22 nt) non-coding RNAs that control protein levels in cells by interfering with gene expression at a post-transcriptional level [1]. MiRNAs work by binding to complementary regions in their target mRNAs within the RNA-induced silencing complex (RISC), which comprises Argonaute-2 (Ago2) and other proteins [2]. Targeting is typically via a 7-8 nt seed match in the 3' untranslated region (UTR) and this results in translational repression or mRNA degradation [1, 3], although other mechanisms are also known [4, 5]. The brain is particularly enriched in miRNA expression, where roles have been identified in cell division, differentiation and regulation of physiological and pathophysiological processes [6].

MicroRNA-134 (miR-134) was originally discovered in a screen of tissue-specific miRNAs [7], and described as a CNS-specific miRNA that is expressed in neurons both within the soma and dendrites [8, 9]. A key function of miR-134 is to negatively regulate dendritic spine size and morphology, which is achieved via targeting of Limk1 [9]. Other validated targets of miR-134 include the transcription factor CREB (c-AMP response element binding protein) [10] and proteins involved in neuronal migration and apoptosis, such as DCX (doublecortin) [11, 12]. It is likely, however, that some individual miRNAs have hundreds of mRNA targets in the brain [13].

Previous studies reported increased levels of miR-134 in experimental and human epilepsy [8, 14-16]. Functional studies using locked nucleic acid-modified miRNA oligonucleotide inhibitors (antagomirs) have demonstrated that silencing miR-134 reduces seizures in various experimental models of epilepsy [8, 14, 16]. This includes kainic acid (KA)-induced seizures *in vivo,* where suppression of miR-134 reduces the subsequent occurrence of spontaneous

seizures and progressive neuropathology in rats and mice [8, 16]. The molecular mechanisms underlying the antagomir effects are not known but Limk1 and spine changes were implicated [8, 14, 17]. MiR-134 has also been suggested as a potential biomarker of neurological disorders [18, 19], particularly in epilepsy [20-22]. As a result, there is significant interest in identifying further targets of miR-134. The present study sought to identify novel targets of miR-134 in the brain.

Materials and methods

Bioinformatics

Predicted targets of miR-134 were identified through DIANA-MICROT (miRanda) and Targetscan (version 6.2). Only 9 target genes were commonly regulated in both data sets. Subsequently, the seed regions of the 9 plausible target genes were evaluated in microRNA.org. Five target genes had seed regions outside of the 3'UTR, two target genes had a poor seed pairing between the 3'UTR and miR-134 (less than 7 bp), while two genes had a strong conserved seed region (7-8 bp complementarity in positions 2-8 in the miRNA sequence); Tulp1 and Rab27a. Then, we narrowed our search by selecting only mRNAs, which demonstrated limited conservation in targeting by miRNAs other than miR-134; this left Tulp1 as a prime candidate. Finally, PubMed searches were performed to identify association of Tulp1 with neuronal activity, regulation, homeostasis and cellular localization.

RNA extraction and real-time PCR analysis of gene expression

Total RNA was extracted using the Trizol protocol as previously described [23]. The quantity of RNA was measured on a Nanodrop Spectrophotometer (Thermo Scientific). For miR-134 analysis, reverse transcription for individual qPCR was carried out using 1 μg of total RNA and the High-Capacity Reverse Transcription kit (Thermo Fisher). Individual qPCRs were carried out on a 7900HT Fast Realtime System using miR-134 specific Taqman microRNA assays (Thermo Fisher). RNU19 was used for normalization. A relative fold change in expression was determined using the comparative cycle threshold method ($2^{-\Delta\Delta Ct}$).

Western blot analysis

Western blotting was performed as previously described [14]. Briefly, tissue (hippocampus or retina) were homogenized in a lysis buffer containing protease inhibitors and protein concentrations determined. Samples were then boiled in gel-loading buffer, separated by SDS-PAGE, transferred to nitrocellulose membranes and immunoblotted with primary antibodies against Tulp1 (1:500, Abcam), β-actin or α-Tubulin (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA, USA). Finally, membranes were incubated with secondary antibodies and bands visualized using chemiluminescence (Pierce, Rockford, IL, USA) under a Fuji-Film digital gel scanner. Semi-quantitative analysis was performed using Alpha-EaseFC4.0 software [14].

Immunohistochemistry

Immunohistochemistry was carried out as previously described [24]. Briefly, brains were cut at 30 μm and sections incubated with primary antibodies against Tulp1 (1:100, Abcam). Next, the sections were rinsed, incubated with HRP-conjugated secondary antibodies and prepared for DAB staining (VectorLabs). Immunocytochemistry on retinal cryosections from 2 week-old wild type 129 and $Tulp1^{-/-}$ (strain no. 005289, The Jackson Laboratory) mice was performed using polyclonal M-tulp1N antibody [25] as described [26].

Cell culture, transfection, treatment and cell viability assay

SH-SY5Y cell line was cultured in DMEM/F12 (Sigma-Aldrich) supplemented with 10% fetal bovine serum, 2 mM L-glutamine and 100 U/ml penicillin/streptomycin. SH-SY5Y cells were transfected with sh-Scramble or sh-Tulp1 expressing plasmid (Origene) using Metafectamine (Biontex) following the manufacturer's protocol.

For the cell viability assay, sh-Scramble or sh-Tulp1 transfected cells were treated with monosodium glutamate (Sigma-Aldrich) 48 h post-transfection. Next, 50 ml of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) of 0.5% w/v in PBS was added to the cells for 4 h. Finally the medium was discarded, MTT crystals dissolved in dimethyl sulfoxide and absorbance measured at 560 nm.

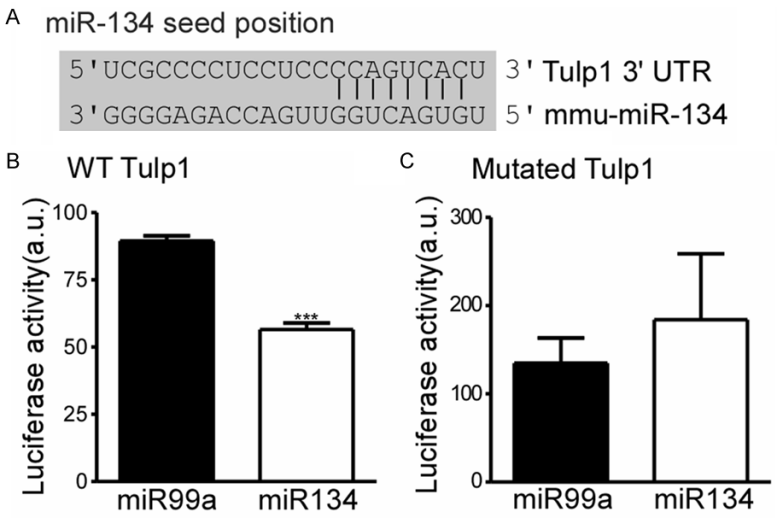

Figure 1. *Tulp1 is a target gene of miR-134.* A. Sequence alignment of Tulp1 3'UTR sequence and miR-134. B. The Tulp1 3'UTR - luciferase plasmid construct was co-transfected with miR-99a (control) or miR-134. The relative luciferase signal was significantly lower in the presence of miR-134 (n = 9, p<0.001). C. A mutated version of the seed region of Tup1 3'UTR was co-transfected with miR-99a (control) or miR-134. The relative levels of the luciferase signal was not affected in the presence of miR-134 (n = 9).

Luciferase assay

Luciferase assays were carried out as previously described [23]. A 350 bp fragment of the Tulp1-3'UTR or a mutated seed region of miR-134 were cloned from pEX-A2 plasmid (Eurofins Genomics) to pGL4-SV40 plasmid (Promega). SH-SY5Y cells were transfected with Metafectamine (Biontex) following the manufacturer's guidelines in a ratio 1:5 DNA:Metafectamine. Cells were transfected with 100 nM of miR-99a (control) or miR-134 precursors (Thermo Fisher), a luciferase plasmid containing the wild-type or a mutant sequence of the Tulp1-3'UTR and an empty Firefly luciferase vector (pGL4-SV40, Promega), the latter as transfection control. The luciferase assays were carried out following the manufacturer's guidelines (Dual Luciferase kit, Promega). Normalized luciferase activity was expressed as a ratio of reporter to control activity.

Seizure model

Animal experiments were performed in accordance with the European Communities Council Directive (86/609/EEC) and were reviewed and approved by the Research Ethics Committee of the Royal College of Surgeons in Ireland under license from the Department of Health, Dublin, Ireland. Experiments were performed as previously described [8]. Briefly, adult (20-22 g) male C57BL/6J mice (Envigo) were used, with food and water provided *ad libitum*. For induction of status epilepticus, mice were first anesthetized with isoflurane and then placed in a mouse-adapted stereotaxic frame. Mice were fitted with three skull-mounted EEG recording screws and a guide cannula (Bilaney Consultants, Sevenoaks, U.K.) (Coordinates from Bregma; AP = -0.94 mm, L = -2.85 mm) [27]. Animals were then placed inside an open Perspex box, which allowed free movement and baseline EEG recorded using a Grass Comet digital EEG. The animals were then lightly restrained and an injection cannula lowered into the amygdala for microinjection of KA (Sigma-Aldrich, Arklow, Ireland) or vehicle (phosphate-buffered saline (PBS)). After 40 min, mice received lorazepam (6 mg kg^{-1}, i.p.) before being disconnected and placed in a recovery chamber. Mice were killed 1, 7 or 20 days later by anaesthetic overdose.

Antagomir treatment

For i.c.v. injections, mice were affixed with a cannula ipsilateral to the side of KA injection. Coordinates from Bregma were: AP = -0.3 mm, L = -1.0 mm, V = -2.0 mm. Twenty-four hours before induction of status epilepticus, mice received 1 µl infusion of either Scr or Ant-134 LNA- and 3'-cholesterol modified oligonucleotides (0.12 nmol; Exiqon) in artificial cerebrospinal fluid (Harvard Apparatus, Kent, U.K.).

Statistical analysis

Data are presented as mean ± standard error of the mean (s.e.m.). Two group comparisons were performed using unpaired Student's t-test, while multi-group comparisons were performed using one-way analysis of variance (ANOVA) and Bonferroni post-hoc test using GraphPad Instat. Significance was accepted at $P<0.05$.

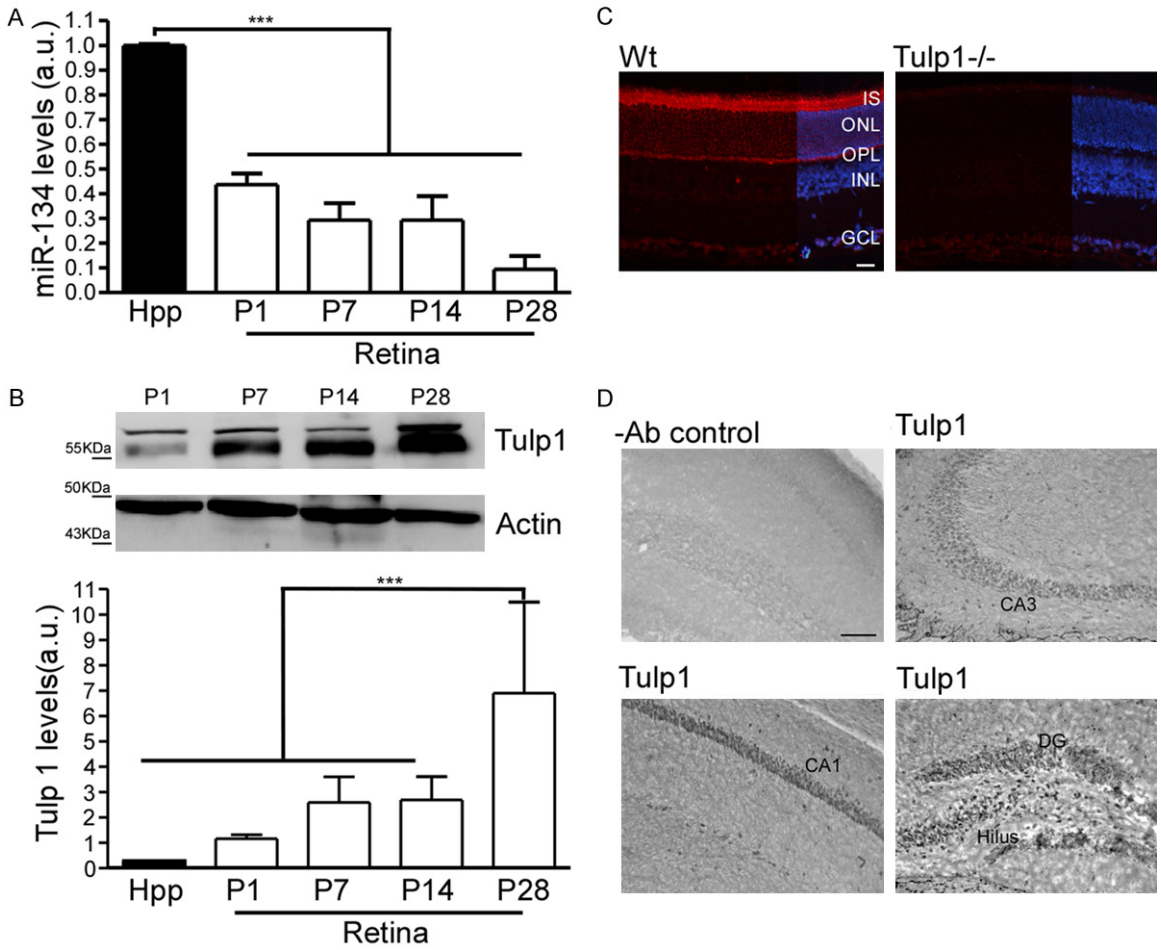

Figure 2. *Inverse expression of Tulp1 and miR-134 in hippocampus and retina.* A. Levels of miR-134 from hippocampus (hpp) of 6 weeks old mice and retina from 1, 7, 14 and 28 days old mice (n = 3, p<0.001). B. Representative Western Blot and quantification of Tulp1 levels from hippocampus (hpp) of 6 weeks old mice and retina from 1, 7, 14 and 28 days old mice (n = 3, p<0.001). C. Representative images of Tulp1 expression in retinas from wild type and *Tulp1*$^{-/-}$ (negative control) mice at 14 days. IS: photoreceptor inner segment layer, ONL: outer nuclear layer, OPL: outer plexiform layer, INL: inner nuclear layer, GCL: ganglion cell layer. Scale bar 25 µm. D. Representative images of Tulp1 from hippocampus from 6 weeks old mice. Note: Positive signal is observed in the *Cornus Ammonis* 1 and 3 (CA1, CA3), hilus and Dentate Gyrus (DG). No signal was observed in the absence of primary antibody (-Ab control). Scale bar: 100 µm.

Results

Tulp1 is a predicted target of miR-134

The major mature form of miR-134 in humans is hsa-miR-134-5p, which comprises of a 22 nt sequence that is identical to mouse mmu-miR-134-5p. Targetscan lists 104 potential target transcripts with conserved target sites for mmu-miR-134 and 175 transcripts for hsa-miR-134, including previously validated targets such as Creb1 [10] and Kras [12]. Targetscan did not predict certain validated targets of miR-134, including Limk1 [9] or doublecortin [11]. After narrowing our search by selecting mRNAs with limited targeting by miRNAs other than miR-134, we identified Tulp1/TULP1 as a potential candidate. In the mouse, the 7 nt seed region lies at position 205-211 in the 3'UTR of Tulp1 and in humans the 7 nt seed lies at 288-294 in the 3'UTR of TULP1. No other conserved miRNA target sites are present in the human and mouse Tulp1 mRNAs in TargetScan (**Figure 1A** and data not shown).

To confirm that miR-134 can target Tulp1 a 3'UTR luciferase assay was performed utilizing a luciferase-Tulp1 3'UTR construct including the mouse miR-134 seed region. Transfection of cells with pre-miR-134 significantly reduced

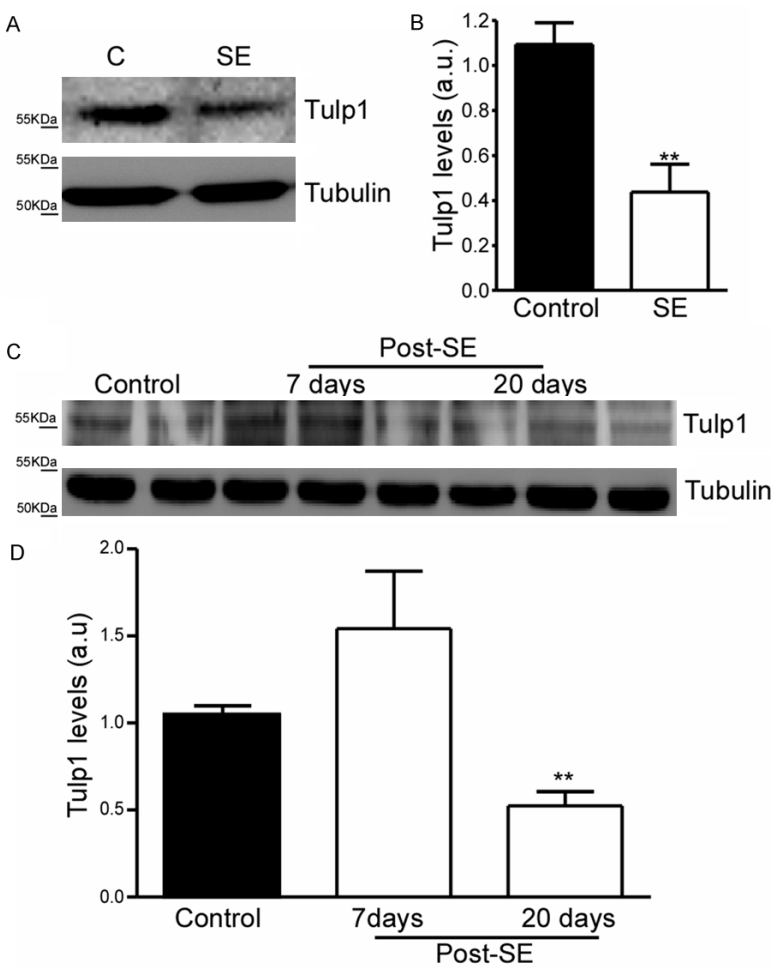

Figure 3. *Tulp1 is downregulated following Status Epilepticus (SE) and in chronic experimental epilepsy.* A. Representative western blot of Tulp1 from hippocampus from control and 24 h post SE. Tubulin was used as a loading control. B. Bars indicate levels of Tulp1 in hippocampus from mice subjected to SE. Note: Levels of Tulp1 are reduced in hippocampus 24 h post SE compared to control group (n = 6, $p<0.01$). C. Representative western blot of Tulp1 from hippocampus from control mice, 7 days and 20 days (chronic epilepsy) post SE. D. Graph indicates levels of Tulp1 in hippocampus from control mice and mice subjected to SE (7 days and 20 days after SE). Note: Tulp1 levels are reduced in hippocampus in experimental epilepsy (20 days after SE) compared to control. No differences are observed at 7 days. (n = 6 per group, $p<0.01$).

luciferase activity compared with the control cells transfected with pre-miR-99a (**Figure 1B**). Further confirming the specificity of miR-134 targeting, mutations within the miR-134 seed region resulted in a loss of the luciferase inhibition by pre-miR-134 (**Figure 1C**). Taken together, these results suggest that Tulp1 is a bona fide target of miR-134.

Expression of miR-134 and Tulp1 in the retina

A known site of Tulp1 expression is in the photoreceptor layer of the retina [28, 29] (**Figure 2C**). We performed real-time quantitative PCRs and western blotting to assess Tulp1 expression in the developing retina (**Figure 2A**, **2B**). Results indicated that expression of Tulp1 increases over time in the mouse retina. Specifically, Tulp1 is present in the inner segments (IS), the synaptic region (outer plexiform layer, OPL) and cell bodies (outer nuclear layer, ONL) of photoreceptors cells (**Figure 2C**). Expression was not found in age-matched *Tulp1*$^{-/-}$ retinas. Furthermore, analysis of miR-134 expression revealed lower expression levels of miR-134 in the retina than the hippocampus (**Figure 2A**, **2B**). Taken together these data support a reciprocal relationship between Tulp1 and miR-134 in these tissues.

Tulp1 is expressed in pyramidal neurons in the adult mouse hippocampus

Previous work reported that Tulp1 was expressed in the adult mouse brain [30]. Therefore, we stained adult mouse brain sections using a Tulp1 specific antibody, which identified low-level expression of Tulp1 within the hippocampus, including pyramidal neurons of the cornu ammonis ((CA) 1 and 3), hilus and dentate gyrus (DG) (**Figure 2D**). Labeling was absent in sections in which the primary antibody had been omitted (**Figure 2D**). Notably, these are the cells previously reported to express miR-134 [8].

Seizure-induced down-regulation of Tulp1

Previously, we reported that miR-134 was upregulated following KA-induced status epilepticus in mice in addition to upregulation in other experimental models and human epilepsy [8, 14, 16]. If Tulp1 is an in vivo target of

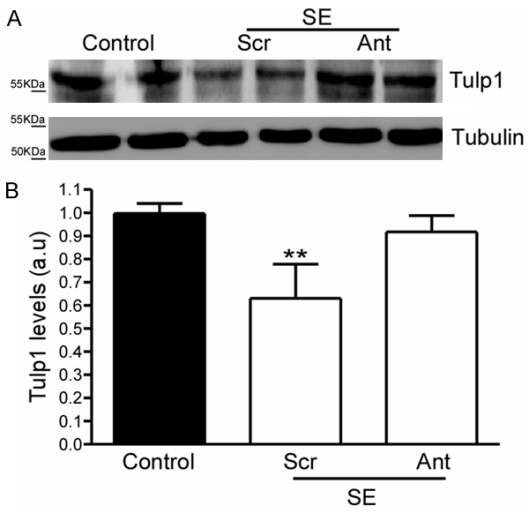

Figure 4. *Inhibition of miR-134 restores levels of Tulp1.* A. Representative western blot of Tulp1 from hippocampus of mice pretreated with scramble control (Scr) or Antagomir-134 (Ant) and subjected to SE 24 h later. Tubulin was used as a loading control. B. Graph indicates levels of Tulp1 in hippocampus from mice subjected to SE and pre-treated with scramble control. Note: levels of Tulp1 are reduced in hippocampus 24 h post SE compared to control group whereas levels of Tulp1 in mice receiving antagomir-134 are similar to control mice ($n = 6$, $p<0.01$).

miR-134 then expression of Tulp1 should be reduced in this model. To test this hypothesis we first measured Tulp1 protein levels following status epilepticus.

Tulp1 protein levels (**Figure 3A**, **3B**) were significantly reduced in hippocampi obtained 24 h post status epilepticus. Next, we examined Tulp1 expression during early and late epilepsy. Western blotting demonstrated that there was no difference in Tulp1 protein levels in samples obtained 7 days after status epilepticus (**Figure 3C**, **3D**), a time which corresponds to when miR-134 expression levels return to baseline in the CA3 subfield of the hippocampus [8]. Tulp1 protein levels were, however, lower in samples obtained 20 days post status epilepticus (**Figure 3C**, **3D**), a time point when animals display recurrent spontaneous seizures (i.e. epilepsy) and increased miR-134 expression levels [8].

Antagomirs targeting miR-134 prevent seizure-induced down-regulation of Tulp1

If miR-134 targets Tulp1 during seizure-induced neuronal death then inhibition of miR-134 should prevent down-regulation of Tulp1 in this model. To test this idea we analyzed hippocampal samples obtained 24 h after status epilepticus in mice previously injected with 0.12 nmol antagomirs targeting miR-134 (Ant-134) or scrambled control antagomir (Scr). Previous work established that Ant-134 produced potent and specific knockdown of miR-134 within 24 h [8]. Mice subjected to status epilepticus and pretreated with Scr displayed down-regulation of Tulp1 protein similar to that observed in mice with status epilepticus induced but without antagomir pre-treatment (**Figure 4A**, **4B**). In contrast, Tulp1 levels in mice given Ant-134 before status epilepticus were similar to that of non-seizure control mice (**Figure 4A**, **4B**). Taken together these data support an in vivo functional relationship between miR-134 and Tulp1.

Knockdown of Tulp1 increases vulnerability to excitotoxicity

The function of Tulp1 in the hippocampus is unclear although Tulp1 protein is known to reside in both cytoplasmic and nuclear compartments in neurons [30]. Therefore we investigated whether knockdown of Tulp1 had any effects on cell survival in a model of glutamate-induced neurotoxicity. Human SH-SY5Y cells were transfected with either non-targeting control or Tulp1 targeting shRNAs and then treated with glutamate. Cell viability analysis revealed that glutamate toxicity resulted in a ~25% reduction of cell viability (**Figure 5A**, **5B**). Cell viability was significantly lower in cells transfected with Tulp1 shRNA prior to glutamate (**Figure 5A**, **5B**).

Discussion

MiRNAs are increasingly recognized as critical determinants of gene expression in the brain and have been implicated in several neurological disorders including epilepsy [8, 14, 16, 24]. To date, a number of miRNAs have been demonstrated to have direct effects on neuronal excitability, including miR-128 [13] and miR-134 [8]. In the intra-amygdala KA model, silencing miR-134 was shown to potently reduce seizures and the subsequent occurrence of spontaneous recurrent seizures [8]. Additionally, silencing miR-134 has been reported to have anti-seizure effects in various other models [14, 16]. Several targets of miR-134 have now been identified, including Limk1 [9], CREB [10],

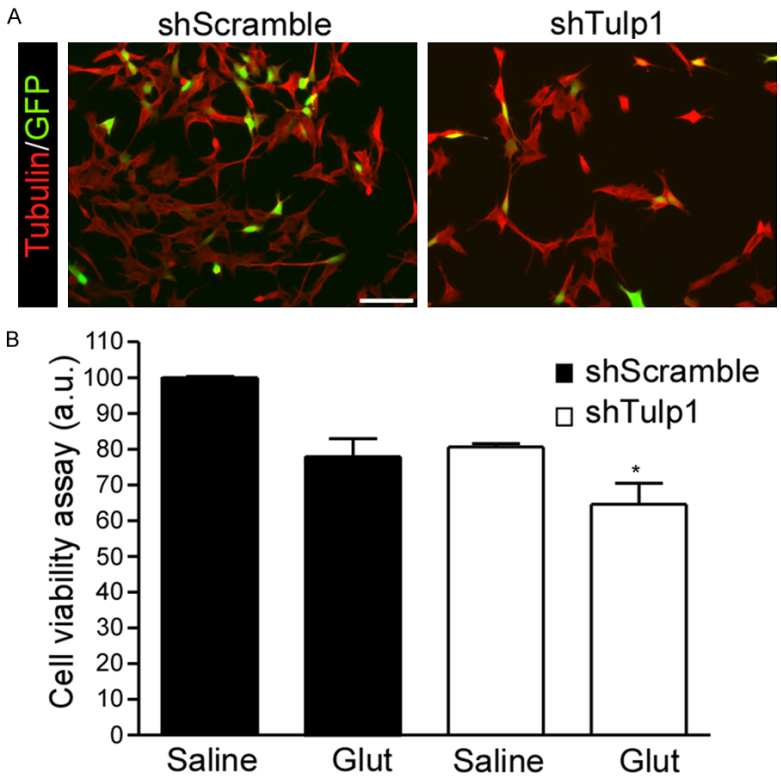

Figure 5. *Suppression of Tulp1 in SH-SY5Y cells reduces cell viability.* A. Representative images of SH-SY5Y cells transfected with sh-Scramble or sh-Tulp1 constructs. Red: Tubulin. Green: GFP (sh-expressing plasmid). B. Graph indicates cell viability in SH-SY5Y cells transfected with sh-Scramble (black bars) or sh-Tulp1 (white bars) and induced with glutamate. Note: Suppression of Tulp1 results in reduction of cell viability. (n = 9, $p<0.05$. *, compared to control (saline). #, compared to glutamate treated SH-SY5Y cells or sh-Tulp1 transfected cells).

DCX [11], KRAS and STAT5B [12]. Given the link between miR-134 and brain excitability, identifying further miR-134 targets is a priority, and may help to elucidate mechanisms of epilepsy and identify potential neuroprotective and anti-seizure treatments.

In the present study we identified Tulp1 as a novel target of miR-134. Tulp1 is a member of the tubby-gene family. Although the function of this gene family is poorly understood, genetic approaches have shown that members of the tubby-gene family are important to maintain cellular homeostasis. The tubby-gene family was first discovered as an obesity-inducer gene over two decades ago [31]. Mutations in the tubby-gene family have been associated with infertility, insulin resistant, hearing loss and retinal degeneration. Tubby-gene family members are localized in the internal layers of the plasma membrane and nucleus, where they may act as transcription factors [32].

While Tulp1 function remains elusive, recent evidence suggests that Tulp1 regulates internal trafficking at the photoreceptor segments and synapses in the retina. Tulp1 interacts with Dynamin-1, which generates a polymeric scaffold at the interface between the membrane and the cytoskeleton and to control vesicular transport [33]. In the presence of Tulp1, neurotransmitter-carrier vesicles are taken up by endocytosis and get recycled in the cytoplasm. In contrast, in the absence of Tulp1, vesicles go through exocytosis and release their neurotransmitter(s) into the synaptic cleft [32].

While the role of Tulp1 in the retina has in part been elucidated, the role for Tulp1 in the hippocampus is even less clear. Our data support earlier work, which indicated that Tulp1 is present in the main neuronal layers of the hippocampus in the adult mouse [30]. Additionally, we demonstrate that Tulp1 is down-regulated following status epilepticus and in chronic epilepsy. Given evidence that Tulp1 is a target for miR-134 and miR-134 expression increases following status epilepticus in both experimental and human epilepsy, we speculate that Tulp1 down-regulation is mediated by miR-134. We also observed a reciprocal relationship whereby high miR-134 expression was usually matched by low Tulp1 expression. A direct relationship between miR-134 and Tulp1 is also supported by our finding that silencing miR-134 using antagomirs partially reverted the down-regulation of Tulp1 following status epilepticus. One caveat of this result is that suppression of miR134 also reduces seizure severity [8]. The down-regulation of Tulp1 may therefore be secondary to effects on seizures. Regardless, these results

indicate that Tulp1 is regulated in experimental epilepsy and implicate miR-134 targeting in this process.

Future experiments will be needed to establish the functional role of Tulp1 in the hippocampus and whether the down-regulation following status epilepticus has any consequences for epileptogenesis. In this study, we found that knocking down Tulp1 in a neuronal cell line resulted in an increased vulnerability to glutamate toxicity supporting a protective function for Tulp1. Previously it was shown that antagomirs targeting miR-134 prevent hippocampal neuronal death induced by KA treatment in vitro, although this effect was mainly attributed to Limk1 [8]. Such a pro-apoptotic effect of miR-134 has also been reported in other cell types [12]. The mechanism(s) of the protective effect(s) via Tulp1 pathway(s) are unknown. However, recent data suggests that Tulp1 may activate phagocytosis in the retina in order to remove apoptotic cells and cellular debris, and reduce propagation of neuronal death in the retina [34, 35].

In summary, miRNAs such as miR-134 are important contributors to shaping the gene expression landscape in the brain and may be therapeutic targets for the treatment of neurological disorders such as epilepsy. In this study, we have identified a novel target gene for miR-134, i.e. Tulp1 and we demonstrated that antagomir suppression of miR-134 results in an increase in hippocampal levels of Tulp1, which is consistent with de-repression. Taken together, our data suggest that Tulp1 is a bone fide target for miR-134. Along with previously identified miR-134 targets (such as Limk1), the Tulp1-miR-134 axis may be involved in seizure control and neuroprotection.

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