Aus dem Institut für Physiologische Chemie
Kommissarischer Leiter: Prof. Dr. Marco Rust
des Fachbereichs Medizin der Philipps-Universität Marburg

Post-transcriptional regulation of microRNA biogenesis and localization in mammalian neurons

Inaugural-Dissertation
zur Erlangung des Doktorgrades
der gesamten Naturwissenschaften
(Dr. rer. nat.)

dem Fachbereich Medizin der Philipps-Universität Marburg
vorgelegt von

Kerstin Weiß
aus Witten

Marburg, 2019

Angenommen vom Fachbereich Medizin der Philipps-Universität Marburg am:
21.10.2019

Gedruckt mit Genehmigung des Fachbereichs

Dekan: Herr Prof. Dr. Helmut Schäfer
Referent: Herr Prof. Dr. Gerhard Schratt
1. Korreferent: Herr Prof. Dr. Carsten Culmsee

Für Papa

in Liebe und Dankbarkeit

Table of Contents

Summary

The remarkable cognitive capabilities of our brain require a complex and dynamic network of neurons that is able to quickly and precisely react to changes. Adapting to an ever-changing environment and the storage of information requires that sensory information is transformed into long-lasting structural changes. At the molecular level, highly sophisticated and tightly regulated gene expression programs are necessary to alter synaptic connections in the brain without disrupting the existing network.

MicroRNAs are important regulators in this neuronal network as they are able to precisely regulate local gene expression. These small non-coding RNAs bind complementary sequences in target mRNAs, thereby repressing their translation into protein. This plays an important role in activity-dependent synapse development, where local protein synthesis in dendrites is required to implement long-lasting changes in synaptic strength. The latter serves as the molecular basis for learning and memory processes.

This cumulative dissertation presents two studies that investigate how neuronal microRNAs are regulated at the level of biogenesis and localization. The first publication *"The DEAH-box helicase DHX36 mediates dendritic localization of the neuronal precursor-microRNA-134"* investigates the transport of miRNA-134 to its final destination in the synapto-dendritic compartment. The study describes that already the precursor (pre-miRNA) is located at the synapse and identifies DHX36 as a protein that specifically binds pre-miR-134 and is important for its transport. Knockdown of DHX36 further shows that the localization of pre-miR-134 to the dendrite is of functional importance. The absence of DHX36 leads to elevated expression of known miR-134 targets, accompanied by an increase in dendritic spine volume.

The second study presented in this thesis entitled *"The nuclear matrix protein Matr3 regulates processing of the synaptic microRNA-138-5p"* investigates the expression of microRNA-138. Two distinct precursor forms are known for miR-138, pre-miR-138-1 and pre-miR-138-2. In our study, we demonstrate that pre-miR-138-2 is the primary source for mature miR-138 in neurons. Using pulldown assays we identify the nuclear matrix protein Matrin-3 (Matr3) as a specific interactor of the hairpin structure of both the primary and precursor form of miR-138-2 (pri-/pre-miR-138-2). Knockdown of its expression demonstrates an inhibitory function of Matr3 in the nuclear processing of pri-miR-138-2, resulting in decreased mature miR-138 levels.

In summary, this thesis describes novel post-transcriptional regulatory mechanisms that control the expression and sub-cellular localization of two neuronal microRNAs, miR-134 and miR-138. Both microRNAs have important roles in synaptic plasticity and a precise regulation of their expression is crucial for maintaining a stable and functional neuronal network. A further understanding of the regulation of these microRNAs and their downstream processes is an important step to gain insight into the complex regulatory processes involved in learning and memory, as well as into malfunctions of these systems that occur in neurological diseases.

Zusammenfassung

Die bemerkenswerten kognitiven Prozesse, die unser Gehirn bewältigen muss, erfordern ein komplexes und dynamisches Netzwerk von Neuronen, das in der Lage ist, schnell und präzise auf Veränderungen zu reagieren. Um sich an veränderte Umwelteinflüsse anzupassen und Informationen zu speichern, müssen sensorische Informationen als langanhaltende struturelle Veränderungen in unserem Gehirn verankert werden. Auf molekularer Ebene sind hierzu hochentwickelte und streng regulierte Genexpressionsprogramme erforderlich, die die synaptischen Verbindungen in unserem Gehirn verändern und ausbauen, ohne das bereits bestehende Netzwerk zu stören. MicroRNAs sind wichtige Regulatoren in diesem neuronalen Netzwerk, da sie in der Lage sind, Genexpression lokal und präzise zu regulieren. Die kleinen, nicht-kodierenden RNAs binden dabei an komplementäre Sequenzen in Ziel-mRNAs und unterdrücken deren Proteinsynthese. Eine Rolle spielt dies bei der aktivitätsabhängigen Synapsenentwicklung, bei der neue Proteine lokal benötigt werden, um nachhaltige Änderungen der Synapsenstärke herbeizuführen, die die molekulare Grundlage für Prozesse wie Lernen und Gedächtnisbildung sind.

Die vorliegende kumulative Dissertation präsentiert zwei Studien, die sich damit beschäftigen, wie neuronale microRNAs in ihrer Expression und Lokalisierung reguliert werden. Die erste Publikation *"The DEAH-box helicase DHX36 mediates dendritic localization of the neuronal precursor-microRNA-134"* untersucht dabei den Transport von miRNA-134 zu ihrem Wirkungsort an der Synapse. Die Studie beschreibt, dass bereits die Vorstufe der microRNA (die pre-miRNA) an der Synapse lokalisiert ist und identifiziert mit DHX36 ein Protein, das pre-miR-134 spezifisch bindet und für den Transport von Bedeutung ist. Durch Knockdown von DHX36 wird gezeigt, dass die

Lokalisierung von pre-miR-134 im Dendriten von funktioneller Bedeutung ist. Abwesenheit von DHX36 führt so zu einer verstärkten Expression von bekannten miR-134 Zielgenen und einem damit einhergehenden vergrößerten Volumen dendritscher Dornfortsätze, dem postsynaptischen Teil der Synapse.

In der zweiten Studie dieser Arbeit mit dem Titel *"The nuclear matrix protein Matr3 regulates processing of the synaptic microRNA-138-5p"*, wird die Expression der microRNA miR-138 untersucht. Für miR-138 sind zwei verschiedene pre-miRNAs bekannt, pre-miR-138-1 und pre-miR-138-2. In unserer Studie zeigen wir auf, dass pre-miR-138-2 die Vorstufe ist, die hauptsächlich für die Expression der reifen miR-138 in Neuronen verantwortlich ist. Mittels eines Pulldown-Assays identifizieren wir außerdem mit Matr3 ein Protein der nukleären Matrix, das spezifisch an die Haarnadelstruktur des Primärtranskripts pri-/pre-miRNA-138-2 bindet. Hemmung der Matr3 Expression zeigt auf, dass Matr3 die Prozessierung der pri-miR-138-2 im Zellkern hemmt und somit zu einer verminderten Expression von reifer miR-138 führt.

Zusammenfassend beschreibt diese Arbeit neuartige regulatorische Mechanismen für zwei neuronale microRNAs, miR-134 und miR-138. Beide microRNAs spielen eine wichtige Rolle für Prozesse der synaptischen Plastiziät und eine präzise Regulation ihrer Expression ist von großer Bedeutung, um ein stabiles und funktionierendes neuronales Netzwerk aufrechtzuerhalten. Ein größeres Verständnis der Regulation dieser microRNAs und ihrer nachgeschalteten Prozesse stellt daher einen wichtigen und weiteren Schritt dar, um komplexe kognitive Prozesse wie Lernen und Gedächtnis zu verstehen und weitere Einblicke in deren Fehlfunktionen, wie etwa bei neurologischen Erkrankungen, zu erhalten.

1 Introduction

1.1 Synaptic plasticity

The brain is formed by a complex and dynamic network of neurons and glia cells that are highly interconnected. In humans ∼100 trillion synapses are formed between the ∼100 billion neurons and it is therefore no surprise that highly sophisticated regulatory pathways are necessary not only to form this network but also to allow its constant adaptation to an ever-changing environment.

On the level of individual neurons, synaptic plasticity is one of the fundamental processes that controls neuronal network formation and forms the molecular basis of learning and memory. It is defined as the ability of neurons to strengthen and weaken individual synapses based on an increase or decrease in their activity and to thereby modulate their response. A multitude of molecular mechanisms contribute to these plasticity mechanisms that occur at the presynaptic as well as the postsynaptic part of the synapse and include changes of neurotransmitter release and efficiency as well as the responsiveness of the postsynaptic site to these neurotransmitters. To manifest the longer lasting changes in structure and composition of the synapse, translation of new proteins is required (Flavell, Greenberg 2008). Ensuring that this protein expression is occurring not only rapidly in response to changing signaling but also exclusively at the involved synapses is a demanding task that requires specific and sophisticated regulation. A class of molecules that has been shown to play an important role in this regulation of protein expression are microRNAs (miRNAs).

1.2 Biogenesis and function of miRNAs

The ∼22 nucleotide (nt) long miRNAs are non-coding RNAs that bind to complementary sequences mostly within the 3' untranslated regions (3' UTR) of their target messenger RNAs (mRNAs), thereby repressing their translation and/or facilitating mRNA degradation (Filipowicz et al. 2008). Over the last years they have emerged as important post-transcriptional regulators of protein expression in a variety of cellular processes, including cell proliferation, differentiation and metabolism (Bushati, Cohen 2007).

MiRNAs are transcribed by RNA polymerase II either from individual genes, larger gene clusters containing multiple miRNAs or introns of host genes (Lee et al. 2004). These primary transcription products (pri-miRNA) are hundreds to thousands of nucleotides long and fold into at least one hairpin structure that serves as substrate for the "microprocessor" complex consisting of the nuclear type-III RNase Drosha and the RNA-binding protein DGCR8 (DiGeorge Syndrome Critical Region 8). This complex cleaves the pri-miRNA into a ∼70 nt long hairpin with a 2 nt overhang at the 3' end, the so-called precursor miRNA (pre-miRNA) (Lee et al. 2004). The characteristic overhang is then recognized by Exportin-5 and the pre-miRNA is transported out of the nucleus into the cytoplasm (Lund et al. 2004). Here, the pre-miRNA is further cleaved by a complex containing the RNAseIII endonuclease Dicer and TRBP (transactivation-responsive RNA binding protein) into a ∼22 nt long double stranded miRNA duplex (Ha, Kim 2014). For most miRNAs, the so-called guide strand of this duplex (the functional miRNA) is separated from the passenger strand (miRNA*) and loaded into the miRNA induced silencing complex (miRISC), while for some miRNA species both strands are loaded and act as functional miRNAs (Okamura et al. 2008). The miRISC targets sequences complementary to the "seed" (nucleotide 2-7) of the incorporated miRNA which are often located in the 3'UTR of the effected mRNAs and thereby mediates silencing of their translation. The two core components of this complex are one of the members of the Argonaute (AGO) protein family and GW182 (glycine-tryptophan protein of 182 kDa), which acts as a scaffolding protein to further recruit factors that assist in the silencing of translation and the modulation of miRISC activity (Pfaff et al.

2013).

As miRNAs act as important regulators within a complex network, their own expression is heavily regulated throughout all these stages of their life cycle, including transcription (Fiore et al. 2009), pri-miRNA processing (Davis et al. 2010), pre-miRNA processing (Obernosterer et al. 2006), miRISC incorporation (Ashraf et al. 2006, Banerjee et al. 2009) and decay of the mature miRNA (Krol et al. 2010a, Sethi, Lukiw 2009), indicating the importance of their precise spatiotemporal regulation (reviewed by Krol et al. (2010b), Siomi, Siomi (2010) and Trabucchi et al. (2009b)). Impaired miRNA expression has increasingly been associated with neurodevelopmental, neuropsychiatric and neurodegenerative diseases (as reviewed by Bicker, Schratt (2008), Fiore et al. (2011), Wang et al. (2012)), underscoring the importance of the proper regulation of the expression of miRNAs and their targets.

1.3 The role of miR-134 and miR-138 in synaptic plasticity

One of the first neuronal miRNAs that has been shown to fulfill an important role in synaptic plasticity is miR-134. It was found to localize within the dendrites of mature hippocampal neurons, where it locally controls the translation of the mRNA encoding LIM domain kinase 1 (Limk1), a regulator of actin filament dynamics. Thus, by repressing LimK1 expression, miR134 is able to restrict spine size at the resting synapse. Upon stimulation with BDNF (brain-derived neurotrophic factor), a neurotrophin that is released upon synaptic stimulation, the repression is reversed and LimK1 can be expressed locally, allowing for actin remodeling and growth of the stimulated synapses (Schratt et al. 2006). Using atomic force microscopy (AFM), a recent study shows that mir-134 levels actually vary among different types of dendritic spines (Park et al. 2019). More specifically, miR-134 levels are inversely correlated with the maturity of individual spines and local BDNF stimulation was able to reduce miR-134 expression in the respective spines. These findings further indicate the role of miR-134 in the local regulation of individual synapses.

In addition to its function in the regulation of dendritic spine size, miR-134 expression (together with other miRNAs from the miR-379–410 cluster) is induced by BDNF and required for activity-dependent dendritogenesis in hippocampal neurons (Fiore et al. 2009). The RNA-binding protein Pumilio 2 (PUM2) was identified as a key target of miR-134-dependent repression in this context and shown to mediate the effects on dendritogenesis. The role of miR-134 in dendritogenesis is further supported by a study investigating a truncated mRNA transcript of the E3 ubiquitin ligase (Ube3a1) (Valluy et al. 2015). The dendritically localized Ube3a1 mRNA was shown to sequester miR-134 and other miRNAs from the miR379-410 cluster and thereby negatively regulate dendritic outgrowth in neuronal cultures and in the hippocampus of rats *in vivo*. Importantly, Ube3a1 function in dendritogenesis was not dependent on its coding function, but instead required the presence of miR-134, consistent with a role of Ube3a1 as a competing endogenous RNA (ceRNA) for miR-134 and other miRNAs of the miR379-410 cluster. The important function of miR-134 in the brain has been further endorsed by studies describing its role in long-term memory formation (Gao et al. 2010), epilepsy (Jimenez-Mateos et al. 2012) as well as social and anxiety-related behaviour (Lackinger et al. 2019).

Another miRNA extensively studied in relation to synaptic plasticity mechanisms is miR-138. Similar to miR-134, it is located within dendrites and was shown to inhibit spine size and miniature excitatory synaptic transmission (Siegel et al. 2009). These effects of miR-138 expression were shown to be mediated by miR-138-dependent repression of its target gene acyl-protein-thioesterase 1 (APT1), an enzyme that, among other targets, depalmitoylates the Gα_{13} subunits of G proteins thereby promoting their dissociation from the membrane. Decreased APT1 expression and function therefore leads to an increased membrane association of Gα_{13} subunits and activation of the downstream RhoA pathway, which induces actomyosin contraction and spine shrinkage. Additional studies have confirmed these findings and shown that upon NMDA stimulation the RISC component MOV10 is degraded which in turn leads to the local translation of APT1 and other known miRNA targets (Banerjee et al. 2009).

Since these findings emerged, other miR-138 functions and targets have been identified, among these cell migration (Kisliouk, Meiri 2013) and axon regeneration (Liu et al.

2013). In this context, the extracellular matrix glycoprotein Reelin (RELN) has been identified as another direct target of miR-138 repression (Liu et al. 2013). RELN is well-known for its role in neuronal migration in the developing brain and the formation of cell–cell contacts (Lakatosova, Ostatnikova 2012, Stranahan et al. 2013) and has been associated with a variety of different neurological diseases such as Alzheimer's disease (Herring et al. 2012), Schizophrenia (Verbrugghe et al. 2012) and bipolar disorder (Goes et al. 2010). Since miR-138 levels have been frequently found to be misregulated in some of these diseases (Bicker et al. 2014), it is tempting to speculate that perturbed miR-138 activity might be causally involved in defective RELN expression observed in these diseases. Further evidence for an important functional role of miR-138 in the neuronal network is provided by a genome wide screening for single nucleotide polymorphisms (SNPs) associated with memory performance in humans. The study identified a SNP leading to alterations in miR-138 expression as well as a SNP in the miR-138 binding site of the DCP1B 3'UTR, consistent with a role of miR-138 in the regulation of long-term memory formation (Schröder et al. 2014).

Overall both miRNA have been shown to exhibit important functions in mechanism of synaptic plasticity and thereby in forming and maintaining a functional and stable neuronal network that is dynamically regulated. A more comprehensive summary of miR-134 and miR-138 expression and their implications in brain function and disease can be found in Bicker et al. (2014).

1.4 Aims of this thesis

The role of miRNAs as regulators of spatiotemporal protein translation in synaptic plasticity has been well established since their initial discovery. More recent studies showing miRNA misregulation in a variety of neurological diseases further emphasize their importance in neuronal network function and homeostasis. They are highly abundant in the mammalian brain (McNeill, van Vactor 2012) and due to their mode of function, each miRNA is able to target a large subset of mRNAs. Each mRNA can in turn be regulated by a variety of different miRNAs. It is therefore not surprising that miRNAs

themselves are subject to a tight regulation. In this thesis, I present two studies that both aim to investigate how miRNAs themselves are subject to regulatory processes.

The first project I collaborated in, investigates the enrichment of miR-134 in dendrites. Data from previous studies has demonstrated that not only the mature form of miR-134 is present at synaptic sites, but also its precursor form, which is located and enriched along the dendrites. There, it potentially provides a source for mature miRNA that can be readily made available at specific synaptic sites. This study therefore investigated a novel mechanism that facilitates dendritic transport of precursor miRNA and its role in the regulation of synaptic spine size.

For the main study presented in this thesis, I aimed to gain insight into the cell-type specific biogenesis of miR-138. MiR-138 is a miRNA that originates from two distinct genomic loci. Whereas one of its precursor forms can be found throughout a variety of different tissues, the mature form seems to be specific to the brain. I therefore investigated the role of both precursor forms and their contribution to the pool of mature miR-138 and how post-transcriptional regulatory mechanisms allow miR-138 to be expressed in neurons, but not other cell types.

2 Summary of publications

2.1 The DEAH-box helicase DHX36 mediates dendritic localization of the neuronal precursor-microRNA-134

2.1.1 Scientific summary

As previously discussed, miR-134 has been shown to be located in the dendritic compartment of neurons and to play an important role in processes involved in synaptic plasticity. The present publication, I collaborated on, aimed to investigate how miR-134 reaches its destination in the dendrite and performs its function in local protein synthesis at the synapse.

Interestingly, initial experiments showed that not only the mature miR-134, but also pre-miR-134, localizes to synaptic sites. Pre-miR-134 is significantly enriched in RNA derived from synaptosome preparations compared to RNA from whole brain, as shown by qRT-PCR. Enrichment levels for pre-miR-134 were comparable to the previously known dendritic RNA BC1 and significantly higher compared to other neuronal pre-miRNAs, demonstrating the specificity of the effect (Fig.1 A, Bicker et al. (2013)). Additional experiments using fluorescent in situ hybridization (FISH) with loop-specific probes (Fig. 1 B, C) and compartmentalized hippocampal cultures (Fig. 1D) further confirmed that pre-miR-134 is specifically localized to dendrites.

Our study then focused on determining which part of the pre-miR-134 sequence is responsible for its dendritic localization. Pre-miRNAs were *in vitro* transcribed and fluorescently labeled with Cy3, before being transfected into rat hippocampal neurons.

Localization of pre-miR-134 and the control pre-miR-150 resembled the earlier findings of a specific localization of pre-miR-134 but not pre-miR-150 in dendrites (Fig. 2 A, B, C). This effect could be reversed when the loop region was exchanged between the two pre-miRNAs. Carrying the miR-134 loop region, the stem of miR-150 now showed an increased dendritic presence, while the chimera of a pre-miR-134 stem with the loop of pre-miR-150 localized mostly within the cell body (Fig. 2 D, E). These results together, suggested that the loop region of pre-miR-134 is the main determinant of pre-miR-134 localization.

We reasoned that specific RNA binding proteins (RBPs) would recognize the sequence of the pre-miR-134 loop to promote dendritic transport. To identify these RBPs we performed pull-down experiments. Therefore, streptavidin beads coated with in vitro biotin-tagged synthetic pre-miR-134 or control sequences were incubated with whole brain protein extracts (Fig. 3 A). Analysis of the associated proteins on a coomassie gel revealed a band around 110 kDa that could be exclusively detected when pre-miR-134 coupled beads were used (Fig. 3 B). Mass spectrometry identified this protein as DHX36. The specific interaction of DHX36 with pre-miR-134 was further confirmed by Western Blot (Fig. 3 C, D, E). To further characterize the interaction between DHX36 and pre-miR-134, in vitro pre-miRNA cleavage assays were preformed that showed reduced cleavage of synthetic pre-miR-134 by recombinant Dicer in the presence of DHX36, an effect that could not be observed for cleavage of pre-miR-150 (Fig. 3 F, G). Since DHX36 binds to the pre-miR-134 loop and interferes with further Dicer-dependent processing, we next sought to determine the effect of DHX36 on the transport of pre-miR-134. We performed shRNA-based knockdown of DHX36 (Fig. 4 C) followed by the visualization of transfected Cy3-labeled pre-miRNAs in hippocampal neurons. The dendritic localization of pre-miR-134 was significantly reduced in DHX36 knockdown neurons compared to control-transfected cells (Fig. 4 D), indicating that DHX36 is necessary for pre-miR-134 localization to dendrites.

Finally, we investigated the impact of DHX36 knockdown on miR-134 function. As previously described, miR-134 negatively regulates dendritic spine size by repressing the local synthesis of LIMK1 in dendrites. We therefore assessed luciferase reporter gene activity of a construct that contains a perfect miR-134-binding site in the context

of the Limk1 3' UTR. Reporter gene levels were increased significantly upon DHX36 knockdown (Fig. 5 A), suggesting that DHX36 positively controls miR-134 function. A similar effect was observed using a Pum2 3'UTR reporter gene, another known miR-134 target gene (Fig. 5 B). Having shown that DHX36 inhibits LIMK1 by promoting miR-134 activity, we went on to study its role in the regulation of dendritic spine size, a process known to be regulated by miR-134 mediated repression of LIMK1. Using confocal microscopy of dendrites from neurons co-transfected with GFP (Fig. 5 C), we found that DHX36 knockdown significantly increased the average size of dendritic spines (Fig. 5 D) consistent with our previous results that showed a positive role of DHX36 in the control of miR-134 function.

Taken together, our study elucidated a novel pathway consisting of DHX36 and pre-miR-134 that ensures spatiotemporal control of miR-134 activity and dendritic spine morphogenesis. As miRNAs are important regulators of protein translation, implementing a correct subcellular distribution of pre-miRNAs is important to allow them to fulfill their role in the precise spatiotemporal regulation of local translation mechanisms that are important for synaptic plasticity.

2.1.2 Description of own contribution

For the described publication, I personally contributed experiments showing the role of DHX36 in the regulation of miR-134 activity. I performed the analysis of spine shape and size upon shRNA-mediated knockdown of DHX36 in hippocampal neurons using confocal microscopy **(Fig. 5 C and D)**.

2.2 The nuclear matrix protein Matr3 regulates processing of the synaptic microRNA-138-5p

2.2.1 Scientific summary

MiRNA-138 is another neuronal miRNA that has been studied in the context of synaptic plasticity and that has been implicated in a variety of neuronal processes. Intriguingly, it has been found that one of the two precursor forms (pre-miR-138-2) that gives rise to mature miR-138 is ubiquitously expressed in a variety of tissues, despite the fact that mature miR-138 expression is mostly restricted to the brain. This led to the hypothesis, that miR-138 processing is regulated at the post-transcriptional level (Obernosterer et al. 2006), possibly involving an inhibitory activity that blocks pre-miR-138-2 processing in non-neuronal cells. This study followed up on this hypothesis and tried to identify post-transcriptional regulatory mechanisms of miR-138 biogenesis and their potential role in synaptic plasticity.

Mature miR-138 can be derived from two precursor forms, pri/pre-miR-138-1 and pri/pre-miR-138-2 which are transcribed from distinct loci in the genome. While both carry the identical sequence for the mature miR-138, they differ in the sequence and size of their loops and other surrounding sequences that are relevant for regulated processing. I initially determined the expression of both precursors as well as the mature miR-138 in developing hippocampal neurons in culture by qRT-PCR. Younger neurons show a low expression of mature miR-138 initially, which increases during the second week of development when synapses are formed in culture (Weiss et al. (2019), Fig. 1 A), consistent with the role of miR-138 in the regulation of synaptic spine size (Siegel et al. 2009). At the level of the precursor, pri/pre-miR-138-1 was mostly undetectable throughout development, while pri/pre-miR-138-2 expression could be detected throughout the developmental time course of the experiment (Fig. 1 B). However, in contrast to the observed increasing levels of mature miR-138, pri/pre-miR-138-2 expression decreased during development. To further investigate differential processing of the two precursors, I *in vitro* transcribed both pre-miRNAs and transfected them into cultured neurons of different developmental stages to determine the amount of

processed mature miR-138 by qRT-PCR. In younger neurons (DIV 0), where endogenous levels of miR-138 are low, transfection of both pre-miRs led to a comparable increase in mature miR-138 levels, indicating that both precursors are processed with similar efficiency (Fig. 1 C). However, more mature neurons (DIV 12) preferentially processed pre-miR-138-2 leading to higher expression of mature miR-138 when the respective pre-miR was transfected compared to pre-miR-138-1 (Fig. 1 D). This finding is consistent with the fact that pre-miR-138-2 also showed a stronger interaction with Dicer in pull-down experiments (Fig. 1 E). Overall this set of experiments indicated that pre-miR-138-2 is the predominant source for mature mir-138 in neurons and that increased processing efficacy is at least partially responsible for the increased levels of miR-138 in older neurons.

Next, we sought to determine whether differential processing of precursors also occurs already at the level of the pri-miRNA. I constructed luciferase reporter constructs containing a fragment of the respective primary miR-138 transcript, which included the pre-miRNA hairpin structure and 100-130 nt flanking region on each side. Drosha-dependent pri-miRNA cleavage would be expected to lead to a loss of the luciferases 3'UTR and polyA-tail, which destabilizes the mRNA and decreases reporter expression. We found that the pri-miR-138-2 reporter had significantly reduced expression (and thus an increased processing rate) compared to the pri-miR-138-1 reporter, while a chimera in which only the loop of pri-miR-138-2 was replaced with the pri-miR-138-1 loop resembled the expression of pri-miR-138-1 (Fig. 2 A). These findings imply that, similar to our observations with pre-miR-138-2, pri-miR-138-2 is more efficiently processed compared to pri-miR-138-1, and that the pri-miR-138-2 loop structure is primarily responsible for increased processing efficiency.

Having determined the loop of the pri/pri-miRNA hairpin structure as an important sequence element that plays a role in processing, we wanted to find and characterize interacting proteins that specifically bind to the loop of pri/pre-miR-138-2. We performed pull-down experiments with the help of streptavidin-coated agarose beads to which in vitro synthesized pre-miRNAs were attached via biotinylated adapter oligonucleotides and which were incubated with neuronal cell lysates. As previous studies had already described potential pre-miR-138-2 binding proteins (Treiber et al. 2017), we

initially investigated two promising candidates from that list, Y box binding protein 1 (YB1) and Matrin3 (Matr3). For both proteins we detected binding to pre-miR-138-2, but only Matr3 showed specificity for pre-miR-138-2, whereas YB1 associated with all pre-miRNAs tested (Fig 2 B). Due to the specific nature of Matr3 binding to the pre-miR-138-2 loop, we decided to investigate its role in miRNA processing in further detail. Matr3 contains four distinct RNA binding domains, among them two zinc finger domains (ZnF1 and ZnF2) and two RNA recognition motifs (RRM1 and RRM2). To assess the binding of Matr3 to the pre-miR-138-2 loop further, we expressed wildtyp (wt) Matr3 and Matr3 deletion mutants lacking the individual binding domains in HEK293 cells, prepared protein extracts and used them for pre-miRNA pull-down assays as described. The deletion of the ZnF2 domain showed the biggest and most consistent reduction in binding to the pre-miR-138-2 loop, suggesting that this domain is required for Matr3 pre-miR-138-2 interation. In contrast, both RRM1 and RRM2 deletions showed slightly reduced binding whereas the ZnF1 deletion even slightly enhanced binding, indicating that these domains are likely not involved in the Matr3/pre-miR-138-2 interaction (Fig. 3 A).

Based on previous studies from non-neuronal cells, Matr3 is constituent of the nuclear matrix and functions in the regulation of nucleotide metabolism in the nucleus (Coelho et al. 2016). However, miRNA processing is a multi-step process that takes place not only within the nucleus, but also in the cytoplasm. To obtain more insight into possible roles of Matr3 in neurons, we first determined its spatiotemporal expression in developing neurons. Using qRT-PCR, we found that Matr3 is expressed throughout neuronal development with levels increasing over time (Fig. 3 B). Next, we studied the subcellular localization of Matr3 by immunocytochemistry (Fig. 3 C) and biochemical fractionations followed by Western Blot (Fig. 3 D). In accordance with previous studies, we found that Matr3 was expressed exclusively within the nucleus, suggesting that in the context of miRNA biogenesis, Matr3 likely participates in early steps (processing/nucleo-cytoplasmic shuttling) that involve the nuclear compartment.

To investigate the effect of Matr3 on pri-miR-138-2 processing, I generated specific shRNAs to knockdown Matr3 expression in cultured rat cortical cells (Fig. 4 A). When co-transfected with the constructs of the previously described pri-miRNA processing

assay (Fig. 2 A), we observed that Matr3 knockdown further increased processing efficiency of the pri-miR-138-2 construct, but not pri-miR-138-1 or the pri-miR-138-2loop1 chimera (Fig. 4 B). This indicates that binding of Matr3 specifically inhibits pri-miR-138-2 processing in neurons. However, as shown before (Fig. 2 A), processing of pri-miR-138-2 in the presence of Matr3 is generally much more efficient in neurons compared to pri-miR-138-1, suggesting that other factors contribute to the regulation of pri-miR-138-2 processing.

As described above, cells of various other tissues, while containing pre-miR-138-2 precursor, lack mature miR-138 expression. We therefore hypothesized that deleting Matr3 from non-neuronal cells should relieve the miR-138 processing block, thereby leading to ectopic expression of miR-138 in such cells. To test this, we used a stable HEK293 cell line that lacks the Matr3 open reading frame via CRISPR/Cas9-mediated knockdown (Matr KO cells). After validating that Matr3 is indeed not expressed in the KO cells (Fig. 4 C), we compared mature miR-138 levels (Fig. 4 D) and pri/pre-miR-138-2 levels (Fig. 4 E) between Matr3 KO and control (ctr)- cell lines by qRT-PCR. Indeed, we found highly increased miR-138 levels in HEK293 cells lacking Matr3, while pre-miR-138-2 levels showed a (non-significant) trend towards a decrease. Lastly, we transfected previously described pri-miR-138-2 constructs into Matr3 KO and ctr cells and measured the resulting mature miR-138 expression by qRT-PCR (Fig. 4 F). Here, we observed a significant increase of mature miR-138 expression in Matr3 KO cells, an effect that could be reversed by plasmid-based expression of Matr3. This result indicates that the lack of Matr3 in these cells is responsible for exaggerated miR-138-2 processing. Importantly, this effect was specific to the pri-miR-138-2 construct since transfection of the pri-miR-138-2loop1 chimera did not cause an increase in miR-138 levels (Fig. 4 G).

Taken together, we could identify a novel regulatory mechanism of miR-138 processing in neurons involving the nuclear matrix protein Matr3. While further studies will be necessary to identify the scope of this regulation and its functional importance, the already established roles of miR-138 and Matr3 in neurons indicate that this mechanism might be of importance in activity-dependent neuronal development, synaptic plasticity and memory-related processes.

2.2.2 Description of own contribution

I am the first author of this publication and performed all the experiments whose results were included in the paper. The Matr3 KO and control cells used in Fig. 4 C-G were generated and kindly provided for experiments in the laboratory of our collaborator G. Meister (University of Regensburg).

3 Discussion

3.1 Dendritic transport of pre-miR-134 and local miRNA processing

MiR-134 has been one of the first neuronal miRNAs for which a role in the local translation of synaptic proteins has been established (Schratt et al. 2006). Our study now demonstrates that not only mature miR-134 is present at the synapse but also its precursor pre-miR-134 is localized in the synapto-dendritic compartment. The enrichment in synaptosome preparations was only observed for pre-miR-134 and a subset of other pre-miRNAs, while other neuronal pre-miRNAs were depleted from the synaptic compartment, indicating that the dendritic localization is achieved by an active transport mechanism rather than a passive diffusion effect.

Subsequently, the helicase DHX36 was identified to interact specifically with the pre-miR-134 loop. DHX36 is a RNA helicase that belongs to the DEAH-box protein family. It contains the evolutionary conserved helicase core region and a unique N-terminus that has been shown to be critical for mRNA binding and that mediates its re-localization from the nucleus into stress granules (Chalupníková et al. 2008). In accordance with this data, we found that binding of DHX36 to pre-miR-134 also occurs via the same domain as demonstrated by the electrophoretic mobility shift assay (EMSA) using the isolated N-terminal domain (Supplemental Fig. S8, Bicker et al. (2013)). We further found that DHX36 binding to pre-miR-134 interferes with Dicer processing, indicating that they might compete for the same binding site. Previous studies have already shown the presence of DHX36 in AGO-2-containing protein complexes and importantly have demonstrated that DHX36 is present in a subset of complexes that do not contain Dicer

(Höck et al. 2007). It can therefore be hypothesized that DHX36 binding protects pre-miR-134 from premature Dicer processing in the cell body and during transport to the synapto-dendritic compartment.

The presence of unprocessed and thus inactive pre-miRNA at the synapse suggests that this pool of pre-miRNA can be made available locally at specific synapses upon activation of pre-miRNA processing. This of course requires that, in addition to the pre-miRNA(s), also the miRNA biogenesis machinery responsible for pre-miRNA processing is located at synaptic sites. Dicer has previously been found to be present at the synapse (Lugli et al. 2005) and a more recent study actually demonstrated that pre-miRNA processing into mature miRNA can indeed occur locally at stimulated synapses and thereby lead to the spatially restricted inhibition of target protein translation at these activated synapses (Sambandan et al. 2017). These findings are consistent with our observation that knockdown of DHX36 and the resulting reduced dendritic localization of pre-miR-134 leads to a decrease in miR-134 activity, as demonstrated by an increase in target gene expression and the effect on spine volume that mimics miR-134 loss of function (Fig. 5, Bicker et al. (2013)).

Since changes in synapse morphology are often required in response to changes in activity, it is not surprising that miRNAs which are involved in synaptic plasticity processes are themselves subject to activity-dependent regulation. For miR-134 it has previously been shown that the transcriptional activation of the miR379-410 cluster, which among 38 other miRNAs contains miR-134, is regulated by BDNF signaling via the transcription factor Mef2 (Myocyte enhancing factor 2). In the context of Mef2-induced miR-134 expression, the miRNA has been shown to promote activity-dependent dendritogenesis by regulation the translation of the RNA-binding protein PUM2 (Fiore et al. 2009). In addition, in the study that first described a function of miR-134 in the translational repression of LIMK1 at the synapse, it was further demonstrated that the miR-134-mediated repression of translation can be relieved upon BDNF stimulation (Schratt et al. 2006). Since the publication of this study, it has therefore also been investigated whether the DHX36-mediated transport of pre-miR-134 is subject to an activity-dependent regulation. Zampa et al. (2018) found that BDNF promotes the dendritic localization of pre-miR-134 and demonstrated that activation of NMDA

receptors (NMDAR) is required in this process. Notably, application of the NMDAR antagonist APV reduced pre-miR-134 localization in the dendrite without altering pre-miR-134 transcription, indicating that NMDAR activation is specifically required for pre-miR-134 transport. Additionally, APV treatment and DHX36 knockdown both antagonized the BDNF- and miR-134-mediated repression of the dendritically localized Pum2 and dendritic outgrowth. This effect was likely due to reduced pre-miR-134 dendritic localization, since elevating dendritic miR-134 levels either via transfection of miR-134 duplex RNA or a pre-miR-134 RNA that could bypass DHX36 mediated transport, alleviated the repression of dendritic outgrowth (Zampa et al. 2018).

Taken together, the DHX36-mediated localization of pre-miR-134 that we identified in our publication, has important implications for miR-134 function in the larger picture of activity-dependent regulation of synaptic plasticity.

3.2 Regulation of nuclear pri-/pre-miR-138 processing

MiR-138 has two distinct precursor forms (miR-138-1 and miR-138-2) that are transcribed from different loci in the genome. In our study we investigated the contribution of both pri- and pre-miRNAs to miR-138 expression and found that precursors originating from the miR-138-2 gene are the main source of miR-138 expression in rat cortical neurons. This is in agreement with a previous study that performed Northern blots on brain tissues and was able to detect a robust expression of pre-miR-138-2, whereas pre-miR-138-1 was undetectable (Obernosterer et al. 2006). However, expression of pre-miR-138-1 has been reported in other studies that focused on oligodendrocyte differentiation (Dugas et al. 2010) or axon regeneration in young mouse cortical neurons (Liu et al. 2013). The latter study further demonstrated that miR-138-1 transcription is regulated via a negative feedback loop involving its own target Sirtuin-1 (SIRT1). Together, these results suggest that the different miR-138 precursors can be utilized for miR-138 production in a developmental stage- and cell-type-specific manner.

We observed not only differences in the transcription of miR-138-1 and -2, but also found differential processing of the two pri-miRNAs that seemed mostly dependent

on their terminal loop. The importance of various structure and sequence elements of pri-miRNAs for their subsequent processing into pre-miRNA has been described in several studies. Multiple features have been identified that enhance cleavage by the microprocessor complex. These features include the hairpin stem length and bulge positions within the stem (Roden et al. 2017), as well as sequence motifs near the Drosha cleavage sites (Rouleau et al. 2018) and downstream of the hairpin (Auyeung et al. 2013), among others. In addition, RBPs have been identified that can either enhance or inhibit the processing of specific miRNAs. Binding of Lin-28 to the loop region of pri-let7 was found to inhibit Drosha processing (Viswanathan, Daley 2010), whereas KSRP binding to the same loop of let-7 leads to an increase in processing (Trabucchi et al. 2009a), allowing for a precise regulation of miRNA expression.

Our study identified Matr3 as a negative regulator of miR-138-2 processing. Matr3 is an RBP located in the nuclear matrix and is part of the SFPQ-NONO complex (Salton et al. 2010). It is involved in mRNA quality control by retaining defective RNAs in the nuceleus (DeCerbo, Carmichael 2005). Furthermore, it functions as a co-factor of Rev, a protein that controls the export of partially spliced and unspliced viral RNAs from the nucleus in the context of HIV-1 (Kula et al. 2011). Given the described roles of Matr3 in export mechanisms, we aimed to understand whether Matr3's repressive effect on miR-138 processing might be mediated by pre-miR-138-2 retention in the nucleus. We conducted some initial experiments comparing the efficiency of export of pre-miR-138-1 and pre-miR-138-2 in a Xenopus laevis oocyte export assays, but could not observe a significant difference (in collaboration with T. Ziegenhals and U. Fischer, unpublished). Nevertheless it is tempting to speculate that the retention of pre-miRNA in the nucleus by Matr3 could be a potential mechanism to regulate miRNA expression under more specific conditions. While data from several publications and this thesis argue for a nuclear function of Matr3, a C-terminally truncated isoform of Matr3 that localizes to mRNA processing bodies in the cytoplasm has been described (Rajgor et al. 2016). Interestingly, this cytoplasmic form of Matr3 was shown to be involved in the regulation of miRISC function, raising the possibility that regulation of miR-138 via Matr3 might also occur at later stages of miR-138 maturation which take place in the cytoplasm.

Consistent with the established role of miR-138 as a repressor of dendritic spine growth, mir-138 has been shown to be negatively regulated by neuronal activity. For example, KCl-mediated depolarization of hippocampal cultures reduces miR-138 expression (Siegel et al. 2009). Similarly, electroconvulsive shock treatment led to a decrease of miR-138 levels in the hippocampus of mice (Eacker et al. 2011). Reduced miR-138 levels in the dorsal hippocampus have also been described after contextual fear conditioning (CFC) (Li et al. 2018). In addition, the same study showed that manipulating miR-138 expression using knockdown and overexpression approaches enhanced or impaired memory formation in CFC, respectively, emphasizing that that the regulation of miR-138 expression is functionally important in synaptic plasticity. It is therefore noteworthy that also Matr3 has been shown to be degraded in neurons in response to NMDAR activation in a PKA-dependent fashion (Giordano et al. 2005). Degradation of Matr3 could thereby also lead to a relief of the repression of miR-138 processing and thus enable the synthesis of more mature miR-138. Although these observations seem contradictory at first glance, activity-dependent inhibition and promotion of miR-138 activity could operate at different time scales, e.g. those related to Hebbian (few hours) and homeostatic (several hours to days) forms of synaptic plasticity. Thus, future studies will have to investigate if and how Matr3-mediated repression of miR-138 processing is regulated by neuronal activity, and what might be the functional implications.

Our study found that the ZnF2 domain is particularly important for the binding of Matr3 to the terminal loop structure of pri-/pre-miR-138-2. Notably, the effect of the different Matr3 RNA binding domains has recently been studied regarding Matr3-mediated neurotoxicity in the context of amyotrophic lateral sclerosis (ALS), and it was shown that the ZnF2 domain modulates Matr3-overexpression-related toxicity (Malik et al. 2018). This correlation of miR-138-2 binding capacity and the involvement of the domain in neurotoxicity raises the possibility that dysregulation of mir-138 maturation might play a role in ALS. In the context of ALS, Matr3 has also been shown to interact with TDP-43 (Johnson et al. 2014). TDP-43 was identified as a major disease protein in ALS and has been extensively studied in this context (Prasad et al. 2019). Interestingly, TDP-43 has also been reported to play a role in miRNA biogenesis, binding to the loops of several miRNAs and regulating their nuclear and cytoplasmic processing by Drosha

and Dicer, respectively (Kawahara, Mieda-Sato 2012). A connection of miR-138 to ALS might be further supported by miR-138's antagonizing role in axon regeneration (Liu et al. 2013) and miR-138 being the second most abundant miRNA in motor neurons (Amin et al. 2015). Further studies will however be necessary to investigate a potential involvement of the Matr3-miR-138 interaction in ALS. A first step could be to test whether any of the ALS-associated Matr3 point mutations (Johnson et al. 2014) affect the association between Matr3 and pri-miR-138-2.

In conclusion, the presented study provides insight into miR-138 biogenesis and identifies Matr3 as a novel regulator of miRNA processing. Since both miR-138 and Matr3 emerge as important factors in cognition and neurological diseases, a more detailed understanding of this regulatory mechanism could provide important insight into learning, memory and neurodegeneration.

References

Amin Neal D., Bai Ge, Klug Jason R., Bonanomi Dario, Pankratz Matthew T., Gifford Wesley D., Hinckley Christopher A., Sternfeld Matthew J., Driscoll Shawn P., Dominguez Bertha, Lee Kuo-Fen, Jin Xin, Pfaff Samuel L. Loss of motoneuron-specific microRNA-218 causes systemic neuromuscular failure // Science (New York, N.Y.). 2015. 350, 6267. 1525–1529.

Ashraf Shovon I., McLoon Anna L., Sclarsic Sarah M., Kunes Sam. Synaptic protein synthesis associated with memory is regulated by the RISC pathway in Drosophila // Cell. 2006. 124, 1. 191–205.

Auyeung Vincent C., Ulitsky Igor, McGeary Sean E., Bartel David P. Beyond secondary structure: primary-sequence determinants license pri-miRNA hairpins for processing // Cell. 2013. 152, 4. 844–858.

Banerjee Sourav, Neveu Pierre, Kosik Kenneth S. A coordinated local translational control point at the synapse involving relief from silencing and MOV10 degradation // Neuron. 2009. 64, 6. 871–884.

Bicker Silvia, Khudayberdiev Sharof, Weiß Kerstin, Zocher Kathleen, Baumeister Stefan, Schratt Gerhard. The DEAH-box helicase DHX36 mediates dendritic localization of the neuronal precursor-microRNA-134 // Genes & development. 2013. 27, 9. 991–996.

Bicker Silvia, Lackinger Martin, Weiß Kerstin, Schratt Gerhard. MicroRNA-132, -134, and -138: a microRNA troika rules in neuronal dendrites // Cellular and molecular life sciences : CMLS. 2014. 71, 20. 3987–4005.

Bicker Silvia, Schratt Gerhard. microRNAs: tiny regulators of synapse function in development and disease // Journal of cellular and molecular medicine. 2008. 12, 5A. 1466–1476.

Bushati Natascha, Cohen Stephen M. microRNA functions // Annual review of cell and developmental biology. 2007. 23. 175–205.

Chalupníková Katerina, Lattmann Simon, Selak Nives, Iwamoto Fumiko, Fujiki Yukio, Nagamine Yoshikuni. Recruitment of the RNA helicase RHAU to stress granules via a unique RNA-binding domain // The Journal of biological chemistry. 2008. 283, 50. 35186–35198.

Coelho Miguel B., Attig Jan, Ule Jernej, Smith Christopher W. J. Matrin3: connecting gene expression with the nuclear matrix // Wiley interdisciplinary reviews. RNA. 2016. 7, 3. 303–315.

Davis Brandi N., Hilyard Aaron C., Nguyen Peter H., Lagna Giorgio, Hata Akiko. Smad proteins bind a conserved RNA sequence to promote microRNA maturation by Drosha // Molecular cell. 2010. 39, 3. 373–384.

DeCerbo Joshua, Carmichael Gordon G. Retention and repression: fates of hyperedited RNAs in the nucleus // Current opinion in cell biology. 2005. 17, 3. 302–308.

Dugas Jason C., Cuellar Trinna L., Scholze Anja, Ason Brandon, Ibrahim Adiljan, Emery Ben, Zamanian Jennifer L., Foo Lynette C., McManus Michael T., Barres Ben A. Dicer1 and miR-219 Are required for normal oligodendrocyte differentiation and myelination // Neuron. 2010. 65, 5. 597–611.

Eacker Stephen M., Keuss Matthew J., Berezikov Eugene, Dawson Valina L., Dawson Ted M. Neuronal activity regulates hippocampal miRNA expression // PloS one. 2011. 6, 10. e25068.

Filipowicz Witold, Bhattacharyya Suvendra N., Sonenberg Nahum. Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight? // Nature reviews. Genetics. 2008. 9, 2. 102–114.

Fiore Roberto, Khudayberdiev Sharof, Christensen Mette, Siegel Gabriele, Flavell Steven W., Kim Tae-Kyung, Greenberg Michael E., Schratt Gerhard. Mef2-mediated transcription of the miR379-410 cluster regulates activity-dependent dendritogenesis by fine-tuning Pumilio2 protein levels // The EMBO journal. 2009. 28, 6. 697–710.

Fiore Roberto, Khudayberdiev Sharof, Saba Reuben, Schratt Gerhard. MicroRNA function in the nervous system // Progress in molecular biology and translational science. 2011. 102. 47–100.

Flavell Steven W., Greenberg Michael E. Signaling mechanisms linking neuronal activity to gene expression and plasticity of the nervous system // Annual review of neuroscience. 2008. 31. 563–590.

Gao Jun, Wang Wen-Yuan, Mao Ying-Wei, Gräff Johannes, Guan Ji-Song, Pan Ling, Mak Gloria, Kim Dohoon, Su Susan C., Tsai Li-Huei. A novel pathway regulates memory and plasticity via SIRT1 and miR-134 // Nature. 2010. 466, 7310. 1105–1109.

Giordano Gennaro, Sánchez-Pérez Ana María, Montoliu Carmina, Berezney Ronald, Malyavantham Kishore, Costa Lucio G., Calvete Juan José, Felipo Vicente. Activation of NMDA receptors induces protein kinase A-mediated phosphorylation and degradation of matrin 3. Blocking these effects prevents NMDA-induced neuronal death // Journal of neurochemistry. 2005. 94, 3. 808–818.

Goes F. S., Willour V. L., Zandi P. P., Belmonte P. L., MacKinnon D. F., Mondimore F. M., Schweizer B., DePaulo J. R., Gershon E. S., McMahon F. J., Potash J. B. Sex-specific association of the Reelin gene with bipolar disorder // American journal of medical genetics. Part B, Neuropsychiatric genetics : the official publication of the International Society of Psychiatric Genetics. 2010. 153B, 2. 549–553.

Ha Minju, Kim V. Narry. Regulation of microRNA biogenesis // Nature reviews. Molecular cell biology. 2014. 15, 8. 509–524.

Herring Arne, Donath Anja, Steiner Katharina M., Widera Manuel P., Hamzehian Samira, Kanakis Dimitrios, Kölble Konrad, ElAli Ayman, Hermann Dirk M., Paulus

Werner, Keyvani Kathy. Reelin depletion is an early phenomenon of Alzheimer's pathology // Journal of Alzheimer's disease : JAD. 2012. 30, 4. 963–979.

Höck Julia, Weinmann Lasse, Ender Christine, Rüdel Sabine, Kremmer Elisabeth, Raabe Monika, Urlaub Henning, Meister Gunter. Proteomic and functional analysis of Argonaute-containing mRNA-protein complexes in human cells // EMBO reports. 2007. 8, 11. 1052–1060.

Jimenez-Mateos Eva M., Engel Tobias, Merino-Serrais Paula, McKiernan Ross C., Tanaka Katsuhiro, Mouri Genshin, Sano Takanori, O'Tuathaigh Colm, Waddington John L., Prenter Suzanne, Delanty Norman, Farrell Michael A., O'Brien Donncha F., Conroy Ronán M., Stallings Raymond L., DeFelipe Javier, Henshall David C. Silencing microRNA-134 produces neuroprotective and prolonged seizure-suppressive effects // Nature medicine. 2012. 18, 7. 1087–1094.

Johnson Janel O., Pioro Erik P., Boehringer Ashley, Chia Ruth, Feit Howard, Renton Alan E., Pliner Hannah A., Abramzon Yevgeniya, Marangi Giuseppe, Winborn Brett J., Gibbs J. Raphael, Nalls Michael A., Morgan Sarah, Shoai Maryam, Hardy John, Pittman Alan, Orrell Richard W., Malaspina Andrea, Sidle Katie C., Fratta Pietro, Harms Matthew B., Baloh Robert H., Pestronk Alan, Weihl Conrad C., Rogaeva Ekaterina, Zinman Lorne, Drory Vivian E., Borghero Giuseppe, Mora Gabriele, Calvo Andrea, Rothstein Jeffrey D., Drepper Carsten, Sendtner Michael, Singleton Andrew B., Taylor J. Paul, Cookson Mark R., Restagno Gabriella, Sabatelli Mario, Bowser Robert, Chiò Adriano, Traynor Bryan J. Mutations in the Matrin 3 gene cause familial amyotrophic lateral sclerosis // Nature neuroscience. 2014. 17, 5. 664–666.

Kawahara Yukio, Mieda-Sato Ai. TDP-43 promotes microRNA biogenesis as a component of the Drosha and Dicer complexes // Proceedings of the National Academy of Sciences of the United States of America. 2012. 109, 9. 3347–3352.

Kisliouk T., Meiri N. MiR-138 promotes the migration of cultured chicken embryonic hypothalamic cells by targeting reelin // Neuroscience. 2013. 238. 114–124.

Krol Jacek, Busskamp Volker, Markiewicz Ilona, Stadler Michael B., Ribi Sebastian, Richter Jens, Duebel Jens, Bicker Silvia, Fehling Hans Jörg, Schübeler Dirk, Oertner Thomas G., Schratt Gerhard, Bibel Miriam, Roska Botond, Filipowicz Witold. Characterizing light-regulated retinal microRNAs reveals rapid turnover as a common property of neuronal microRNAs // Cell. 2010a. 141, 4. 618–631.

Krol Jacek, Loedige Inga, Filipowicz Witold. The widespread regulation of microRNA biogenesis, function and decay // Nature reviews. Genetics. 2010b. 11, 9. 597–610.

Kula Anna, Guerra Jessica, Knezevich Anna, Kleva Danijela, Myers Michael P., Marcello Alessandro. Characterization of the HIV-1 RNA associated proteome identifies Matrin 3 as a nuclear cofactor of Rev function // Retrovirology. 2011. 8. 60.

Lackinger Martin, Sungur A. Özge, Daswani Reetu, Soutschek Michael, Bicker Silvia, Stemmler Lea, Wüst Tatjana, Fiore Roberto, Dieterich Christoph, Schwarting Rainer Kw, Wöhr Markus, Schratt Gerhard. A placental mammal-specific microRNA cluster acts as a natural brake for sociability in mice // EMBO reports. 2019. 20, 2.

Lakatosova Silvia, Ostatnikova Daniela. Reelin and its complex involvement in brain development and function // The international journal of biochemistry & cell biology. 2012. 44, 9. 1501–1504.

Lee Yoontae, Kim Minju, Han Jinju, Yeom Kyu-Hyun, Lee Sanghyuk, Baek Sung Hee, Kim V. Narry. MicroRNA genes are transcribed by RNA polymerase II // The EMBO journal. 2004. 23, 20. 4051–4060.

Li Da-Wei, Liu Jin-Zhi, Li Shu-Chen, Yang Jin-Bin, Sun Huan-Huan, Wang Ai-Hua. Fear conditioning downregulates miR-138 expression in the hippocampus to facilitate the formation of fear memory // Neuroreport. 2018. 29, 16. 1418–1424.

Liu Chang-Mei, Wang Rui-Ying, Saijilafu , Jiao Zhong-Xian, Zhang Bo-Yin, Zhou Feng-Quan. MicroRNA-138 and SIRT1 form a mutual negative feedback loop to regulate mammalian axon regeneration // Genes & development. 2013. 27, 13. 1473–1483.

Lugli Giovanni, Larson John, Martone Maryann E., Jones Ying, Smalheiser Neil R. Dicer and eIF2c are enriched at postsynaptic densities in adult mouse brain and are modified by neuronal activity in a calpain-dependent manner // Journal of neurochemistry. 2005. 94, 4. 896–905.

Lund Elsebet, Güttinger Stephan, Calado Angelo, Dahlberg James E., Kutay Ulrike. Nuclear export of microRNA precursors // Science (New York, N.Y.). 2004. 303, 5654. 95–98.

Malik Ahmed M., Miguez Roberto A., Li Xingli, Ho Ye-Shih, Feldman Eva L., Barmada Sami J. Matrin 3-dependent neurotoxicity is modified by nucleic acid binding and nucleocytoplasmic localization // eLife. 2018. 7.

McNeill Elizabeth, van Vactor David. MicroRNAs shape the neuronal landscape // Neuron. 2012. 75, 3. 363–379.

Obernosterer Gregor, Leuschner Philipp J. F., Alenius Mattias, Martinez Javier. Post-transcriptional regulation of microRNA expression // RNA (New York, N.Y.). 2006. 12, 7. 1161–1167.

Okamura Katsutomo, Phillips Michael D., Tyler David M., Duan Hong, Chou Yu-ting, Lai Eric C. The regulatory activity of microRNA* species has substantial influence on microRNA and 3' UTR evolution // Nature structural & molecular biology. 2008. 15, 4. 354–363.

Park Ikbum, Kim Hyun Jin, Kim Youngkyu, Hwang Hye Sung, Kasai Haruo, Kim Joung-Hun, Park Joon Won. Nanoscale imaging reveals miRNA-mediated control of functional states of dendritic spines // Proceedings of the National Academy of Sciences of the United States of America. 2019. 116, 19. 9616–9621.

Pfaff Janina, Hennig Janosch, Herzog Franz, Aebersold Ruedi, Sattler Michael, Niessing Dierk, Meister Gunter. Structural features of Argonaute-GW182 protein interactions // Proceedings of the National Academy of Sciences of the United States of America. 2013. 110, 40. E3770–9.

Prasad Archana, Bharathi Vidhya, Sivalingam Vishwanath, Girdhar Amandeep, Patel Basant K. Molecular Mechanisms of TDP-43 Misfolding and Pathology in Amyotrophic Lateral Sclerosis // Frontiers in molecular neuroscience. 2019. 12. 25.

Rajgor Dipen, Hanley Jonathan G., Shanahan Catherine M. Identification of novel nesprin-1 binding partners and cytoplasmic matrin-3 in processing bodies // Molecular biology of the cell. 2016. 27, 24. 3894–3902.

Roden Christine, Gaillard Jonathan, Kanoria Shaveta, Rennie William, Barish Syndi, Cheng Jijun, Pan Wen, Liu Jun, Cotsapas Chris, Ding Ye, Lu Jun. Novel determinants of mammalian primary microRNA processing revealed by systematic evaluation of hairpin-containing transcripts and human genetic variation // Genome research. 2017. 27, 3. 374–384.

Rouleau Samuel G., Garant Jean-Michel, Bolduc François, Bisaillon Martin, Perreault Jean-Pierre. G-Quadruplexes influence pri-microRNA processing // RNA biology. 2018. 15, 2. 198–206.

Salton Maayan, Lerenthal Yaniv, Wang Shih-Ya, Chen David J., Shiloh Yosef. Involvement of Matrin 3 and SFPQ/NONO in the DNA damage response // Cell cycle (Georgetown, Tex.). 2010. 9, 8. 1568–1576.

Sambandan Sivakumar, Akbalik Güney, Kochen Lisa, Rinne Jennifer, Kahlstatt Josefine, Glock Caspar, Tushev Georgi, Alvarez-Castelao Beatriz, Heckel Alexander, Schuman Erin M. Activity-dependent spatially localized miRNA maturation in neuronal dendrites // Science (New York, N.Y.). 2017. 355, 6325. 634–637.

Schratt Gerhard M., Tuebing Fabian, Nigh Elizabeth A., Kane Christina G., Sabatini Mary E., Kiebler Michael, Greenberg Michael E. A brain-specific microRNA regulates dendritic spine development // Nature. 2006. 439, 7074. 283–289.

Schröder Julia, Ansaloni Sara, Schilling Marcel, Liu Tian, Radke Josefine, Jaedicke Marian, Schjeide Brit-Maren M., Mashychev Andriy, Tegeler Christina, Radbruch Helena, Papenberg Goran, Düzel Sandra, Demuth Ilja, Bucholtz Nina, Lindenberger Ulman, Li Shu-Chen, Steinhagen-Thiessen Elisabeth, Lill Christina M., Bertram

Lars. MicroRNA-138 is a potential regulator of memory performance in humans // Frontiers in human neuroscience. 2014. 8. 501.

Sethi Prerna, Lukiw Walter J. Micro-RNA abundance and stability in human brain: specific alterations in Alzheimer's disease temporal lobe neocortex // Neuroscience letters. 2009. 459, 2. 100–104.

Siegel Gabriele, Obernosterer Gregor, Fiore Roberto, Oehmen Martin, Bicker Silvia, Christensen Mette, Khudayberdiev Sharof, Leuschner Philipp F., Busch Clara J. L., Kane Christina, Hübel Katja, Dekker Frank, Hedberg Christian, Rengarajan Balamurugan, Drepper Carsten, Waldmann Herbert, Kauppinen Sakari, Greenberg Michael E., Draguhn Andreas, Rehmsmeier Marc, Martinez Javier, Schratt Gerhard M. A functional screen implicates microRNA-138-dependent regulation of the depalmitoylation enzyme APT1 in dendritic spine morphogenesis // Nature cell biology. 2009. 11, 6. 705–716.

Siomi Haruhiko, Siomi Mikiko C. Posttranscriptional regulation of microRNA biogenesis in animals // Molecular cell. 2010. 38, 3. 323–332.

Stranahan Alexis M., Erion Joanna R., Wosiski-Kuhn Marlena. Reelin signaling in development, maintenance, and plasticity of neural networks // Ageing research reviews. 2013. 12, 3. 815–822.

Trabucchi Michele, Briata Paola, Filipowicz Witold, Rosenfeld Michael G., Ramos Andres, Gherzi Roberto. How to control miRNA maturation? // RNA biology. 2009a. 6, 5. 536–540.

Trabucchi Michele, Briata Paola, Garcia-Mayoral Mariaflor, Haase Astrid D., Filipowicz Witold, Ramos Andres, Gherzi Roberto, Rosenfeld Michael G. The RNA-binding protein KSRP promotes the biogenesis of a subset of microRNAs // Nature. 2009b. 459, 7249. 1010–1014.

Treiber Thomas, Treiber Nora, Plessmann Uwe, Harlander Simone, Daiß Julia-Lisa, Eichner Norbert, Lehmann Gerhard, Schall Kevin, Urlaub Henning, Meister Gunter.

A Compendium of RNA-Binding Proteins that Regulate MicroRNA Biogenesis // Molecular cell. 2017. 66, 2. 270–284.e13.

Valluy Jeremy, Bicker Silvia, Aksoy-Aksel Ayla, Lackinger Martin, Sumer Simon, Fiore Roberto, Wüst Tatjana, Seffer Dominik, Metge Franziska, Dieterich Christoph, Wöhr Markus, Schwarting Rainer, Schratt Gerhard. A coding-independent function of an alternative Ube3a transcript during neuronal development // Nature neuroscience. 2015. 18, 5. 666–673.

Verbrugghe Phebe, Bouwer Sonja, Wiltshire Steven, Carter Kim, Chandler David, Cooper Matthew, Morar Bharti, Razif Muhammad F. M., Henders Anjali, Badcock Johanna C., Dragovic Milan, Carr Vaughan, Almeida Osvaldo P., Flicker Leon, Montgomery Grant, Jablensky Assen, Kalaydjieva Luba. Impact of the Reelin signaling cascade (ligands-receptors-adaptor complex) on cognition in schizophrenia // American journal of medical genetics. Part B, Neuropsychiatric genetics : the official publication of the International Society of Psychiatric Genetics. 2012. 159B, 4. 392–404.

Viswanathan Srinivas R., Daley George Q. Lin28: A microRNA regulator with a macro role // Cell. 2010. 140, 4. 445–449.

Wang Wenyuan, Kwon Ester J., Tsai Li-Huei. MicroRNAs in learning, memory, and neurological diseases // Learning & memory (Cold Spring Harbor, N.Y.). 2012. 19, 9. 359–368.

Weiss Kerstin, Treiber Thomas, Meister Gunter, Schratt Gerhard. The nuclear matrix protein Matr3 regulates processing of the synaptic microRNA-138-5p // Neurobiology of learning and memory. 2019. 159. 36–45.

Zampa Federico, Bicker Silvia, Schratt Gerhard. Activity-Dependent Pre-miR-134 Dendritic Localization Is Required for Hippocampal Neuron Dendritogenesis // Frontiers in molecular neuroscience. 2018. 11. 171.

Reprints of original publications

DEAH-box helicase DHX36 mediates dendritic localization of the neuronal precursor-microRNA-134

Bicker S, Khudayberdiev S, Weiß K, Zocher K, Baumeister S, Schratt G., Genes & Development, 2013, 27: 991-996

DOI: https://doi.org/10.1101/gad.211243.112

The nuclear matrix protein Matr3 regulates processing of the synaptic microRNA-138-5p

Weiss K, Treiber T, Meister G, Schratt G., Neurobiology of Learning and Memory, 2019, 159: 36-45

DOI: https://doi.org/10.1016/j.nlm.2019.02.008

RESEARCH COMMUNICATION

The DEAH-box helicase DHX36 mediates dendritic localization of the neuronal precursor-microRNA-134

Silvia Bicker,[1] Sharof Khudayberdiev,[1] Kerstin Weiß,[1] Kathleen Zocher,[1] Stefan Baumeister,[2] and Gerhard Schratt[1,3]

[1]Institut für Physiologische Chemie, Biochemisch-Pharmakologisches Centrum Marburg, Philipps-Universität Marburg, 35032 Marburg, Germany; [2]Fachbereich Biologie-Protein Analytik, Philipps-Universität Marburg, 35032 Marburg, Germany

Specific microRNAs (miRNAs), including miR-134, localize to neuronal dendrites, where they control synaptic protein synthesis and plasticity. However, the mechanism of miRNA transport is unknown. We found that the neuronal precursor-miRNA-134 (pre-miR-134) accumulates in dendrites of hippocampal neurons and at synapses in vivo. Dendritic localization of pre-miR-134 is mediated by the DEAH-box helicase DHX36, which directly associates with the pre-miR-134 terminal loop. DHX36 function is required for miR-134-dependent inhibition of target gene expression and the control of dendritic spine size. Dendritically localized pre-miR-134 could provide a local source of miR-134 that can be mobilized in an activity-dependent manner during plasticity.

Supplemental material is available for this article.

Received November 30, 2012; revised version accepted April 5, 2013.

MicroRNAs (miRNAs) are important regulators of morphological and functional plasticity in the developing and mature nervous system (Ashraf et al. 2006; Schratt et al. 2006; Rajasethupathy et al. 2009; Gao et al. 2010). Specific miRNAs control plasticity by binding to dendritic mRNAs (Schuman et al. 2006; Bramham and Wells 2007; Martin and Ephrussi 2009), thereby modulating the local synthesis of the respective proteins at synapses in an activity-dependent manner (Schratt et al. 2006; Banerjee et al. 2009; Fiore et al. 2009; Muddashetty et al. 2011). The biogenesis of miRNAs involves two subsequent RNase-dependent cleavage steps: Drosha-mediated processing of primary miRNA transcripts (pri-miRNAs) in the nucleus followed by Dicer-mediated processing of 70- to 100-nucleotide (nt) stem–loop precursors (precursor-miRNAs [pre-miRNAs]) in the cytoplasm (Kim 2005; Krol et al. 2010). miRNA biogenesis can be regulated by sequence-specific RNA-binding proteins (RBPs) that preferentially interact with the terminal loop structure of pri- and pre-miRNAs (Krol et al. 2010; Newman and Hammond 2010). Interestingly, miR-29b contains a sequence element at the 3′ end of the mature miRNA that directs its nuclear localization (Hwang et al. 2007), but nothing is known about miRNA sequence determinants responsible for localization to specific cytoplasmic compartments, such as the synapto-dendritic compartment of neurons.

We speculated that dendritic localization of specific miRNAs could occur at the pre-miRNA stage, possibly by the presence of sequence elements within pre-miRNA terminal loops that serve as binding sites for RBPs, which are in turn part of transport complexes. Dendritic localization of specific pre-miRNAs would offer the advantage of a local source of miRNAs that could be mobilized, for example, in response to synaptic activity.

[*Keywords*: dendritic transport; DHX36; precursor-microRNA; protein synthesis; synaptic plasticity]
[3]**Corresponding author**
E-mail gerhard.schratt@staff.uni-marburg.de
Article is online at http://www.genesdev.org/cgi/doi/10.1101/gad.211243.112.

Results and Discussion

To interrogate synapto-dendritic localization of pre-miRNAs, we selected 16 previously identified dendritic miRNAs (Schratt et al. 2006; Siegel et al. 2009) and determined corresponding pre-miRNA levels in synaptosomes by quantitative RT–PCR (qRT–PCR). We found that for two of these candidates (pre-miR-7a-2 and pre-miR-134), the degree of synaptic enrichment was significantly higher compared with the nuclear U6 snRNA and at least equal to the known dendritic noncoding RNA BC1 (Brosius and Tiedge 2001), suggesting that these pre-miRNAs are actively transported into the synapto-dendritic compartment (Fig. 1A). Due to the well-documented synaptic functions of miR-134 (Schratt et al. 2006; Fiore et al. 2009; Gao et al. 2010), we decided to focus on pre-miR-134. Synaptic enrichment of pre-miR-134 was validated by Northern blot of synaptosomal and whole-brain RNA preparations (Supplemental Fig. S1; Siegel et al. 2009). Since synaptosomes contain both pre- and postsynaptic compartments, we performed locked nucleic acid (LNA)-based fluorescent in situ hybridization (FISH) in rat hippocampal neurons (7 d in vitro [DIV]) (Fig. 1B) to determine the subcellular localization of pre-miR-134. BDNF (brain-derived neurotrophic factor) treatment of neurons was used to increase pre-miR-134 expression (Fiore et al. 2009). Using a FISH probe specifically recognizing the loop sequence of pre-miR-134 (Supplemental Fig. S2), we detected a specific signal in the neuronal cell body as well as discrete punctate staining in proximal and distal parts of MAP2-positive dendrites (Fig. 1B, top panel, insert at higher magnification). In contrast, expression of pre-miR-137, which was not enriched in synaptosomes, was restricted to the cell body compartment (Fig. 1B, bottom panel). Quantification of FISH signals along the entire length of multiple dendrites confirms the specific dendritic localization of pre-miR-134 (Fig. 1C). The specificity of our FISH protocol was validated with negative (scrambled LNA oligonucleotide) and positive (LNA oligonucleotide recognizing mature miR-134) (Schratt et al. 2006) control probes (Supplemental Fig. S3). The presence of pre-miR-134 and pre-miR-7a-2, but not pre-miR-137, in processes of rat hippocampal neurons was further confirmed using a compartmentalized culture system (Fig. 1D; Supplemental Fig. S4a,b). This system also faithfully recapitulated process enrichment of mature miR-134 but not of the nondendritic miR-133b (Supplemental

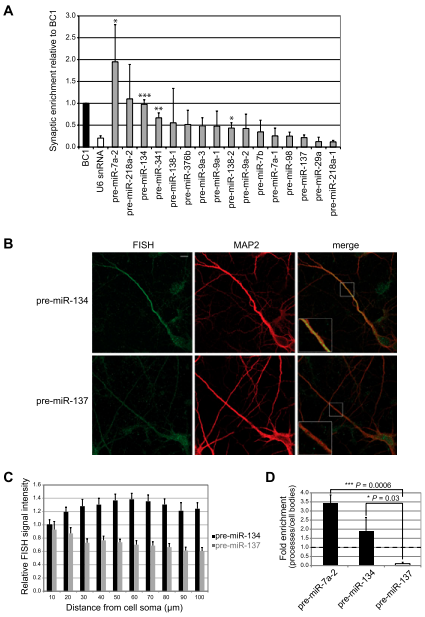

Figure 1. pre-miR-134 localizes to neuronal dendrites and synapses. (*A*) Levels of the indicated pre-miRNAs, BC1, and U6 snRNA in rat postnatal day 15 (P15) synaptosomes relative to whole forebrain measured by qRT–PCR (mean ± SD, $n = 3$). (*) $P < 0.05$; (**) $P < 0.01$; (***) $P < 0.001$). BC1 was set to one. (*B*, *left*) Representative images from FISH on BDNF-treated hippocampal neurons (7 DIV) using LNA probes directed against the terminal loop of either pre-miR-134 (*top*) or pre-miR-137 (*bottom*). (*Middle*) MAP2 immunostaining. (*Right*) Merge. *Inserts* at higher magnification illustrate the presence of pre-miR-134 puncta and the absence of pre-miR-137 puncta in distal dendrites. Bar, 10 μm. (*C*) Quantification of FISH analysis performed in *B*. Relative signal intensities of dendritic segments derived from 20 neurons of each condition ±SD. (*D*) Levels of indicated pre-miRNAs in the process compartment of hippocampal neurons relative to cell bodies measured by qRT–PCR (mean ± SD, $n = 3$).

Fig. S4c). Together, our data obtained with synaptosomes, FISH, and compartmentalized neuron cultures support a specific localization of endogenous pre-miR-134 in dendrites of primary hippocampal neurons and at synapses in vivo.

We next sought to determine the specific sequence elements required for dendritic localization of pre-miR-134 in hippocampal neurons. We transfected in vitro transcribed, fluorescently (Cy3) labeled pre-miRNAs into hippocampal neurons and analyzed dendritic localization by fluorescence confocal microscopy. All synthetic pre-miRNAs contain a 2-nt 3′ overhang, thereby resembling endogenous Drosha cleavage products (Fig. 2A). The majority of puncta formed by wild-type Cy3-pre-miR-134 localized to dendrites (Fig. 2B, top panel, arrows), demonstrating that exogenous pre-miR-134 recapitulates dendritic localization of the endogenous counterpart. The subcellular localization of Cy3-pre-miR-134 puncta was specific, since the majority of a nondendritic pre-miRNA, Cy3-pre-miR-150, localized to the neuronal cell body (Fig. 2B, bottom panel, arrowheads). Quantification of Cy3-positive puncta from at least 60 neurons for each experimental condition confirmed the specific dendritic localization of Cy3-pre-miR-134 (Fig. 2C). Only Cy3-positive puncta that were intracellularly localized were considered, as judged by the overlap with the dendritic marker protein MAP2 along the entire confocal Z-stack (Supplemental Fig. S5a). In contrast, we did not observe localization of Cy3-pre-miR-134 to TAU-1-positive axons (Supplemental Fig. S5b). Importantly, the vast majority of pre-miR-134 within these puncta was not yet processed at the time of analysis (2.5 h after transfection), as shown by FISH (Supplemental Fig. S6). The loop region is the most accessible structure within pre-miRNAs and often serves as a binding platform for regulatory protein complexes (Krol et al. 2010; Newman and Hammond 2010). Replacing the loop sequence of pre-miR-134 with the loop of the nondendritic pre-miR-150 (pre-miR-134L150) abolished dendritic accumulation of pre-miR-134 puncta (Fig. 2D; Supplemental Fig. S7a). Conversely, putting the pre-miR-134 loop sequence within the context of the pre-miR-150 stem (pre-miR-150L134) completely restored dendritic localization (Fig. 2D; Supplemental Fig. S7a). Further mutagenesis analysis revealed that exchanging the five central loop nucleotides (Fig. 2A, bold letters) within the context of pre-miR-134 (pre-miR-134C150) abolished dendritic enrichment, whereas a pre-miR-150 mutant containing the central loop nucleotides of pre-

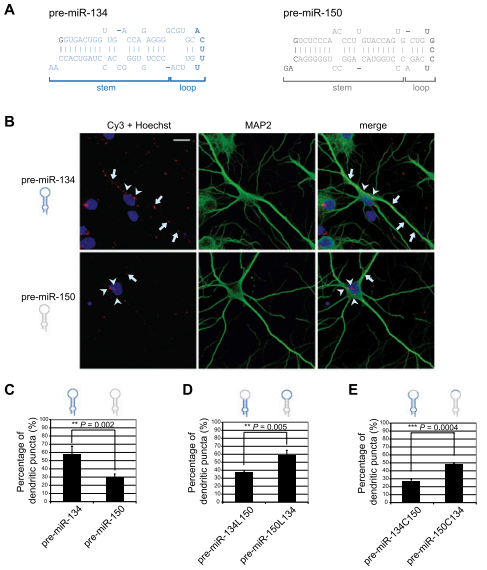

Figure 2. The pre-miR-134 terminal loop is necessary and sufficient for dendritic targeting. (*A*) Sequences of in vitro transcribed pre-miR-134 (blue) and pre-miR-150 (gray). The five central nucleotides of the terminal loop are shown in bold. Sequence changes required for efficient in vitro transcription (G at position +1) and for correct 3′ termini (2-nt overhang) are indicated in black. (*B*) Representative images of hippocampal neurons transfected with indicated Cy3-pre-miRNAs. (*Left*) Cy3-pre-miRNA (red) and Hoechst (blue). (*Middle*) MAP2 (green). (*Right*) Merge. Arrows and arrowheads indicate pre-miRNA puncta localized in dendrites or the cell body, respectively. Bar, 10 μm. (*C–E*) Percentage of dendritic puncta for the indicated pre-miRNAs or mutants (mean ± SD, $n = 4$, 64 neurons per condition). (L) Loop; (C) central loop.

miR-134 (pre-miR-150C134) largely regained the capacity to localize to dendrites (Fig. 2E; Supplemental Fig. S7b). Together, these experiments suggest that the pre-miR-134 loop sequence is an important determinant of pre-miR-134 dendritic localization.

We speculated that specific RBPs that interact with the pre-miR-134 loop might be responsible for directed transport to the synapto-dendritic compartment. To identify proteins that selectively interact with the pre-miR-134 loop, we performed pull-down experiments by incubating rat forebrain protein extract with biotin-tagged pre-miR-134 or respective mutants coupled to streptavidin beads (Fig. 3A; Heo et al. 2008, 2009). Thereby, we identified one band above 100 kDa that was specifically present in eluates from pull-downs with wild-type pre-miR-134 but not with pre-miR-150 or pre-miR-134C150 (Fig. 3B, arrow). We identified this protein as DHX36 by mass spectrometry

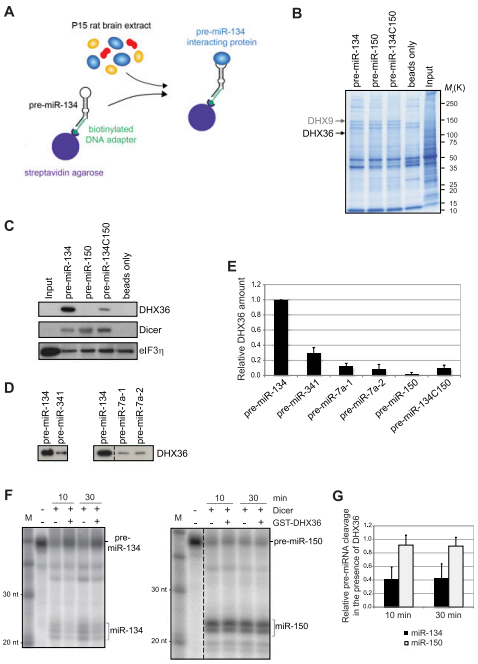

Figure 3. DHX36 interacts with the pre-miR-134 terminal loop. (*A*) Scheme of the purification strategy used to isolate pre-miR-134-interacting proteins from P15 rat brain extract. (*B*) Coomassie gel of pull-downs using rat P15 brain extract and the indicated pre-miRNAs. Arrows point to bands that were identified by mass spectrometry as DHX9 (~140 kDa) and DHX36 (~110 kDa). (*C*) Western blot against the indicated proteins with extracts from pull-downs shown in *B*. (*D*) Western blot against DHX36 with extracts from the indicated pre-miRNA pull-downs. (*E*) Quantification of DHX36 binding in pre-miRNA pull-down experiments (pre-miR-134 = 1; mean ± SD, $n = 3$). (*F*) In vitro pre-miRNA cleavage assay. pre-miRNAs were incubated with recombinant Dicer for the indicated time in either the presence or absence of GST-DHX36. The position of pre-miRNAs and mature miRNAs is indicated. (*G*) Ratio of mature miRNA to pre-miRNA as an index for Dicer cleavage activity (mean ± SD, $n = 4$–5; conditions without DHX36 were set to 1 for both time points).

(Supplemental Table T1). In addition, we identified DHX9 (Fig. 3B, arrow) as a protein that interacts with all tested pre-miRNAs, in accordance with a described function of DHX9 within RNA-induced silencing complex (RISC) (Robb and Rana 2007). The specific interaction of pre-miR-134 with DHX36 could be confirmed by Western blot using an anti-DHX36 antibody (Fig. 3C, top panel). Importantly, Dicer interacted with all three pre-miRNAs (Fig. 3C, middle panel), demonstrating that all of them were per se competent in protein binding. An unrelated protein, eIF3η, did not specifically interact with any of the tested pre-miRNAs (Fig. 3C, bottom panel). In the case of other dendritically localized pre-miRNAs, the interaction with DHX36 was strongly reduced (Fig. 3D,E), suggesting that DHX36 preferentially interacts with pre-miR-134. Affinity-purified full-length DHX36 and the isolated DHX36 N-terminal domain, which contains an RNA-binding motif (Chalupnikova et al. 2008), both associated with radiolabeled pre-miR-134 in electrophoretic mobility shift assay (EMSA) in vitro (Supplemental Fig. S8), demonstrating a direct interaction between DHX36 and pre-miR-134. Using in vitro pre-miRNA cleavage assays (Leuschner and Martinez 2007), we found that DHX36 interfered with cleavage of synthetic pre-miR-134 by recombinant Dicer but not with Dicer-mediated pre-miR-150 cleavage (Fig. 3F,G). Together, our experiments demonstrate that DHX36 directly interacts with pre-miR-134 and reduces Dicer-mediated pre-miR-134 cleavage in vitro.

We next addressed whether DHX36 functions in dendritic localization of pre-miR-134. DHX36 was shown to resolve secondary nucleic acid structures in the nucleus (Vaughn et al. 2005) and regulate RNA metabolism in the cytoplasm in nonneuronal cells (Tran et al. 2004; Chalupnikova et al. 2008; Zhang et al. 2011). However, DHX36 has not yet been studied in neurons. Using compartmentalized primary rat hippocampal neuron cultures, we detected DHX36 expression in both the cell body and, to a lesser extent, the process compartment (Fig. 4A). In further support of a function of DHX36 in neuronal processes, ectopically expressed GFP-DHX36 fusion protein localized to dendrites and axons (Supplemental Fig. S9). To test whether pre-miR-134 interacts with DHX36 in neurons, we transfected cortical neurons at 6 DIV with either pre-miR-134 or pre-miR-150 and performed an RNA immunoprecipitation (RNA-IP) assay. We found that transfected pre-miR-134, in contrast to pre-miR-150, was specifically enriched in anti-DHX36 compared with IgG immunoprecipitates, as judged by qRT–PCR (Fig. 4B). This finding suggests that endogenous DHX36 and synthetic pre-miR-134 interact in neurons. To investigate the function of DHX36, we designed shRNA expression constructs targeting different regions of the rat DHX36 mRNA and assessed their efficiency by nucleofection (Fig. 4C). To assess the role of DHX36 in dendritic transport of pre-miR-134, we combined plasmid-based knockdown of DHX36 (3 d of expression) with short-term transfection of Cy3-labeled pre-miRNAs. To rule out potential effects of DHX36 knockdown on miR-134 processing, we decided to study subcellular distribution of synthetic Cy3-pre-miR-134 shortly after transfection. At 2.5 h after transfection, Cy3-pre-miR-134 was found to be excluded from the nucleus, and the vast majority of Cy3-pre-miR-134 was still not processed (Supplemental Fig. S6). Neurons transfected with the two most effective DHX36 shRNAs displayed a significant reduction in the percentage of

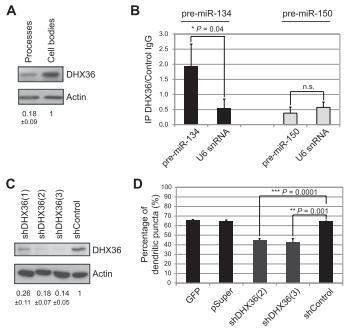

Figure 4. DHX36 is required for dendritic localization of pre-miR-134 in hippocampal neurons. (*A*) Western blot against the indicated proteins with extracts from process or cell body fractions of hippocampal neurons. Quantification of band intensities is indicated *below* (mean ± SD, $n = 3$). (*B*) RNA-IP of cortical neurons transfected with the indicated pre-miRNAs using a DHX36 or control IgG antibody. Ratio of RNA amounts from DHX36 immunoprecipitates to control IgG immunoprecipitates as determined by qRT–PCR is shown (mean ± SD, $n = 3$) (*C*) Western blot against the indicated proteins with extracts from hippocampal neurons nucleofected with the indicated shRNA-expressing constructs. Quantification of band intensities is indicated *below* (mean ± SD, $n = 2$). (*D*) Percentage of dendritic Cy3-pre-miR-134 puncta observed in hippocampal neurons transfected with the indicated constructs (mean ± SD, $n = 3$, ANOVA: $F(4;10) = 84$, $P < 0.001$; 30 neurons per condition).

dendritic Cy3-pre-miR-134 puncta (Fig. 4D; Supplemental Fig. S10a). DHX36 knockdown did not affect global levels of pre-miR-134 and mature miR-134 (Supplemental Fig. S10b,c). These results suggest that DHX36 is specifically required for the accumulation of pre-miR-134 in neuronal dendrites.

Finally, we addressed whether DHX36-dependent localization of pre-miR-134 might contribute to the activity of miR-134 in dendrites. For these experiments, we first used a luciferase reporter gene that contains a perfect miR-134-binding site in the context of the Limk1 3′ untranslated region (UTR; Limk1 134pbds) (Christensen et al. 2010). The Limk1 3′ UTR confers dendritic localization of the reporter (Schratt et al. 2006), allowing us to study the role of DHX36 in local translation. Upon DHX36 knockdown, expression of the Limk1 134pbds reporter gene was significantly increased compared with control conditions, whereas expression of a reporter gene containing a mutated miR-134-binding site was unaffected (Fig. 5A). In addition, both a Pum2 luciferase reporter gene containing a single miR-134-binding site (Fiore et al. 2009) and endogenous Limk1 protein expression were similarly induced by DHX36 knockdown in neurons (Fig. 5B; Supplemental Fig. S10d). Together, these results demonstrate that DHX36 knockdown results in an increased expression of known dendritic miR-134 target genes, possibly due to reduced dendritic pre-miR-134 accumulation. Nevertheless, based on our data, we cannot rule out that derepression of miR-134 targets in the cell body might also contribute to the observed effects of DHX36 knockdown. Finally, we interrogated whether DHX36 knockdown had an impact on neuronal function, possibly via regulation of miR-134 activity. Toward this end, we analyzed dendritic spine morphology, since we previously found that treatment of hippocampal neurons with miR-134 function-blocking antisense oligonucleotides led to elevated spine size (Schratt et al. 2006). Consistent with reduced dendritic miR-134 activity upon DHX36 knockdown, we found that neurons transfected with a DHX36 shRNA construct contained a higher fraction of large mushroom-shaped spines compared with neurons transfected with a control shRNA (Fig. 5C; Supplemental Fig. S11). Accordingly, the average spine volume of neurons transfected with two independent DHX36 shRNA constructs was significantly higher compared with control conditions (Fig. 5D). This result demonstrates that DHX36 negatively regulates dendritic spine morphogenesis in hippocampal neurons. It is further consistent with the idea that DHX36-dependent pre-miR-134 accumulation in dendrites could be important for neuronal function.

Taken together, our results show that DHX36 plays a role in dendritic localization of pre-miR-134 and spine

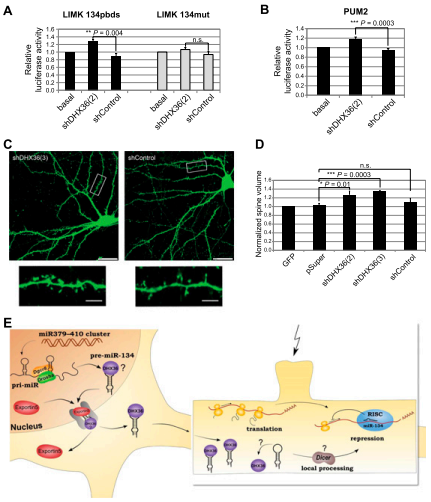

Figure 5. DHX36 is required for miR-134-regulated neuronal functions. (*A*,*B*) Reporter gene activity in primary neurons transfected with the indicated shRNA constructs and firefly luciferase reporters containing Limk1 (*A*; 15 DIV hippocampal neurons) and Pum2 (*B*; 15 DIV cortical neurons) 3′ UTRs. Cotransfection of empty *Renilla* reporter served as transfection control. Relative luciferase activity represents the ratio of firefly to *Renilla* activity; basal condition was set to 1 (mean ± SD, $n = 3$, each experimental condition measured in duplicates within one experiment). (*C*) Representative images of hippocampal neurons transfected with either shDHX36(3) (*left*) or shControl (*right*) shRNAs. *Inserts* at higher magnification illustrate increased proportion of large, mushroom-shaped spines in the shDHX36 condition compared with shControl. Bars: main panels, 20 µm; *inserts*, 5 µm. (*D*) Quantification of average normalized spine volume as determined by the ratio of GFP signal in dendritic spines to total cell intensity. GFP-only condition was set to 1 (mean ± SD, $n = 3$; ANOVA: $F(3;8) = 15{,}921$, $P = 0.001$; 18 neurons per condition; 150–200 spines per neuron). (*E*) Working model for the role of DHX36 in pre-miR-134 dendritic transport and processing. The pre-miR-134 loop contains a *cis*-acting dendritic targeting element that is recognized by DHX36, resulting in pre-miR-134 transport to the dendrite. In the postsynaptic compartment, stimulation of specific dendritic spines (flash) may trigger Dicer-dependent pre-miRNA processing (possibly involving release of DHX36), thereby enabling an enhanced production of mature miR-134 and repression of miR-134 target mRNAs.

morphogenesis, a process regulated by miR-134 (Fig. 5E). This adds subcellular localization to the growing list of functions for proteins that associate with the terminal loop of specific pre-miRNAs (Krol et al. 2010). The presumptive DHX36-binding site within the pre-miR-134 central loop is not present in the loop of other dendritic pre-miRNAs, which, together with our in vitro binding assays, suggests that distinct localization mechanisms are used by specific dendritic pre-miRNAs. Previously, we found that mature miR-134 is delivered to dendrites when transfected into neurons (Schratt et al. 2006). The underlying mechanism is unknown but could involve cotransport of miR-134 with its dendritic target mRNAs, such as Limk1. We speculate that dendritically localized pre-miR-134 might, in parallel, provide a pool of miR-134 that can be rapidly mobilized in response to activity. This local pool could be important to reinforce miR-134-mediated repression of gene expression, for example, in conditions of chronically elevated activity that are known to induce homeostatic synaptic scaling (Turrigiano 2008). The molecular mechanisms involved in a possible local control of miR-134 production in dendrites are currently unknown. Dicer has been detected in synaptosomes (Lugli et al. 2005), suggesting that pre-miR-134 could undergo local Dicer-dependent processing at synapses. Since DHX36 competes with Dicer in vitro (Fig. 3F) and Argonaute complexes that contain DHX36 are devoid of Dicer activity (Hock et al. 2007), we speculate that pre-miR-134 has to be released from DHX36 upon activity to allow efficient pre-miR-134 processing at the synapse (Fig. 5E). Future work will address the mechanism of activity-dependent local processing of pre-miR-134 and its significance for dendritic protein synthesis and synaptic plasticity.

Materials and methods

Cell culture

Primary hippocampal and cortical embryonic (embryonic day 18) neuron cultures were prepared and cultured as described (Schratt et al. 2006). For compartmentalized neuron cultures, dissociated hippocampal neurons were plated onto 1-μm pore and 30-mm diameter polyethylene tetraphthalate (PET) membrane filter inserts (Millipore) that were matrix-coated with poly-L-lysine (Sigma-Aldrich) and Laminin (BD Biosciences) on the top and bottom (Poon et al. 2006).

Transfections

Transfections of primary neurons using Lipofectamine 2000 (Invitrogen) were performed as described (Siegel et al. 2009). For validation of shRNA efficacy, primary cortical neurons of rat embryos (E18) were nucleofected using the P3 Primary Cell 4D-Nucleofector kit (Lonza), program DC-104, according to the manufacturer's instructions. Transfections of primary hippocampal or cortical neurons with in vitro transcribed pre-miRNAs (25–75 nM) were performed using siPORT NeoFX transfection agent (Ambion).

Preparation of synaptosomes

Synaptosomes were prepared from postnatal day 15 (P15) Sprague-Dawley rat pups as previously described (Schratt et al. 2004).

In vitro transcription of pre-miRNAs

Pre-miRNAs were in vitro transcribed from dsDNA templates (Supplemental Material) using the MEGAshortscript T7 kit (Ambion) according to the manufacturer's instructions, with a template concentration of 150 nM and an incubation time of 4 h at 37°C. pre-miRNAs were purified using illustra MicroSpin G-25 columns (GE Healthcare).

RNA labeling

Labeling of pre-miRNAs by Cy3 was performed using the Label IT nucleic acid labeling kit (Mirus) according to the manufacturer's instructions. For Dicer assays, pre-miRNAs were 5′ end-labeled using [γ-^{32}P] ATP (PerkinElmer) as previously described (Leuschner and Martinez 2007).

Subcellular localization of in vitro transcribed pre-miRNAs

Rat hippocampal neurons were transfected at 6 DIV with 30 nM Cy3-labeled precursors using siPORT NeoFX transfection agent (Ambion) for 2 h. Fifteen minutes post-transfection, cells were treated with Cell Scrub buffer (Genlantis) for 5 min to remove extracellular Cy3 complexes, fixed, and stained for MAP2 to visualize dendrites. Nuclei were counterstained with Hoechst (Invitrogen). Data sets were blinded to the experimental conditions, and Z-stack images (seven consecutive optical sections per stack) were taken with an LSM 5 Pascal confocal microscope (Zeiss). ImageJ software ("analyze particles" option) was used for subsequent analysis of intracellular Cy3 puncta distribution. For quantification, the average percentage of dendritic puncta compared with the total number of intracellular puncta was determined for each pre-miRNA.

RNA pull-down

The RNA pull-down approach used in this study was based on a previously described protocol for isolation of pre-let-7-binding proteins concerning the general procedure (Heo et al. 2008) and on a protocol for FMRP immunoprecipitation concerning brain lysate preparation (Edbauer et al. 2010). See the Supplemental Material for further details.

RNA-IP

For RNA-IP, cortical neurons were transfected with in vitro transcribed pre-miRNAs and immunoprecipitated using rabbit anti-DHX36 antibody (Novus Biologicals). RNA-IP was performed using the same buffer system as described for the RNA pull-down approach. A detailed protocol can be found in the Supplemental Material.

FISH

Dissociated hippocampal neurons were treated with BDNF (PeproTech, 50 ng/mL) for 2 h before fixation. FISH was performed as previously described (Fiore et al. 2009) using FITC-labeled LNA probes (5 pmol per well in the 24-well format; Exiqon) directed against the pre-miR-134 loop, the pre-miR-137 loop, the mature miR-134 sequence, and a control probe of unrelated sequence (scramble). Signals were further amplified by a two-step antibody procedure. Neurons were costained for the dendritic protein MAP2.

Luciferase assay

Primary neurons were transfected in duplicates with 50 ng of pGL3 firefly reporter constructs and equal amounts of empty *Renilla* reporter as transfection control. For DHX36 knockdown, neurons were cotransfected with 20 ng of the respective pSuper construct. Luciferase assays were performed after 3 d of expression using the Dual-Luciferase reporter assay system (Promega) on the GloMax R96 Microplate Luminometer (Promega).

Spine analysis

For spine analysis, images were taken on an LSM5 Zeiss Pascal confocal microscope and analyzed with ImageJ software as previously described (Siegel et al. 2009). For each condition, spines from 18 representative hippocampal neurons (150–200 spines per neuron) derived from three independent experiments were measured.

Antibodies

Primary antibodies used in this study were as follows: mouse anti-β-actin antibody (Sigma), rabbit anti-DHX36 antibody (Novus Biologicals; immunoprecipitation, Western), goat anti-DHX36 antibody (Santa Cruz Biotechnology; Western), rabbit anti-Dicer antibody (D-349; provided by Witold Filipowicz, FMI Basel), goat anti-eIF3η antibody (Santa Cruz Biotechnology), Alexa488 rabbit anti-FITC antibody (Molecular Probes), rabbit

anti-GFAP antibody (DAKO), mouse anti-MAP2 antibody (Sigma), and rabbit anti-NUP153 antibody (Abcam).

Plasmids

shRNA sequences (summarized in the Supplemental Material) were designed using the online available Dharmacon siRNA design center and cloned into pSuper (Oligoengine). Reporter plasmids pGL3 Limk 134pbds (Christensen et al. 2010), pGL3 Limk1 134mut (Schratt et al. 2006), and pGL3 Pum2(long) (Fiore et al. 2009) have been described. pAcG3XE-RHAU-His6, pGEX-2T-GST-Nter, and pEGFP-C1-DHX36 were provided by Yoshikuni Nagamine (Friedrich Miescher Institute, Basel, Switzerland).

Dicer assay

[^{32}P] 5′ end-labeled pre-miRNAs (20,000 counts per minute [cpm]) were incubated in a processing reaction with 1 U of recombinant Dicer (Recombinant Dicer Enzyme kit, Genlantis) for 10–30 min at 37°C in either the absence or presence (preincubation for 1 h at room temperature) of 30 nM GST-DHX36 (full-length). Reaction products were resolved on a 15% denaturing PAGE and visualized by autoradiography.

Mass spectrometry analysis

MALDI was performed using an Autoflex Bruker Daltonics mass spectrometer. Data analysis was done with the MASCOT program (Matrix Science). A detailed protocol can be found in the Supplemental Material.

Statistics

For each data set, three independent biological experiments were performed if not stated otherwise. Error bars represent standard deviation. *P*-values were calculated with Student's *t*-test (two-tailed, type 2) for one-way comparisons and with ANOVA followed by post-hoc test (Student's *t*-test with Bonferroni correction) for multi-way comparisons.

Acknowledgments

We thank W. Filipowicz and Y. Nagamine for providing reagents; U. Beck, E. Becker, N. Braun, K. Burk, R. Gondrum, G. Jarosch, H. Kaiser, B. Kowalski, and T. Wüst for excellent technical assistance; and R. Fiore for critically reading the manuscript. This work was funded by the DFG (SFB593) and the EMBO Young Investigator Program. S.B. is a recipient of a DOC-fFORTE fellowship of the Austrian Academy of Sciences.

References

Ashraf SI, McLoon AL, Sclarsic SM, Kunes S. 2006. Synaptic protein synthesis associated with memory is regulated by the RISC pathway in *Drosophila*. *Cell* **124:** 191–205.

Banerjee S, Neveu P, Kosik KS. 2009. A coordinated local translational control point at the synapse involving relief from silencing and MOV10 degradation. *Neuron* **64:** 871–884.

Bramham CR, Wells DG. 2007. Dendritic mRNA: Transport, translation and function. *Nat Rev Neurosci* **8:** 776–789.

Brosius J, Tiedge H. 2001. Neuronal BC1 RNA: Intracellular transport and activity-dependent modulation. *Results Probl Cell Differ* **34:** 129–138.

Chalupnikova K, Lattmann S, Selak N, Iwamoto F, Fujiki Y, Nagamine Y. 2008. Recruitment of the RNA helicase RHAU to stress granules via a unique RNA-binding domain. *J Biol Chem* **283:** 35186–35198.

Christensen M, Larsen LA, Kauppinen S, Schratt G. 2010. Recombinant adeno-associated virus-mediated microRNA delivery into the postnatal mouse brain reveals a role for miR-134 in dendritogenesis in vivo. *Front Neural Circuits* **3:** 16.

Edbauer D, Neilson JR, Foster KA, Wang CF, Seeburg DP, Batterton MN, Tada T, Dolan BM, Sharp PA, Sheng M. 2010. Regulation of synaptic structure and function by FMRP-associated microRNAs miR-125b and miR-132. *Neuron* **65:** 373–384.

Fiore R, Khudayberdiev S, Christensen M, Siegel G, Flavell SW, Kim TK, Greenberg ME, Schratt G. 2009. Mef2-mediated transcription of the miR379-410 cluster regulates activity-dependent dendritogenesis by fine-tuning Pumilio2 protein levels. *EMBO J* **28:** 697–710.

Gao J, Wang WY, Mao YW, Graff J, Guan JS, Pan L, Mak G, Kim D, Su SC, Tsai LH. 2010. A novel pathway regulates memory and plasticity via SIRT1 and miR-134. *Nature* **466:** 1105–1109.

Heo I, Joo C, Cho J, Ha M, Han J, Kim VN. 2008. Lin28 mediates the terminal uridylation of let-7 precursor microRNA. *Mol Cell* **32:** 276–284.

Heo I, Joo C, Kim YK, Ha M, Yoon MJ, Cho J, Yeom KH, Han J, Kim VN. 2009. TUT4 in concert with Lin28 suppresses microRNA biogenesis through pre-microRNA uridylation. *Cell* **138:** 696–708.

Hock J, Weinmann L, Ender C, Rudel S, Kremmer E, Raabe M, Urlaub H, Meister G. 2007. Proteomic and functional analysis of Argonaute-containing mRNA-protein complexes in human cells. *EMBO Rep* **8:** 1052–1060.

Hwang HW, Wentzel EA, Mendell JT. 2007. A hexanucleotide element directs microRNA nuclear import. *Science* **315:** 97–100.

Kim VN. 2005. MicroRNA biogenesis: Coordinated cropping and dicing. *Nat Rev Mol Cell Biol* **6:** 376–385.

Krol J, Loedige I, Filipowicz W. 2010. The widespread regulation of microRNA biogenesis, function and decay. *Nat Rev Genet* **11:** 597–610.

Leuschner PJ, Martinez J. 2007. In vitro analysis of microRNA processing using recombinant Dicer and cytoplasmic extracts of HeLa cells. *Methods* **43:** 105–109.

Lugli G, Larson J, Martone ME, Jones Y, Smalheiser NR. 2005. Dicer and eIF2c are enriched at postsynaptic densities in adult mouse brain and are modified by neuronal activity in a calpain-dependent manner. *J Neurochem* **94:** 896–905.

Martin KC, Ephrussi A. 2009. mRNA localization: Gene expression in the spatial dimension. *Cell* **136:** 719–730.

Muddashetty RS, Nalavadi VC, Gross C, Yao X, Xing L, Laur O, Warren ST, Bassell GJ. 2011. Reversible inhibition of PSD-95 mRNA translation by miR-125a, FMRP phosphorylation, and mGluR signaling. *Mol Cell* **42:** 673–688.

Newman MA, Hammond SM. 2010. Emerging paradigms of regulated microRNA processing. *Genes Dev* **24:** 1086–1092.

Poon MM, Choi SH, Jamieson CA, Geschwind DH, Martin KC. 2006. Identification of process-localized mRNAs from cultured rodent hippocampal neurons. *J Neurosci* **26:** 13390–13399.

Rajasethupathy P, Fiumara F, Sheridan R, Betel D, Puthanveettil SV, Russo JJ, Sander C, Tuschl T, Kandel E. 2009. Characterization of small RNAs in aplysia reveals a role for miR-124 in constraining synaptic plasticity through CREB. *Neuron* **63:** 803–817.

Robb GB, Rana TM. 2007. RNA helicase A interacts with RISC in human cells and functions in RISC loading. *Mol Cell* **26:** 523–537.

Schratt GM, Nigh EA, Chen WG, Hu L, Greenberg ME. 2004. BDNF regulates the translation of a select group of mRNAs by a mammalian target of rapamycin-phosphatidylinositol 3-kinase-dependent pathway during neuronal development. *J Neurosci* **24:** 9366–9377.

Schratt GM, Tuebing F, Nigh EA, Kane CG, Sabatini ME, Kiebler M, Greenberg ME. 2006. A brain-specific microRNA regulates dendritic spine development. *Nature* **439:** 283–289.

Schuman EM, Dynes JL, Steward O. 2006. Synaptic regulation of translation of dendritic mRNAs. *J Neurosci* **26:** 7143–7146.

Siegel G, Obernosterer G, Fiore R, Oehmen M, Bicker S, Christensen M, Khudayberdiev S, Leuschner PF, Busch CJ, Kane C, et al. 2009. A functional screen implicates microRNA-138-dependent regulation of the depalmitoylation enzyme APT1 in dendritic spine morphogenesis. *Nat Cell Biol* **11:** 705–716.

Tran H, Schilling M, Wirbelauer C, Hess D, Nagamine Y. 2004. Facilitation of mRNA deadenylation and decay by the exosome-bound, DExH protein RHAU. *Mol Cell* **13:** 101–111.

Turrigiano GG. 2008. The self-tuning neuron: Synaptic scaling of excitatory synapses. *Cell* **135:** 422–435.

Vaughn JP, Creacy SD, Routh ED, Joyner-Butt C, Jenkins GS, Pauli S, Nagamine Y, Akman SA. 2005. The DEXH protein product of the DHX36 gene is the major source of tetramolecular quadruplex G4-DNA resolving activity in HeLa cell lysates. *J Biol Chem* **280:** 38117–38120.

Zhang Z, Kim T, Bao M, Facchinetti V, Jung SY, Ghaffari AA, Qin J, Cheng G, Liu YJ. 2011. DDX1, DDX21, and DHX36 helicases form a complex with the adaptor molecule TRIF to sense dsRNA in dendritic cells. *Immunity* **34:** 866–878.

Neurobiology of Learning and Memory 159 (2019) 36–45

Contents lists available at ScienceDirect

Neurobiology of Learning and Memory

journal homepage: www.elsevier.com/locate/ynlme

The nuclear matrix protein Matr3 regulates processing of the synaptic microRNA-138-5p

Kerstin Weiss[a], Thomas Treiber[b], Gunter Meister[b], Gerhard Schratt[a,*]

[a] Institute of Physiological Chemistry, Philipps-University Marburg, Marburg, Germany
[b] Faculty for Biology and Preclinical Medicine, Biochemistry I, University of Regensburg, Regensburg, Germany

ARTICLE INFO

Keywords:
microRNA
miR-138
Matrin-3
RNA-binding protein
Synaptic plasticity

ABSTRACT

microRNA-dependent post-transcriptional control represents an important gene-regulatory layer in post-mitotic neuronal development and synaptic plasticity. We recently identified the brain-enriched miR-138 as a negative regulator of dendritic spine morphogenesis in rat hippocampal neurons. A potential involvement of miR-138 in cognition is further supported by a recent GWAS study on memory performance in a cohort of aged (> 60 years) individuals. The expression of miR-138, which is encoded in two independent genomic loci (*miR-138-1 and -2*), is subject to both cell-type and developmental stage-specific regulation, the underlying molecular mechanisms however are poorly understood. Here, we show that miR-138-2 is the primary source of mature miR-138 in developing rat hippocampal neurons. Furthermore, we obtained evidence for the regulation of miR-138-2 biogenesis at the level of primary miRNA processing. Using biochemical pull-down assays, we identified the nuclear matrix protein Matrin-3 as pri/pre-miR-138 interacting protein and mapped the interaction to the pri/pre-miR-138-2 loop region. Matrin-3 loss-of-function experiments in HEK293 cells and primary neurons together with protein localization studies suggest an inhibitory function of Matrin-3 in nuclear pri-miR-138-2 processing. Together, our experiments unravel a new mechanism of miR-138 regulation in neurons, with important implications for miR-138 regulation during neuronal development, synaptic plasticity and memory-related processes.

1. Introduction

Synaptic plasticity, experience-dependent modifications of chemical synapses in response to changes in neuronal activity, is one of the major cellular substrates of learning and memory-related processes. Synaptic plasticity encompasses both changes in the pre- and postsynaptic efficacy. The latter in particular involve morphological and functional changes at the level of dendritic spines, the major sites of postsynaptic contact on neuronal dendrites. For example, the storage of memory traces is accompanied by a long-lasting enhancement of excitatory synaptic transmission, which in turn correlates with spine enlargement and increased surface expression of ionotropic glutamate receptor complexes. Mechanistically, long-lasting forms of synaptic plasticity require the *de novo* synthesis of plasticity-related proteins, e.g. structural proteins of the postsynaptic density and neurotransmitter receptors, in response to synaptic stimulation.

We have recently uncovered an important role of microRNA (miRNA)-dependent control of synaptic protein synthesis in dendritic spine morphogenesis related to learning and memory. miRNAs are a large group (about 1000 different species in mammals) of small (21–24 nt) non-coding RNAs that act as negative regulators of protein synthesis by inhibiting mRNA translation and/or promoting mRNA decay. In order to associate with cellular mRNAs, the miRNA sequence has to display a certain degree of complementarity to the so-called target mRNA, which is particularly pronounced with the miRNA seed region (nt 2–8). Thereby, miRNAs control the expression of a specific subset (up to a few hundred) of target mRNAs. The miRNA target spectrum in a given cell type ultimately dictates the functional outcome of miRNA-dependent gene expression control. Using microarray-based microRNA profiling, we recently identified 10 mature miRNAs that are highly enriched in rat forebrain synaptosomes, a biochemical preparation that mostly contains re-sealed excitatory pre- and postsynaptic membranes. One of the synaptic candidate miRNAs was miR-138-5p, a miRNA that is highly expressed in the central and peripheral nervous system, as well as in other tissues (e.g. muscle). In the rodent brain, miR-138-5p was shown to negatively regulate the formation of fear memories (Li et al.,

* Corresponding author at: Lab of Systems Neuroscience, Institute for Neuroscience, Department of Health Science and Technology, Swiss Federal Institute of Technology, Zurich, Switzerland.
E-mail address: Gerhard.schratt@hest.ethz.ch (G. Schratt).

https://doi.org/10.1016/j.nlm.2019.02.008
Received 26 November 2018; Received in revised form 16 January 2019; Accepted 17 February 2019
Available online 18 February 2019

2018) and to have a pro-oncogenic role in glioblastoma (Chan et al., 2012). At the cellular level, brain miR-138-5p appears to be expressed in both neurons and glia cells, with particularly high expression in cortical and hippocampal pyramidal neurons (Siegel et al., 2009), motor neurons (Amin et al., 2015) and oligodendrocytes (Dugas et al., 2010). In mature hippocampal pyramidal neurons, miR-138-5p negatively regulates dendritic spine growth by targeting the depalmitoylase APT-1 (Lypla1) (Siegel et al., 2009). The repressive effect of miR-138-5p can be reversed by activity-dependent decay of Mov10, a component of the miRNA-associated gene regulatory complex (Banerjee, Neveu, & Kosik, 2009). In addition to this postsynaptic function, miR-138-5p also plays a role in axon regeneration via its interaction with the mRNA encoding the histone deacetylase SIRT1 (Liu, Wang, Saijilafu, Zhang, & Zhou, 2013). Intriguingly, higher levels of miR-138-5p in the hippocampus correlated with better memory performance in mice (Tatro et al., 2013) and humans (Schröder et al., 2014), suggesting an important role of miR-138-5p-dependent regulation of synaptic plasticity in cognition.

In addition to a regulation at the level of transcription, a number of studies have now provided robust evidence for the relevance of post-transcriptional regulatory mechanisms in the control of miRNA expression. In this regard, miRNA processing of miRNA precursor molecules, either the nuclear-localized primary miRNA transcript (pri-miRNA) or the cytoplasm-localized precursor miRNA (pre-miRNA), appear to play a particularly important role. In addition to the core processing enzymes Drosha and Dicer, a number of auxiliary proteins that either positively or negatively affect miRNA processing in different cellular contexts have been identified. Furthermore, these processing complexes are subject to intense post-translational modifications in response to extracellular stimuli. In the case of miR-138-5p, a role for precursor processing in the regulation of miR-138 expression was suggested by an earlier study by Obernosterer et al. The authors showed that miR-138-5p was expressed ubiquitously in the fetus, but was restricted to the brain in adult mice. In contrast, pre-miR-138-2, the predominant miR-138-5p precursor form during mouse development, was widely expressed in different tissues even at the adult stage. Further biochemical experiments using cellular extracts suggested that the processing of pre-miR-138-2 is blocked in tissues of non-neuronal origin, although the identity of this inhibitory activity was not further explored. In contrast, studies in the oligondendrocyte lineage suggested that miR-138-5p is mostly generated from miR-138-1 precursors in this specific cell type (Obernosterer, Leuschner, Alenius, & Martinez, 2006). Altogether, these reports indicate intricate cell-type and developmental-stage specific regulation of miR-138-5p expression at the level of precursor processing.

In this study, we attempted to explore regulatory mechanisms of miR-138-5p processing in differentiating rat hippocampal neurons with potential relevance for the control of dendritic spine development and synaptic plasticity. Using a combination of biochemical and cell biological assays, we identified the nuclear matrix protein Matrin-3 (Matr3) as novel regulator of miR-138-5p biogenesis in vitro and *in vivo*. Our findings have implications for our understanding of processes in which miR-138-5p plays an important role, including learning and memory, brain tumorigenesis and neurodegeneration.

2. Materials and methods

2.1. Primary neuronal and HEK293 cell culture

Primary neuron cultures of hippocampal and cortical cells were prepared from embryonic day 18 (E18) Sprague-Dawley rats (Charles River Laboratories) and cultured as previously described (Schratt, Nigh, Chen, Hu, & Greenberg, 2004).

Matr3 KO and control HEK293 cells were generated by gene editing using CRISPR/Cas9 technology as previously described (Treiber et al., 2017). The control is a non-edited HEK293 cell clone from the same experiment that was used to generate the Matr3 CRISPR knockout cell line. The sequence of the gRNA for the deletion of Matr3 gene is as follows: 5′-GATCTCTCTTGTAAGGGGCC. HEK293 cells were cultivated in minimum essential medium (MEM) supplemented with 10% FBS, 2 mM L-Glutamine and Penicillin/Streptomycin (Gibco) at subconfluent density.

2.2. Transfections of primary neurons and HEK293 cells

For luciferase reporter assays primary neurons were transfected using Lipofectamine 2000 (Invitrogen) as previously described (Siegel et al., 2009). The validation of shRNA efficacy and the transfection of in vitro transcribed pre-miRNAs for DIV 0 cortical neurons was performed on primary cortical neurons (E18) with the P3 Primary Cell 4D-Nucleofector kit (Lonza) using program DC-104, according to manufacturer's instruction. Transfection of DIV 12 cortical neurons with in vitro transcribed pre-miRNAs was performed using the siPORT NeoFX transfection agent (Ambion) according to manufacturer's instructions.

HEK293 cells were transfected using the calcium phosphate method as previously described (Christensen, Larsen, Kauppinen, & Schratt, 2010).

3. RNA extraction and real-time qRT-PCR

RNA extraction was performed with the mirVana miRNA isolation kit (Ambion) following the manufacturer's instructions. RNA samples were then treated with TURBO DNase (Ambion) according to manufacturer's instructions and quantity and quality of the RNA was determined using the NanoDrop 2000c spectrophotometer (Thermo Scientific). Reverse transcription was performed using the iScript cDNA synthesis kit (Bio-Rad) according to manufacturer's instructions. For detection of mature miR-138 the respective TaqMan MicroRNA Assay kit (Applied Biosystems) was used, while pre-miRNAs were detected using iTaq SYBR Green Supermix with ROX (Bio-Rad). Primer sequences are summarized below. All real-time PCRs were performed on the StepOnePlus Real-Time PCR System (Applied Biosystems).

3.1. Dual luciferase reporter assay

Luciferase assay in primary rat cortical neurons was performed with the Dual-Luciferase Reporter Assay System (Promega). DIV 12 primary cortical neurons were transfected with 50 ng of the respective pGL4 Firefly reporter and equal amount of Renilla reporter as a transfection control. Lysis was performed 2 days after transfection (DIV 14) and luciferase activity of both Firefly and Renilla was measured on a GloMax R96 Microplate Luminometer (Promega). Relative luciferase activity was then calculated as a ratio of Firefly to Renilla signal.

3.2. In vitro transcription of pre-miRNAs

Pre-miRNA for processing and pulldown experiments were in vitro transcribed using the MEGAshortscript T7 kit (Ambion) according to the manufacturer's instructions. The template sequences are summarized below. A concentration of 150 nM of the dsDNA templates was used with an incubation time of 4 h at 37 °C before RNA was purified using Illustra MicroSpin G-25 columns (GE Healthcare). Quantity and quality of the RNA was determined using the NanoDrop 2000c spectrophotometer (Thermo Scientific).

3.3. RNA pulldown

RNA pulldown experiments were performed as previously described (Bicker et al., 2013). For the identification of pre-miR-138-2 specific binding proteins the pulldown was performed with lysate from DIV5 primary cortical neurons. Pulldown with Matr3 deletion constructs was performed with lysate from HEK293 cells transfected with 1 μg/10 cm^2

dish of the respective Flag-Matr3 deletion constructs. Plasmids of Flag-Matr3 deletions were a gift from Yossi Shiloh (Addgene plasmids #32880, #32881, #32882, #32883 and #32884).

3.4. Western Blot

All Western Blots were performed as previously described (Siegel et al., 2009). All antibodies that have been used are summarized below. Subcellular fractionation was performed as previously described (Störchel et al., 2015).

3.5. Immunohistochemistry

Immunocytochemistry was performed as previously described (Siegel et al., 2009) and images were acquired with a LSM 5 Pascal (Zeiss) microscope. Antibodies that have been used are summarized below.

3.6. Plasmids and cloning

The pGL4 pri-miRNA processing sensors were created by amplifying a sequence spanning at least 100 bp on either side of the pre-miRNA-138-1 and pre-miRNA-138-2 location of the rat genome and inserting it into the 3′UTR of the pGL4.13 firefly luciferase vector (Promega) using its XbaI restriction sides. To create the pri-miR-138-2L1 construct with an exchanged loop, overlapping primers of the loop region of pri-miR-138-1 were designed and used in combination with pri-miR-138-2 amplification primers. The resulting construct was cloned into pGL4.13 as described above. All primers are summarized below.

The shRNA sequences for the knockdown of Matr3 have been designed using the Dharmacon siRNA design center (available online) and the sequences of the utilized oligonucleotides are summarized below. The annealed oligonucleotides were inserted into pSuper vector (Oligoengine), according to manufacturer's instructions.

3.7. Oligonucleotides

All sequences are given in 5′ to 3′ direction. Oligonucleotides were purchased lyophilized from Invitrogen and diluted to a stock concentration of 100 µM with nuclease-free ddH2O.

qRT-PCR:

pre-miR-138-1/2 fw	AGCTGGTGTTGTGAATCAGGC
pre-miR-138-1 rev	TGTTGTGAAGTAGCCGTTCTCTGA
pre-miR-138-2 rev	GTGAAATAGCCGGGTAAGAGGAT

templates for in vitro transcription:

pre-miR-138-1/2 promoter fw	AATTTAATACGACTCACTATAGGCTGGTGTTGTGAATCAGGCCG
pre-miR-138-1/2 promoter rev	CTGATTCACAACACCAGCCTATAGTGAGTCGTATTAAATT
pre-miR-138-1 fw	TTGCCAATCAGAGAACGGCTACTTCACAACACCAGGG
pre-miR-138-1 rev	CCCTGGTGTTGTGAAGTAGCCGTTCTCTGATTGGCAACGGC
pre-miR-138-2 fw	ACGAGCAACGCATCCTCTTACCCGGCTATTTCACGACACCAGGGT
pre-miR-138-2 rev	ACCCTGGTGTCGTGAAATAGCCGGGTAAGAGGATGCGTTGCTCGTCGGC
TAG-promoter-adapter fw	AATTTAATACGACTCACTATAGGGAGAATAGATAGT
TAG promoter-adapter rev	TCTATTCTCCCTATAGTGAGTCGTATTAAATT
TAG-pre-miR-138-1 fw 1	AGCTGGTGTTGTGAATCAGGCCGTTGCCAA
TAG-pre-miR-138-1 fw 2	TCAGAGAACGGCTACTTCACAACACCAGGGT
TAG-pre-miR-138-1 rev 1	CAACGGCCTGATTCACAACACCAGCTACTA
TAG-pre-miR-138-1 rev 2	ACCCTGGTGTTGTGAAGTAGCCGTTCTCTGATTGG
TAG-pre-miR-138-2 fw 1	AGCTGGTGTTGTGAATCAGGCCGACGAGCAACGC
TAG-pre-miR-138-2 fw 2	ATCCTCTTACCCGGCTATTTCACGACACCAGGGT
TAG-pre-miR-138-2 rev 1	GCGTTGCTCGTCGGCCTGATTCACAACACCAGCTACTA
TAG-pre-miR-138-2 rev 2	ACCCTGGTGTCGTGAAATAGCCGGGTAAGAGGATTTGG
TAG-pre-miR-150 fw 1	TCTCCCAACCCTTGTACCAGTGCT
TAG-pre-miR-150 fw 2	GTGCCTCAGACCCTGGTACAGGCCTGGGGGACA
TAG-pre-miR-150 rev 1	CTGGTACAAGGGTTGGGAGAACTA
TAG-pre-miR-150 rev 2	TGTCCCCCAGGCCTGTACCAGGGTCTGAGGCACAGCA

Matr3 shRNAs:

shRNA#1 fw 1	GATCCCCGGAAGAAGCTGCAGCACTATTCAAGAGA
shRNA#1 fw 2	TAGTGCTGCAGCTTCTTCCTTTTTA
shRNA#1 rev 1	GGGCCTTCTTCGACGTCGTGAT
shRNA#1 rev 2	AAGTTCTCTATCACGACGTCGAAGAAGGAAAAATTCGA
shRNA#2 fw 1	GATCCCCGAAGGAAACTTAAGATGTATTCAAGAGA
shRNA#2 fw 2	TACATCTTAAGTTTCCTTCTTTTTA
shRNA#2 rev 1	GGGCTTCCTTTGAATTCTACAT
shRNA#2 rev 2	AAGTTCTCTATGTAGAATTCAAAGGAAGAAAAATTCGA

Amplification primers for cloning of pri-miRNA processing constructs:

pri-138-1 fw	CGATTCTAGAAGCTCTGCAGAGCCACCTTTGGAT
pri-138-1 rev	GATAGCTAGCTGGTGTGGGGTAGGGTGGGATAAT
pri-138-2 fw	ATGCTCTAGAGGCTCCTGGTTTGGCAATCCTAGA
pri-138-2 rev	ATACGCTAGCCCCAGGTGACTATGAGGTGAGGTA
loop1 fw	ATGCCTCGAGGGCTCCTGGTTTGGCAATCCTAGA
loop1 rev	ATACGTCGACCCCAGGTGACTATGAGGTGAGGTA

3.8. Antibodies

Primary antibodies used in this study and their dilutions for Western Blot (WB) and Immunocytochemistry (IC): rabbit anti-Dicer (Sigma-Aldrich, WB 1:1000), mouse anti-Flag (Sigma-Aldrich, WB: 1:1000), chicken anti-GFP (Abcam, WB: 1:10000), rabbit anti-Matrin3 (Abcam, IC: 1:500, WB: 1:5000), mouse anti-MAP2 (Sigma-Aldrich, IC: 1:2000), rabbit anti-α-Tubulin (Cell Signaling, WB: 1:10000), rabbit anti-YBX1 (Novus Biologicals, WB: 1:5000).

Secondary antibodies for WB were species-specific, HRP-conjugated (Calbiochem) and applied at 1:20,000 dilutions.

3.9. Statistics

For each data set, three independent biological experiments were performed if not stated otherwise. Error bars represent standard deviation. P-values were calculated with Student's *t*-test (two-tailed, type 2).

4. Results

Mature miR-138-5p plays an important regulatory role in dendritic spine morphogenesis in developing rat hippocampal neurons (Siegel et al., 2009). To obtain first insight into a potential stage-specific regulation of miR-138-5p biogenesis during neuronal development, we

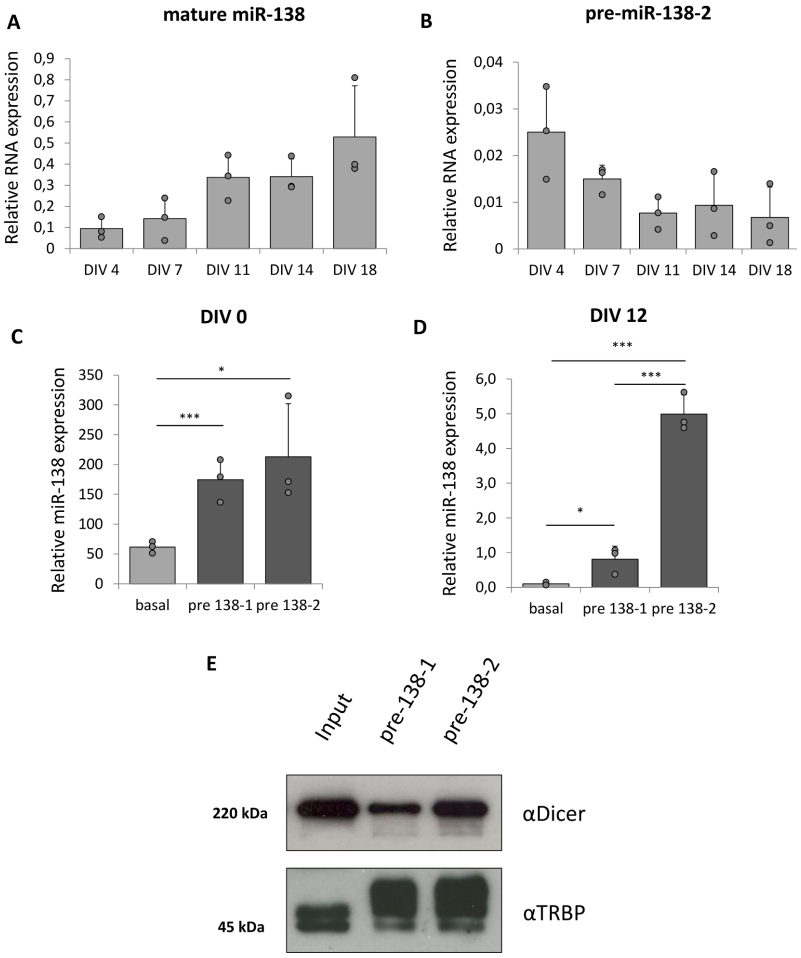

Fig. 1. pri/pre-miR-138-2 is the main source of mature miRNA-138 in mature neurons. (A, B) Expression of mature miR-138-5p (A) and pre-miR-138-2 (B) in primary cortical neurons at the indicated days in vitro (DIV) as measured by quantitative real-time PCR (qRT-PCR). Relative expression levels were calculated as a ratio of miR-138 and pre-miR-138-2 to the endogenous control U6 snRNA. N = 3 independent experiments (C) Expression levels of mature miR-138-5p in DIV 0 cortical neurons 7 h after the nucleofection of in vitro transcribed miR-138 precursors (100 nM) as measured by qRT-PCR. Relative expression was calculated as a ratio to the endogenous control U6 snRNA. (D) Expression levels of mature miR-138-5p in DIV 12 cortical neurons 7 h after in vitro transcribed miR-138 precursors (100 nM) were transfected with the siPORT NeoFX Transfection Agent (Ambion). MiR-138 levels were measured by qRT-PCR and relative expression was calculated as a ratio to the endogenous control U6 snRNA. In (C) and (D), values represent averages + standard deviations from three independent experiments. Student's *t*-test: $^{*}p < 0.05$, $^{***}p < 0.0001$. (E) Pull-down was performed with tagged in vitro transcribed pre-miR-138-1 and pre-miR-138-2 as illustrated in Fig. 2B. The Western Blot shows the binding affinity of Dicer (upper panel) and TRBP (lower panel) towards the two pre-miRNAs.

monitored the expression of mature and precursor (pre-)miR-138-1/2 expression by qPCR. We used either a stem-loop primer that is specific for mature miR-138-5p, the major product of miR-138 biogenesis based on RNAseq data, or conventional primer pairs that selectively amplify both the pre- and pri-miRNA forms (pri/pre) of miR-138-1 or miR-138-2, respectively. We observed a gradual increase of mature miR-138-5p expression in hippocampal neuron cultures, consistent with the reported function of miR-138-5p in dendritic spine development. Based on our previous fluorescent in situ hybridization experiments, miR-138-5p expression is restricted to neurons and not detectable in GFAP-positive glial cells (Siegel et al., 2009). At the level of pri/pre-miR-138, we readily detected pri/pre-miR-138-2, while pri/pre-miR-138-1 was basically undetectable (Fig. 1B). Consistent with these results, rare reads of miR-138-3p observed in RNAseq exclusively mapped to the miR-138-2, but not to the miR-138-1 gene (unpublished RNAseq experiments). We conclude that miR-138-2 is the predominant, if not exclusive source of miR-138-5p in developing rat hippocampal neurons. In contrast to mature miR-138-5p, expression of pre-miR-138-2 gradually declined during neuron development (Fig. 1A, B), consistent with enhanced processing of miR-138-2 in more mature neurons.

To further investigate potential differences in processing efficiencies between pre-miR-138-1 and pre-miR-138-2 dependent on the developmental stage of the neurons, we transfected in vitro synthesized pre-miR-138 into primary rat hippocampal neurons at different developmental stages and measured the levels of the resulting mature miR-138-5p by qPCR. In young neurons (DIV0) that express little endogenous mature miR-138-5p (Fig. 1A), mature miR-138-5p levels were indistinguishable between pre-miR-138-1 and -2 transfected neurons, indicating equal processing efficiencies of the two precursor molecules at this stage (Fig. 1C). In contrast, pre-miR-138-2 transfection yielded significantly more mature miR-138-5p compared to pre-miR-138-1 in more differentiated neurons (12 DIV; Fig. 1D). Thus, pre-miR-138-2 processing efficiency is specifically enhanced in more mature neurons. Consistent with this enhanced pre-miR-138-2 processing activity, pre-miR-138-2 interacted more strongly with Dicer in pull-down assays compared to pre-miR-138-1 (Fig. 1E). Equal pull-down efficiency of TRBP served as a loading control in these experiments.

Next, we investigated whether different miR-138 processing efficiencies in neurons might be already evident at the level of the primary miR-138-1 and -2 transcripts. Therefore, we employed a previously described *in vivo* pri-miRNA processing assay (Allegra & Mertens, 2011). In brief, primary rat hippocampal neurons were transfected with firefly luciferase reporter gene plasmids (pGL4 pri-138-1 and -2) that contain a fragment of the respective primary miR-138 transcript consisting of the pre-miRNA stem-loop and at least 100nt flanking region on each side, including the hairpin structure and adjacent sequence elements required for Drosha-dependent pri-miRNA cleavage. In addition, a plasmid was transfected in which the loop sequence within pri-miR-138-2 was replaced with the one from pri-miR-138-1 (pGL4 pri-138-2-loop1). To normalize for potential variations in transfection efficiency, a pGL4 Renilla luciferase reporter gene was co-transfected for all conditions. We observed a significantly reduced relative firefly/renilla activity for pGL4 pri-miR-138-2 compared to pri-miR-138-1, suggesting that, similar to our results obtained with pre-miR-138, pri-miR-138-2 is more efficiently processed in primary rat hippocampal neurons compared to pri-miR-138-1 (Fig. 2A). This enhanced processing of pri-miR-138-2 is due to the presence of the unique miR-138-2 loop sequence, since the pri-miR-138-2-loop1 chimera was not processed more efficiently compared to pri-miR-138-1 in this assay. Thus, miR-138 precursors containing the miR-138-2 loop structure (both pre- and pri-miR-138-2) are preferentially processed in mature rat hippocampal neurons.

Given the pivotal role of the miR-138-2 loop structure in miR-138 processing, we decided to characterize miR-138-2 interacting proteins using biochemical pull-down assays. Thereby, we hoped to obtain additional mechanistic insight into the regulation of miR-138 biogenesis in developing neurons. For pull-downs, we used streptavidin-coated agarose beads to which in vitro synthesized pre-miRNAs containing unique loop sequences (pre-miR-138-1, -2 and pre-miR-150) were attached via biotinylated adapter oligonucleotides (see Fig. 2B, left panel). Subsequently, pre-miR-coupled beads were incubated with lysate of DIV 5 primary cortical neurons, and interacting proteins were visualized by Western blot. For our initial experiments, we focused on YB1 and Matr3, since these two proteins had been shown to interact with pre-miR-138-2 in a previously published proteomics-based pull-down approach in non-neuronal cell lines (Treiber et al., 2017). Surprisingly, although we could confirm an interaction of YB1 with pre-miR-138-2, this interaction did not display any sequence-specificity, since YB1 was pulled down with equal efficiency when using pre-miR-138-1 or the unrelated pre-miR-150 as a bait (Fig. 2B, right panel). In contrast, Matr3 was highly selectively pulled down with pre-miR-138-2, but not with any of the other pre-miRNAs (Fig. 2B, right panel). Therefore, we decided to study a potential involvement of Matr3 in miR-138 biogenesis in neurons in more detail. Matr3 is a sequence-specific RNA-binding protein of the nuclear matrix. As part of the SFPQ-NONO complex, it plays a role in the nuclear retention of defective RNAs in the context of mRNA quality control (DeCerbo & Carmichael, 2005). Interestingly, MATR3 mutations are found in patients with familial ALS/FTD, a rare neurodegenerative diseases of genetic origin (Johnson et al., 2014; Malik et al., 2018). Matr3 contains a total of four RNA-binding domains, two RRM domains (RRM1&2) and two zinc finger motifs (ZnF1). In order to determine the contribution of these domains to the observed Matr3-pre-miR-138-2 interaction, we performed pull-down experiments with cell lysates from HEK293T cells that had been transfected with either wt Matr3 or Matr3 mutants lacking individual RNA-binding domains (dRRM1,2; dZnF1,2; Fig. 3A). Compared to wt Matr3, the dZnF2 mutant showed the strongest reduction in pre-miR-138-2 pull-down efficiency, indicating that the presence of an intact ZnF2 is required for the interaction. In contrast, deletion of either RRM domain had only little effect, and deletion of ZnF1 even slightly enhanced the Matr3 pre-miR-138-2 interaction (Fig. 3A).

Since the function of Matr3 in neurons is still poorly understood, we next assessed the expression of Matr3 protein at different times of neuronal differentiation in culture by Western blot. Thereby, we observed a gradual increase in Matr3 expression over time (Fig. 3B), similar to the observed time-course of mature miR-138-5p expression. This data is consistent with a potential regulatory role of Matr3 in miR-138 biogenesis in hippocampal neurons. Next, we studied the subcellular Matr3 localization in neurons using immunocytochemistry. Consistent with its reported function as nuclear matrix constituent, Matr3 was exclusively detected in neuronal nuclei (Fig. 3C). The absence of Matr3 from the cytosolic compartment was further confirmed by subcellular fractionation followed by Western blot (Fig. 3D). Our results strongly argue for an involvement of Matr3 in nuclear steps of miR-138 biogenesis, either at the level of pri-miR-138-2 processing/stability and/or pre-miR-138-2 nuclear export.

To investigate the role of Matr3 in miR-138 biogenesis more directly, we used shRNA-mediated knockdown to interfere with Matr3 function in primary rat hippocampal neurons. One out of two tested Matr3 shRNA constructs (sh1) showed a highly specific and efficient knockdown of Matr3 when nucleofected (efficiency > 90%) into primary rat cortical neurons (Fig. 4A). Co-transfection of this functional Matr3 shRNA (sh1), but not an unrelated control shRNA sequence (ctr sh), significantly reduced relative luciferase activity of pGL4 pri-miR-138-2 compared to either pGL4 pri-miR-138-1 or pGL4 pri-138-2-loop1 transfected neurons (Fig. 4B). This result suggests that endogenous Matr3 in primary rat neurons selectively inhibits miR-138-2 biogenesis at the level of pri-miR-138-2. We note however that pri-miR-138-2 compared to pri-miR-138-1 processing is more efficient in non-manipulated neurons (Fig. 2A), indicating that additional protein(s) are involved in the suppression of miR-138-1 processing, the facilitation of miR-138-2 processing, or both.

We attempted to obtain independent support for the unexpected negative regulatory role of Matr3 in pri-miR-138-2 processing. Towards this end, we used a stable HEK293T cell line that lacks Matr3 expression due to Crispr/Cas9-mediated disruption of the Mat3 open reading frame (Matr3 KO cells). First, we validated the absence of Matr3 protein expression in these cells by Western blot (Fig. 4C). Next, we assessed the effect of Matr3 deficiency on miR-138-5p expression. HEK293T cells express relatively little endogenous miR-138-5p. However, Matr3 KO cells express about 3-fold higher levels of mature miR-138-5p compared to wildtype (wt) cells (Fig. 4D), consistent with a negative function of Matr3 in miR-138 biogenesis. In contrast, pre-miR-138-2 levels were not significantly different between KO and wt cells (Fig. 4E). This might be explained by an opposite effect of Matr3 KO on the levels of pri-miR-138-2, which is also recognized by qPCR primers directed against pre-miR-138-2. In addition, higher levels of pre-miR-138-2 caused by Matr3 KO might induce cytoplasmic processing, which in turn would lead to a reduction in pre-miR-138-2 steady-state levels. The results obtained with Matr3 KO cells so far did not directly address a role for Matr3 in pri-miR-138-2 processing. Towards this end, we transfected the

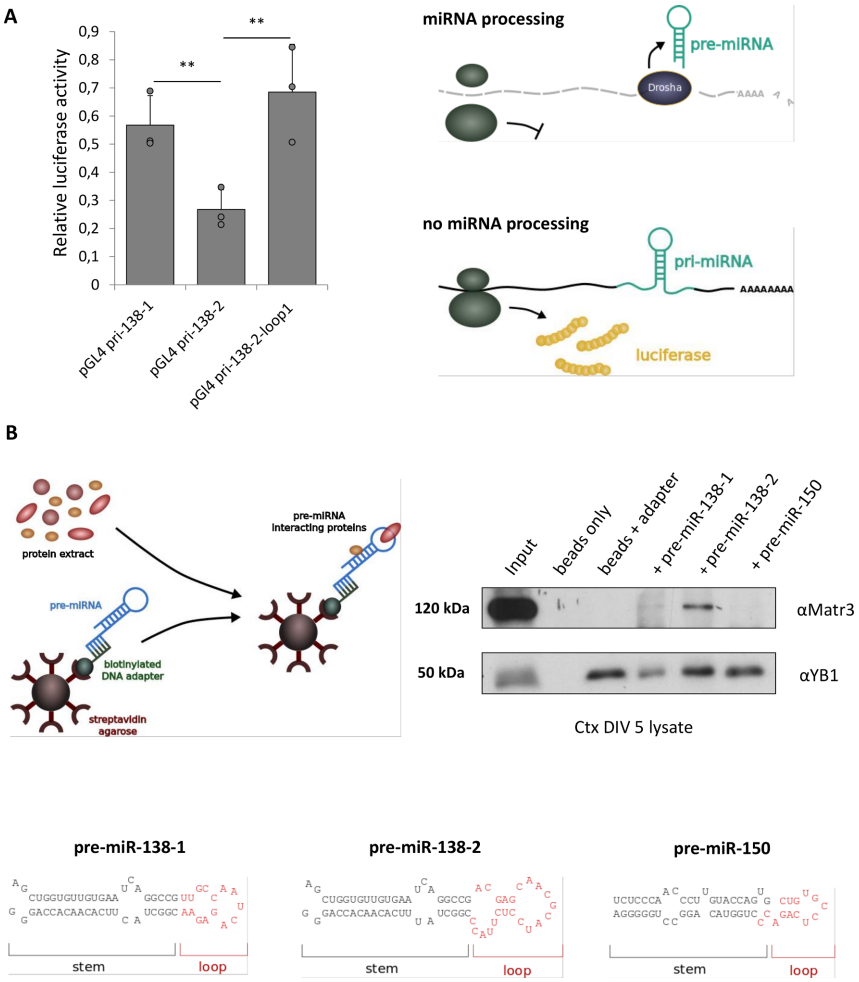

Fig. 2. The role of the pre-miR-138-2 loop in processing and identification of loop interacting proteins. (A) At DIV 12 primary cortical neurons were transfected with pri-miRNA-138 firefly processing sensors that include the indicated primary miRNA in their 3′UTR as illustrated in the right panel and renilla luciferase as a transfection control. Processing of the miRNA involves cleavage of the miRNA stem-loop by Drosha and thereby leads to a loss of the further downstream part of the luciferase 3′UTR and its poly-A tail, resulting in a destabilization of the sensor and reduced firefly luciferase expression. The left panel shows relative firefly expression levels after extraction at DIV 14 for pri-miR-138-1, pri-miR-138-2 and the chimera pri-miR-138-2-loop1, where the loop structure of pri-miR-138-2 has been exchanged with the loop of pri-miR-138-1. Relative luciferase expression is calculated as a ratio of firefly activity to renilla activity and an empty firefly reporter not including any pri-miRNA was set to 1. Values represent averages + standard deviations from three independent experiments. Student's *t*-test: $^{**}p < 0.01$. (B) The upper left panel shows the pulldown strategy used to isolate and identify pre-miRNA interacting proteins. The right panel shows a representative Western Blot for the two candidate proteins Matrin3 and YB1 as pulled down from extract of DIV5 old primary cortical neurons with the indicated pre-miRNAs. The three different pre-miRNAs used for the pulldown experiment are shown in detail in the lower panel.

described pri-miR-138-2 processing reporters – either pri-138-2 or pri-138-2L1 – into Matr3 KO and wt cells and measured relative mature miR-138-5p expression by qPCR. As expected, pri-miR-138-2 transfection into Matr3 KO cells resulted in significantly higher levels of mature miR-138-5p compared to wt cells (Fig. 4F). Importantly, the observed differential expression of miR-138-5p between KO and wt cells was reversed upon plasmid-based Matr3 overexpression (Fig. 4F), demonstrating that increased miR-138 production in Matr3 KO cells was in fact due to the absence of Matr3. The effects of Matr3 on miR-138 biogenesis required the miR-138-2 loop region, since mature miR-138 levels were insensitive to either Matr3 KO or Matr3 overexpression upon transfection of pri-138-2loop1 (Fig. 4G).

In conclusion, we identified here a novel regulatory mechanism for the control of miR-138 expression in neurons (Fig. 5). This mechanism has potential implications for activity-dependent neuronal development, synaptic plasticity and memory-related processes.

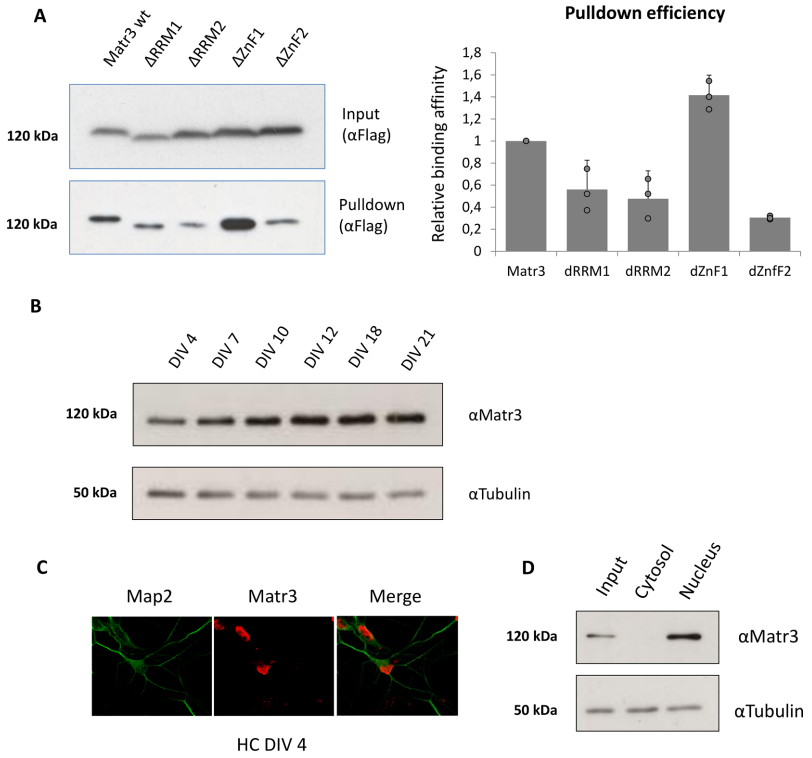

Fig. 3. Mapping of Matrin3 binding and expression of Matrin3 in neurons. (A) Full-length Matr3-Flag construct and Matr3 deletion mutants missing the indicated RNA-binding domains (RRM, ZnF) were transfected into HEK293 cells. Two days after transfection the extracts were used in a pulldown experiment with in vitro transcribed pre-miR-138-2. The left panel shows a representative anti-Flag Western Blot showing the input levels of Matr3 mutants and the bound Matr3 after pulldown. The right panel shows the quantification of the relative binding affinity, calculated as a ratio of bound Matr3 after pulldown and the input with full-length Matr3 set to 1. The values represent averages + standard deviations from three independent experiments. (B) anti-Matr3 Western Blot in primary cortical neurons at the indicated days in vitro (DIV). Tubulin was used as a loading control. (C) Immunohistochemistry showing Matr3 expression (red) in hippocampal neurons DIV 4. Map2 (green) was used to visualize the neurons. (D) anti-Matr3 Western Blot using whole-cell extract (input) and cytosolic and nuclear fractions of primary hippocampal neurons harvested at DIV 7. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

5. Discussion

miR-138-5p is encoded in two different genomic loci (miR-138-1, miR-138-2), and our results from qPCR with precursor-specific primers indicate that the miR-138-2 gene is the primary source of miR-138-5p expression in developing rat hippocampal neurons. Our results are in agreement with previous findings (Obernosterer et al., 2006), who were able to detect robust expression of the miR-138-2 pre-miRNA in brain tissue by Northern blot, whereas pre-miR-138-1 was almost undetectable. On the other hand, two other recent studies (axon regeneration, oligodendrocyte function) detected abundant expression of pre-miR-138-1 and the regulation of miR-138-1 transcription in different types of brain cells (Dugas et al., 2010; Liu et al., 2013). In general, these studies provide further support for the idea that duplication of miRNA loci might endow organisms with the capability of cell-type and developmental-stage specific control of miRNA expression due to the presence of specific regulatory elements that flank the respective miRNA gene. In the future, it will be important to elucidate the key regulatory elements that control miR-138-1/2 transcription in a cell-type/developmental stage-specific manner. In addition, miR-138-5p might be subject to regulation at the level of the mature miRNA by targeted-directed miRNA degradation (TDMD) (de la Mata et al., 2015).

A previous study postulated the existence of negative regulatory factors, likely proteins, that inhibit the processing of pre-miR-138-2 into mature miR-138-5p in non-neuronal cells (Obernosterer et al., 2006). While our results obtained from primary neurons and HEK293T cells are mostly consistent with these findings, we in addition observed intrinsic differences in the processing efficiencies between pre/pri-miR-138-1 and -2 in developing neurons. Based on these results, we suggest that, in addition to non-neuronal factor(s) inhibiting pre-miR-138-2 processing, positive regulatory factors which bind to the miR-138-2 loop promote processing of miR-138-2 in mature neurons, e.g. by preferentially recruiting Dicer to the pre-miR-138-2 sequence. This could explain the observation that miR-138-5p levels increase during hippocampal neuron synaptogenesis. Whether and how this regulatory mechanism is subject to control by neuronal activity remains to be determined.

Our biochemical quest for proteins interacting specifically with the miR-138-2 loop sequence surprisingly led to the discovery of a nuclear protein, Matr3. Matr3 loss-of-function studies in both neurons and HEK293T cells provided robust evidence for a negative regulatory role of Matr3 in the processing of pri-miR-138-2 in the nucleus. Given the

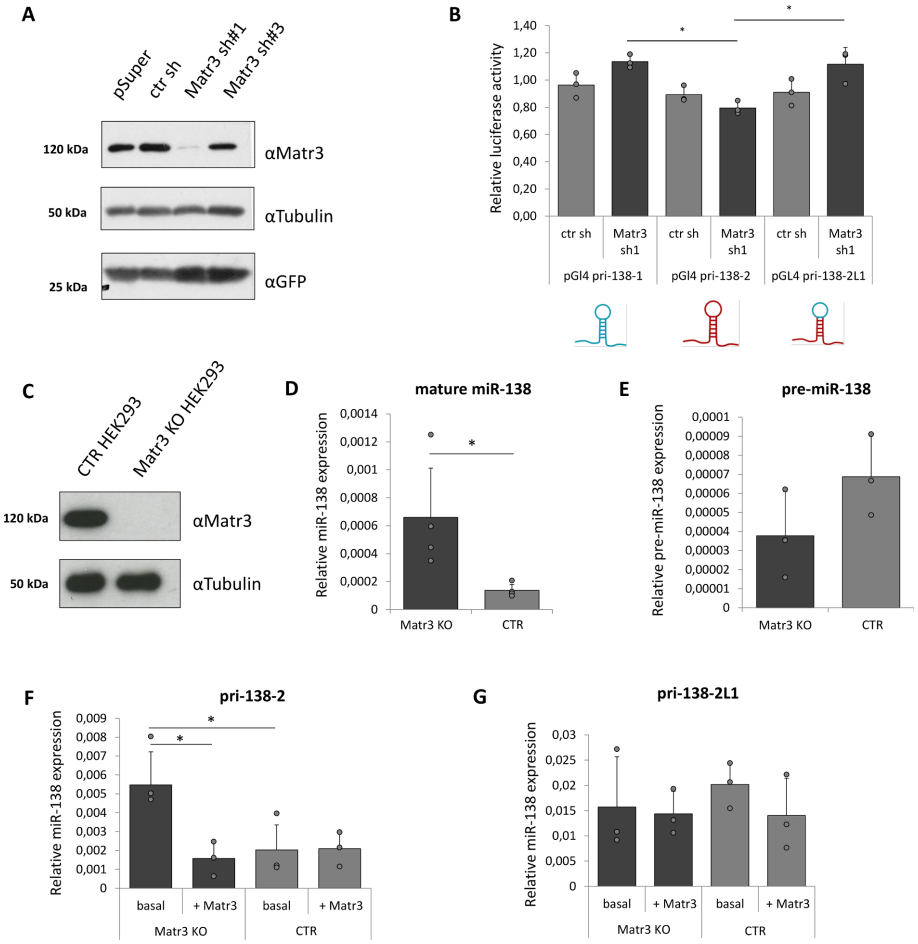

Fig. 4. The function of Matr3 in miR-138 processing. (A) anti-Matr3 Western Blot for the validation of Matr3 shRNAs. Cortical neurons were nucleofected with shRNA expressing pSuper plasmids and GFP as a transfection control. Protein extracts were prepared three days after nucleofection (DIV 3). Tubulin was used as a loading control. (B) Matr3 shRNA#1 (which showed an efficient knockdown) and a control shRNA were co-transfected with the pre-miR-138 firefly processing sensors and renilla as a transfection control into primary cortical neurons at DIV 12. Extracts were prepared after 2 days of expression on DIV 14. Relative luciferase expression was calculated as the firefly/renilla ratio and a condition containing empty pSuper vector was set to 1 for each pre-miR-138 processing sensor. The values represent averages + standard deviations from three independent experiments. (C) Validation of Matr3 knockout in HEK293 Matr3-Ko cells by anti-Matr3 Western Blot. Tubulin was used as a loading control. (D, E) Expression of mature miR-138 (D) and pre-miR-138-2 (E) in Matr3-KO and control cells as measured by quantitative real-time PCR (qRT-PCR). Relative expression levels were calculated as a ratio of miR-138 and pre-miR-138-2 to the endogenous control U6 snRNA. The values represent averages + standard deviations from three independent experiments. Student's *t*-test: $^{*}p < 0.05$. (F, G) Matr3 KO and ctr cells were transfected with pre-miR-138-2 (F) and pre-miR-138-2loop1 (G) expressing plasmids either alone or together with a Matr3 expressing plasmid. Mature miR-138-5p levels were measured by quantitative real-time PCR (qRT-PCR). Relative expression levels were calculated as a ratio of miR-138 to U6 snRNA. Values represent averages + standard deviations from three independent experiments. Student's *t*-test: $^{*}p < 0.05$.

(almost) exclusive localization of Matr3 in the nucleus, we consider it unlikely that Matr3 participates in the regulation of pre-miR-138-2 processing under basal conditions. However, a recent study demonstrated that specific domains of Matr3 are involved in the cytoplasmic localization of the protein (Rajgor, Hanley, & Shanahan, 2016), raising the possibility that Matr3 might participate in cytoplasmic regulation of pre-miR-138-2 under specific conditions, e.g. in response to stress. There are however no constrains in export of pre-miR-138-2 from the nucleus to the cytosol, since pre-miR-138-1 and -2 nuclear export was similarly efficient in *Xenopus laevis* oocyte export assays (K.W., G.S., T. Ziegenhals and U. Fischer, unpublished observation). Nevertheless, Matr3 has been characterized as a component of the SFPQ-Nono RNA quality control pathway, raising the possibility that Matr3 could be involved in the retention of specifically modified pre-miR-138-2 in the nucleus. Matr3 is highly expressed at all stages of neuronal development and also in non-neuronal cells, suggesting that differences in

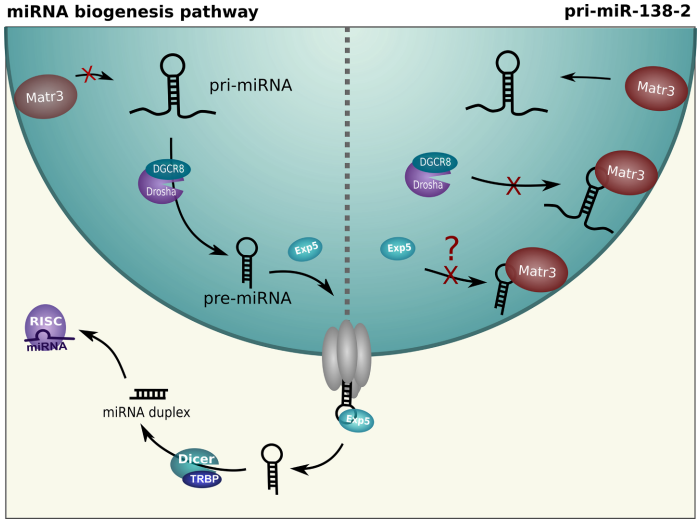

Fig. 5. Model of Matrin-3 function in the regulation of miR-138 expression. The canonical miRNA biogenesis pathway involves cleavage of the pri-miRNA by Drosha/DGCR8 in the nucleus, resulting in the generation of the pre-miRNA stem-loop with a 5′ overhang that is recognized by Exportin5 (Exp5). Upon binding of Exp5, the pre-miRNA is then transported into the cytoplasm where it is further processed by a Dicer complex into a miRNA duplex. One of the strands of this duplex (the mature miRNA) is then incorporated into the RNA-induced-silencing complex (RISC) where it becomes functional and assumes its role in gene silencing. The nuclear matrix protein Matr3 is able to bind the loop of pri- and pre-miR-138-2 specifically and thereby might hinder the processing of the pri-miRNA into the pre-miRNA form by Drosha/DGCR8 and/or prevent the further export of the pre-miRNA-138-2 into the cytoplasm.

Matr3 expression alone do not account for the differences in pre-miRNA processing efficiency and mature miR-138-5p expression between different cell types and developmental stages. However, since Matrin-3 is degraded in neurons in response to NMDA-R activation in a PKA-dependent fashion (Giordano et al., 2005), one could speculate that neuronal-activity-degradation of Matr3 could be involved in the relief of processing inhibition, enabling the synthesis of more mature miR-138-5p that could fulfil its regulatory activity in mature neurons, e.g. during spine development. It will be interesting to test whether Matr3 mutants deficient for specific phosphorylation sites are able to reverse the miR-138 processing block that is observed in Matr3 ko HEK293T cells.

Our observation that Matr3 knockdown in primary neurons only has a relatively subtle effect on pri-miR-138-2 processing suggests that other pri-miRNA interacting proteins participate in the control of processing. To elucidate these proteins, we have already embarked in more unbiased proteomics approaches, which led to the identification of several new candidate proteins that interact with the miR-138-2 sequence in a loop-dependent manner (K.W., G.S., unpublished observation). In addition, a recent large-scale proteomics approach identified a number of pre-miR-138-2 interacting proteins, including ZNF106, DDX49, YBX1-3, USP36, among others. Currently, we are testing a functional involvement of the identified proteins at various stages along the miR-138 biogenesis pathway. With regard to Matr3, we already obtained evidence for a functional importance of specific Matr3 domains. Whereas deletion of both RRM domains only slightly reduced the association with pre-miR-138-2, deletion of ZnF2 almost completely abolished the association, suggesting that this domain is necessary for pri-miR-138-2 processing. Intriguingly, a very recent study explored the function of the different Matr3 RNA-binding domains in the context of neurodegeneration (ALS; (Malik et al., 2018). There, it was shown that Matr3-mediated neurotoxicity was largely dependent on the presence of ZnF2, the same domain that we found to be required for the pre-miR-138-2 Matr3 interaction. Thus, there is a strong correlation between the strength of the Matr3 pre-miR-138-2 interaction and neurotoxicity, raising the possibility that the association of pri-miRNA-138-2 with Matrin-3 and the subsequent inhibition of miR-138-5p production could promote neurotoxicity. In this regard, it is worth mentioning that miR-138-5p antagonizes axon regeneration by targeting SIRT1 (Liu et al., 2013), and that miR-138-5p is the 2nd most abundant miRNA in motor neurons (Amin et al., 2015), both findings that are consistent with a role of miR-138 in motor neuron axons. In the future, it will be interesting to test whether interfering with miR-138 activity, e.g. by antisense approaches, could be beneficial in Matr3-associated neuropathies, in particular ALS. The investigation of several identified Matr3 point mutations with respect to their ability to interact with miR-138 could be an important first step towards this aim.

Finally, it is intriguing to speculate that the regulation of miR-138 processing by Matr3 and potentially other factors could be involved in the activity-dependent regulation of synaptic plasticity and learning and memory. For example, pre-miR-138-2 is significantly enriched in dendrites of rat hippocampal neurons (Bicker et al., 2013), suggesting that local miR-138 production could be controlled at the level of precursor processing, similar to other dendritic miRNAs (Bicker et al., 2013; Sambandan et al., 2017). In humans, miR-138 levels appear to correlate with cognitive abilities (Schröder et al., 2014) and are also affected during cognitive decline (Tatro et al., 2013). Furthermore, miR-138 levels are downregulated in the striatum of transgenic mouse models of Huntington's disease (Lee et al., 2011). We therefore hypothesize that at least part of these expression impairments could be due to defects in miR-138 processing efficiency. Thus, further insight into the mechanisms of miR-138 processing promises to contribute to our understanding of memory-related processes and cognitive impairment. On the other hand, manipulating miR-138 processing efficiency might represent a valuable strategy for the therapy of neurodegenerative diseases, including ALS.

Acknowledgment

We are very grateful to U. Beck, E. Becker, R. Gondrum, G. Jarosch, H. Kaiser and B. Kowalski for excellent technical assistance. This work was funded in part by a grant from the Deutsche Forschungsgemeinschaft DFG to G.S. (SCHR 1136/8-1).

Conflict of interest

The authors declare no conflict of interest

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.nlm.2019.02.008.

References

Allegra, D., & Mertens, D. (2011). In-vivo quantification of primary microRNA processing by Drosha with a luciferase based system. *Biochemical and Biophysical Research Communications, 406*(4), 501–505.

Amin, N. D., Bai, G., Klug, J. R., Bonanomi, D., Pankratz, M. T., Gifford, W. D., et al. (2015). Loss of motoneuron-specific microRNA-218 causes systemic neuromuscular failure. *Science (New York, NY), 350*(6267), 1525–1529.

Banerjee, S., Neveu, P., & Kosik, K. S. (2009). A coordinated local translational control point at the synapse involving relief from silencing and MOV10 degradation. *Neuron, 64*(6), 871–884.

Bicker, S., Khudayberdiev, S., Weiß, K., Zocher, K., Baumeister, S., & Schratt, G. (2013). The DEAH-box helicase DHX36 mediates dendritic localization of the neuronal precursor-microRNA-134. *Genes & Development, 27*(9), 991–996.

Chan, X. H. D., Nama, S., Gopal, F., Rizk, P., Ramasamy, S., Sundaram, G., et al. (2012). Targeting glioma stem cells by functional inhibition of a prosurvival oncomiR-138 in malignant gliomas. *Cell Reports, 2*(3), 591–602.

Christensen, M., Larsen, L. A., Kauppinen, S., & Schratt, G. (2010). Recombinant adeno-associated virus-mediated microRNA delivery into the postnatal mouse brain reveals a role for miR-134 in dendritogenesis in vivo. *Frontiers in Neural Circuits, 3*, 16.

de la Mata, M., Gaidatzis, D., Vitanescu, M., Stadler, M. B., Wentzel, C., Scheiffele, P., et al. (2015). Potent degradation of neuronal miRNAs induced by highly complementary targets. *EMBO Reports, 16*(4), 500–511.

DeCerbo, J., & Carmichael, G. G. (2005). Retention and repression: Fates of hyperedited RNAs in the nucleus. *Current Opinion in Cell Biology, 17*(3), 302–308.

Dugas, J. C., Cuellar, T. L., Scholze, A., Ason, B., Ibrahim, A., Emery, B., et al. (2010). Dicer1 and miR-219 Are required for normal oligodendrocyte differentiation and myelination. *Neuron, 65*(5), 597–611.

Giordano, G., Sánchez-Pérez, A. M., Montoliu, C., Berezney, R., Malyavantham, K., Costa, L. G., et al. (2005). Activation of NMDA receptors induces protein kinase A-mediated phosphorylation and degradation of matrin 3. Blocking these effects prevents NMDA-induced neuronal death. *Journal of Neurochemistry, 94*(3), 808–818.

Johnson, J. O., Pioro, E. P., Boehringer, A., Chia, R., Feit, H., Renton, A. E., et al. (2014). Mutations in the Matrin 3 gene cause familial amyotrophic lateral sclerosis. *Nature Neuroscience, 17*(5), 664–666.

Lee, S.-T., Chu, K., Im, W.-S., Yoon, H.-J., Im, J.-Y., Park, J.-E., et al. (2011). Altered microRNA regulation in Huntington's disease models. *Experimental Neurology, 227*(1), 172–179.

Li, D.-W., Liu, J.-Z., Li, S.-C., Yang, J.-B., Sun, H.-H., & Wang, A.-H. (2018). Fear conditioning downregulates miR-138 expression in the hippocampus to facilitate the formation of fear memory. *NeuroReport, 29*(16), 1418–1424.

Liu, C.-M., Wang, R.-Y., Saijilafu, Jiao Z-X, Zhang, B.-Y., & Zhou, F.-Q. (2013). MicroRNA-138 and SIRT1 form a mutual negative feedback loop to regulate mammalian axon regeneration. *Genes & Development, 27*(13), 1473–1483.

Malik, A. M., Miguez, R. A., Li, X., Ho, Y.-S., Feldman, E. L., & Barmada, S. J. (2018). Matrin 3-dependent neurotoxicity is modified by nucleic acid binding and nucleocytoplasmic localization. *eLife, 7*.

Obernosterer, G., Leuschner, P. J. F., Alenius, M., & Martinez, J. (2006). Post-transcriptional regulation of microRNA expression. *RNA (New York, NY), 12*(7), 1161–1167.

Rajgor, D., Hanley, J. G., & Shanahan, C. M. (2016). Identification of novel nesprin-1 binding partners and cytoplasmic matrin-3 in processing bodies. *Molecular Biology of the Cell, 27*(24), 3894–3902.

Sambandan, S., Akbalik, G., Kochen, L., Rinne, J., Kahlstatt, J., Glock, C., et al. (2017). Activity-dependent spatially localized miRNA maturation in neuronal dendrites. *Science (New York, NY), 355*(6325), 634–637.

Schratt, G. M., Nigh, E. A., Chen, W. G., Hu, L., & Greenberg, M. E. (2004). BDNF regulates the translation of a select group of mRNAs by a mammalian target of rapamycin-phosphatidylinositol 3-kinase-dependent pathway during neuronal development. *The Journal of Neuroscience the Official Journal of the Society for Neuroscience, 24*(33), 7366–7377.

Schröder, J., Ansaloni, S., Schilling, M., Liu, T., Radke, J., Jaedicke, M., et al. (2014). MicroRNA-138 is a potential regulator of memory performance in humans. *Frontiers in Human Neuroscience, 8*, 501.

Siegel, G., Obernosterer, G., Fiore, R., Oehmen, M., Bicker, S., Christensen, M., et al. (2009). A functional screen implicates microRNA-138-dependent regulation of the depalmitoylation enzyme APT1 in dendritic spine morphogenesis. *Nature Cell Biology, 11*(6), 705–716.

Störchel, P. H., Thümmler, J., Siegel, G., Aksoy-Aksel, A., Zampa, F., Sumer, S., et al. (2015). A large-scale functional screen identifies Nova1 and Ncoa3 as regulators of neuronal miRNA function. *The EMBO Journal, 34*(17), 2237–2254.

Tatro, E. T., Risbrough, V., Soontornniyomkij, B., Young, J., Shumaker-Armstrong, S., Jeste, D. V., et al. (2013). Short-term recognition memory correlates with regional CNS expression of microRNA-138 in mice. *The American Journal of Geriatric Psychiatry Official Journal of the American Association for Geriatric Psychiatry, 21*(5), 461–473.

Treiber, T., Treiber, N., Plessmann, U., Harlander, S., Daiß, J.-L., Eichner, N., et al. (2017). A compendium of RNA-binding proteins that regulate MicroRNA biogenesis. *Molecular Cell, 66*(2), 270–284.e13.

List of Abbreviations

3'UTR	3' untranslated region
AFM	atomic force microscopy
ALS	amyotrophic lateral sclerosis
APT1	acyl-protein-thioesterase 1
APV	2-amino-5-phosphonopentanoic acid
AGO	Argonaute protein
BC1	brain cytoplasmic RNA 1
BDNF	Brain-derived neurotrophic factor
Cas9	CRISPR-associated protein 9
ceRNA	competing endogenous RNA
CFC	contextual fear conditioning
CRISPR	clustered regularly interspaced short palindromic repeats
ctr	control
DCP1B	mRNA-decapping enzyme 1B
DEAH box	Asp-Glu-Ala-His box
DGCR8	DiGeorge Syndrome Critical Region 8
DHX	DEAH box protein
DIV	days *in vitro*
EMSA	electrophoretic mobility shift assay
GFP	green fluorescent protein
GW182	glycine-tryptophan protein of 182 kDa
HEK293	human embryonic kidney 293
HIV-1	Human immunodeficiency virus 1
KCl	potassium chloride

kDa	kilodaltons
KO	knockout
KSRP	Far upstream element-binding protein 2
LimK1	LIM domain kinase 1
Matr3	Matrin3
Mef2	Myocyte enhancing factor 2
miRISC	miRNA induced silencing complex
miRNA	microRNA
MOV10	Moloney leukemia virus 10
mRNA	messenger RNA
NMDA	N-Methyl-D-aspartic acid
NMDAR	NMDA receptor
NONO	Non-POU domain-containing octamer-binding protein
nt	nucleotide
PKA	protein kinase A
pre-miRNA	precursor miRNA
pri-miRNA	primary miRNA
PUM2	Pumilio 2
qRT-PCR	quantitative reverse transcription polymerase chain reaction
RBP	RNA binding protein
RELN	Reelin
RhoA	Ras homolog gene family member A
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
RRM	RNA recognition motif
shRNA	short hairpin RNA
SIRT1	Sirtuin-1
SFPQ	Splicing factor, proline- and glutamine-rich
TDP-43	TAR DNA-binding protein 43
TRBP	transactivation-responsive RNA binding protein
Ube3a	ubiquitin ligase E3A

SNP	single nucleotide polymorphism
wt	wildtyp
YB1	Y box binding protein 1
ZnF	Zinc finger domain

List of Academic Teachers

My academic teachers at the Ruprecht-Karls University Heidelberg were:

Bading
Bartram
Bengston
Braack
Braunbeck
Brunner
Bukau
Ciccolini
Clayton
Denk
Diederichs
Dobberstein
Doye
Draguhn
Euler
Frings
Großhans
Hell
Helmstädter
Holstein
Klingmüller
Koch
Kuner
Mayer
Möhrlen
Nave
Nickel
Niers
Pollerberg
deKieviet
Rausch
Robinson
Rossner
Schenkel
Schratt
Schreiber
Schuhmacher
Schuster
Spanagel
Stöcklin
Storch
Unsicker
Wittbrodt

Acknowledgments

First and foremost I would like to express my gratitude to Prof. Dr. Gerhard Schratt for offering me a great project and scientific environment, for providing me with enough guidance to learn from his immense experience but also enough freedom and trust to grow on my own.

An invaluable part of this environment was the team I had the pleasure and honor to work with: Roberto, thank you for your professional and personal support from the very beginning, you are a big part of why I started being "a little less useless". A special thank you also goes to Silvia, who allowed me to contribute to her fascinating project that is part of this thesis, who made me an experienced proof-reader and was someone I could always look up to (not only because of her size). Anna, Ayla, Federico, Gabi, Helena, Jeremy, Jule, Marek, Martin, Michi, Peter, Reeta, Reuben, Sharof and Tati it would be hard to find a better group of colleagues and friends to work with. You all are amazing people and each of you helped me to grow professionally and personally in your own unique ways. It was a pleasure to share not only a workplace but also a part of my life with all of you.

The same is true for Christian, Birgit, Cecilia and Bernd, our teaching staff, whose support made the first steps into teaching so much easier to handle, as well as Bettina, Eva, Heinrich, Karl-Heinz, Renate, Ute and Traudel, who welcomed us in Marburg with opened arms, kept everything organized and provided so much more than just excellent cells and technical support. My gratitude also goes to Prof. Marco Rust and Sophie and Lara from his lab for their support, advice and valuable discussions.

On a personal level, I can not express enough thanks to Matthias, who came to Marburg with me, was always by my side and supported me with an incredible patience. I'm extremely grateful to have you in my life.

Zuletzt ein Dank an meine ganze Familie und besonders natürlich meinen Vater, der mir von klein auf immer den Rückhalt gegeben hat meine Träume zu verfolgen, nie an mir gezweifelt hat und mich zu der Person gemacht hat, die ich heute bin. Danke, dass du bis zuletzt immer für mich da warst. Im Herzen bist du heute noch immer hier bei mir und begleitest mich auf meinem Weg.

Thank you! Danke! Grazie! Obrigada! Vďaka! Tesekkür ederim!
Merci! Efcharis‘to! Rahmat! Mulțumesc!