#### PRDM16-DT: A Brain and Astrocyte-Specific lncRNA Implicated in Alzheimer's Disease

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Cells" (MBExC), University of Göttingen, Germany<br>Department of Psychiatry and Psychotherapy, University Medical Center Göttingen, Göttingen,<br>Germany<br>Abstract The Theory of Phychiatry and Psychotherapy,<br>Thermany<br>Thermany<br>Abstract provide crucial support for neur  $\overline{ }$ 

#### Abstract

Dermany<br>Department of Psychotal Center of Psychotal Center of Psychotherapy, University Medical Center Göttingen, Göt<br>Depending and Department of Psychotherapy, Under Databological Conditions deregulation of Semany<br>Abstract<br>Astrocyte<br>maintena |
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| maintenance, and neurotransmitter recycling. Under pathological conditions, deregulation of astrocytes contributes to neurodegenerative diseases such as Alzheimer's disease (AD), highlighting the growing interest in target maintenance, and neurotransmitter recycling. These pathological conditions, actogramously<br>astrocytes contributes to neurodegenerative diseases such as Alzheimer's disease (AD), highlighting<br>the growing interest in targetin astrocyte function to address early phases of AD pathogenesis.<br>While most research in this field has focused on protein-coding genes, non-coding RNAs, particularly<br>long non-coding RNAs (lncRNAs), have emerged as significan While most research in this field has focused on protein-coding genes, non-coding RNAs, particularly<br>long non-coding RNAs (lncRNAs), have emerged as significant regulatory molecules. In this study, we<br>identified the lncRNA Nong non-coding RNAs (IncRNAs), have emerged as significant regulatory molecules. In this study, we<br>identified the IncRNA *PRDM16-DT* as highly enriched in the human brain, where it is almost<br>exclusively expressed in astro identified the lncRNA *PRDM16-DT* as highly enriched in the human brain, where it is almost exclusively expressed in astrocytes. *PRDM16-DT* and its murine homolog, *Prdm16os*, are downregulated in the brains of AD patient exclusively expressed in astrocytes. *PRDM16-DT* and its murine homolog, *Prdm16os*, are<br>downregulated in the brains of AD patients and in AD models. In line with this, knockdown of<br>*PRDM16-DT* and *Prdm16os* revealed its exclusively expressed in astrocytes. *PRDM16-DT* and its mume homolog, *Prum1605*, are<br>downregulated in the brains of AD patients and in AD models. In line with this, knockdown of<br>*PRDM16-DT* and *Prdm16os* revealed its cr down-guide in the brains of AD patients and in AD models. The brains and supporting neuronal function by regulating genes essential for glutamate uptake, lactate release, and neuronal spine density through interactions wit PRDM16-DT and Prom160s revealed its critical role in maintaining astrocyte homeostasis and<br>supporting neuronal function by regulating genes essential for glutamate uptake, lactate release,<br>and neuronal spine density throug supporting neuronal spine density through interactions with the RE1-Silencing Transcription factor (Rest)<br>and Polycomb Repressive Complex 2 (PRC2). Notably, CRISPR-mediated overexpression of *Prdm16os*<br>mitigated functional and Polycomb Repressive Complex 2 (PRC2). Notably, CRISPR-mediated overexpression of *Prdm16os*<br>mitigated functional deficits in astrocytes induced by stimuli linked to AD pathogenesis. These and Polycomb Repressive Complex 2 (PRC2). Notably, CRISPR-mediated overexpression of Pram16os<br>mitigated functional deficits in astrocytes induced by stimuli linked to AD pathogenesis. These<br>findings underscore the importa mingated functional definition in added by contact to  $\mu$  pathogenesis indices in a finding underscore the importance of *PRDM16-DT* in astrocyte function and its potential as a novel<br>therapeutic target for neurodegenerat therapeutic target for neurodegenerative disorders characterized by astrocyte dysfunction therapeutic target for neurodegenerative disorders characterized by astrocyte dysfunction  $\mathcal{L}_{\mathcal{S}}$ 

# $\overline{a}$ Introduction

 $\frac{1}{2}$ Astrocytes are abundant cell types in the central nervous system (CNS). Under physiological conditions, they provide trophic and metabolic support for neurons, and are crucial for synaptogenesis, synapse maintenance, and s example, they provide traphic and metabolic support for neurons, and support synaptogenesis, synapse maintenance, and synaptic pruning. Additionally, they play roles in neurotransmitter recycling and maintaining the bloodsynaptor and maintaining the blood-brain barrier, ion, pH, and fluid homeostasis<br>
[1] [2] [3]. In response to CNS damage, such as injury or disease, astrocytes can undergo<br>
morphological, molecular, and functional changes, neuron mainter recycling and maintaining the blood-brain barrier, i.e., physical characteristics<br>[1] [2] [3]. In response to CNS damage, such as injury or disease, astrocytes can undergo<br>morphological, molecular, and funct morphological, molecular, and functional changes, a process often referred to as 'reactive<br>astrocytosis' [4]. Depending on various factors, including the pathological context, affected astrocyte<br>subpopulations, and environ astrocytosis' [4]. Depending on various factors, including the pathological context, affected astrocyte<br>subpopulations, and environmental conditions, the adopted states can be either detrimental, where<br>astrocytes contribut subpopulations, and environmental conditions, the adopted states can be either detrimental, where subpopulations, and entity distributed conditions, the adopted states can be either detrimental, increased as<br>astrocytes contribute to neuroinflammatory processes while neuronal support function is<br>compromised, or benefici

compromised, or beneficial, acting in a neuroprotective manner [4].<br>There is increasing awareness of the role of astrocytes in neurodegenerative diseases such as<br>Alzheimer's disease (AD), as genes expressed in astrocytes h There is increasing awareness of the role of astrocytes in neuro<br>Alzheimer's disease (AD), as genes expressed in astrocytes have<br>pathogenesis. Moreover, atrophic and reactive astrocytes have been Alzheimer's disease (AD), as genes expressed in astrocytes have been genetically linked to AD pathogenesis. Moreover, atrophic and reactive astrocytes have been described in postmortem brain tissue from AD patients and in pathogenesis. Moreover, atrophic and reactive astrocytes have been described in postmortem brain<br>tissue from AD patients and in AD models [5] [6] [7] [8] [9]. Recent studies suggest a scenario in<br>which activated microglia pathogenesis. Moreover, and in AD models [5] [6] [7] [8] [9]. Recent studies suggest a scenario in which activated microglia release interleukin 1a (IL1a), tumor necrosis factor alpha (Tnfa), and complement component 1q (C which activated microglia release interleukin 1a (IL1a), tumor necrosis factor alpha (Tnfa), and<br>complement component 1q (C1q), driving astrocytes to a neurotoxic phenotype that contributes to<br>synaptic dysfunction and neur complement component 1q (C1q), driving astrocytes to a neurotoxic phenotype that contributes to<br>synaptic dysfunction and neuronal cell death [3]. Similar results have been observed when<br>astrocytes were exposed to amyloid b synaptic dysfunction and neuronal cell death [3]. Similar results have been observed when<br>astrocytes were exposed to amyloid beta 42 (Abeta24) [10] [11]. However, the molecular<br>mechanisms underlying the formation of neurot synaptic dystamlent and neutronal cell death  $[3]$ . Then the death of the molecular mechanisms underlying the formation of neurotoxic astrocytic states are only beginning to emerge.<br>A better understanding of these process mechanisms underlying the formation of neurotoxic astrocytic states are only beginning to emerge.<br>A better understanding of these processes could facilitate the development of therapeutic<br>approaches towards neurodegenerati A better understanding of these processes could facilitate the development of therapeutic<br>approaches towards neurodegenerative diseases.<br>In this context, basic and translational research in the past decades has mainly focu

A better understanding of these processes.<br>
A approaches towards neurodegenerative diseases.<br>
In this context, basic and translational research in the past decades has mainly focused on the coding<br>
part of the genome, henc In this context, basic and translational research in<br>part of the genome, hence genes that are translate<br>genome encodes for proteins while most of In this content, basic and the basic and translated into proteins. However, only 1,5% of the human<br>genome encodes for proteins while most of the transcriptome represents non-coding RNAs<br>(ncRNAs), of which the majority is c part of the general are translated into proteins that are presents non-coding. RNAs<br>genome, encodes for proteins, while most of the transcriptome, represents non-coding, RNAs<br>(ncRNAs), of which the majority is classified a

(ncRNAs), of which the majority is classified as long-non-coding RNAs (lncRNAs) [12].<br>IncRNAs are a heterogenous group of RNA molecules with a length of more than 300 nucleotides<br>that lack the potential to code for protein (ncRNAs are a heterogenous group of RNA molecules with a length of more than<br>that lack the potential to code for proteins [13] [14]. Initially, many of these non-co<br>been regarded as transcriptional noise. However, in the l that lack the potential to code for proteins [13] [14]. Initially, many of these non-coding RNAs have been regarded as transcriptional noise. However, in the last years, it has become apparent that been regarded as transcriptional noise. However, in the last years, it has become apparent that

many lncRNAs are indeed functional RNA molecules that can play important roles in different<br>biological processes [14] [15]. A significant proportion of lncRNAs exhibit tissue- and cell type-<br>specific expression patterns, w biological promoting and cargo [16], especially considering the growing recognition of him  $s_{\text{re}}$  approximately approximately the distribution of the brain-displace to  $\mu$  by  $\mu$ specially considering their critics make<br>them promising drug targets [18], especially considering the growing recognition of RNA<br>therapeutics as a promising avenue for treating various diseases, including brain disorders [ them promising drug targets [18], especially considering the growing recognition of RNA<br>therapeutics as a promising avenue for treating various diseases, including brain disorders [19] [20].<br>However, the knowledge about ln therapeutics as a promising avenue for treating various diseases, including brain disorders [19] [20].<br>However, the knowledge about lncRNAs in the brain is still limited and especially for astrocytes,<br>there is only very li However, the knowledge about lncRNAs in the brain is still limited and especially for astrocytes, there is only very little information about functional involvements in the context of reactive astrocytosis.

In this study, we aimed to identify long non-coding RNAs (lncRNAs) that are specifically enriched in astrocytosis.<br>In this study, we aimed to identify long non-coding RNAs (lncRNAs) that are specifically enriched in<br>astrocytes and deregulated under conditions of reactive astrocytosis and in neurodegenerative astrocytes<br>In this study,<br>astrocytes are<br>diseases. We  $\frac{1}{3}$ In this strocytes and deregulated under conditions of reactive astrocytosis and in neurodegenerative<br>diseases. We hypothesized that such lncRNAs could serve as *bona fide* candidates for targeted drug<br>interventions. By com diseases. We hypothesized that such lncRNAs could serve as *bona fide* candidates for targeted drug<br>interventions. By combining single nucleus RNA sequencing (snucRNAseq) of the human brain with<br>additional transcriptome da interventions. By combining single nucleus RNA sequencing (snucRNAseq) of the human brain with<br>additional transcriptome data from human tissues we identify *PRDM16-DT* as the most highly<br>enriched lncRNA in astrocytes with additional transcriptome data from human tissues we identify *PRDM16-DT* as the most highly<br>enriched lncRNA in astrocytes with a murine homolog, named *Prdm16os. PRDM16-DT* is<br>downregulated in reactive astrocytes and in th additional transcriptome data from human tissues we identify *PRDM16-DT* as the most highly<br>enriched lncRNA in astrocytes with a murine homolog, named *Prdm16os. PRDM16-DT* is<br>downregulated in reactive astrocytes and in th enriched lincRNA in astrocytes with a mailine homolog, named Pram16os. PRDM16-DT is<br>downregulated in reactive astrocytes and in the brains of individuals with AD. Knockdown of<br>*Prdm16os* followed by total RNA sequencing an Prdm16os followed by total RNA sequencing and functional assays revealed its important role in<br>maintaining astrocyte homeostasis and supporting neuronal function. Furthermore, we find that<br>Prdm16os and PRDM16-DT regulate t Pramitious followed by total RNA sequencing and functional assays revealed its important role in<br>maintaining astrocyte homeostasis and supporting neuronal function. Furthermore, we find that<br>*Prdm16os* and *PRDM16-DT* regu *Prdm16os* and *PRDM16-DT* regulate the expression of genes critical for synaptic support by functioning as a decoy for RE1-Silencing Transcription factor (Rest) in conjunction with the H3K27 methyltransferase Polycomb Rep Functioning as a decoy for RE1-Silencing Transcription factor (Rest) in conjunction with the H3K27<br>methyltransferase Polycomb Repressive Complex 2 (PRC2). In line with these observations, reduced<br>*Prdm16os and PRDM16-DT* l methyltransferase Polycomb Repressive Complex 2 (PRC2). In line with these observations, reduced<br>*Prdm16os and PRDM16-DT* levels affect glutamate uptake and lactate release which correlates with<br>impaired neuronal cell viab Prdm16os and PRDM16-DT levels affect glutamate uptake and lactate release which correlates with Impaired neuronal cell viability and spine density. Finally, CRISPR-mediated overexpression of *Prdm16os* can mitigate functional deficits induced in reactive astrocytes. Overall, these results report for the first time a Frdm16os can mitigate functional deficits induced in reactive astrocytes. Overall, these results report<br>for the first time a role of *Prdm16os* and *PRDM16-DT* in the brain and highlight its specific role in<br>astrocyte func for the first time a role of *Prdm16os* and *PRDM16-DT* in the brain and highlight its specific role in astrocyte function. Thus, *PRDM16-DT* is a potential novel therapeutic target for neurological disorders characterize astrocyte function. Thus, PRDM16-DT is a potential novel therapeutic target for neurological disorders characterized by astrocyte dysfunction.<br>
Results<br>
Single Nucleus RNA Sequencing identifies *PRDM16-DT* as an astrocyte-specific lncRNA in the

#### Results

disorders characterized by astrocyte dysidiction.<br>Results<br>Single Nucleus RNA Sequencing identifies *PRL*<br>human brain Single Nucleus RNA Sequencing identifies PRDM16-DT as an astrocyte-specific lncRNA in the human brain

The disperse was to identify analytic promoted interaction in the brain and dysregulation<br>neurodegenerative disorders (Fig. 1A). We hypothesized that studying such lncRNAs functionally<br>could provide insights into the role neurodegenerative disorders (Fig. 1A). We hypothesized that studying such lictives randcolonity<br>could provide insights into the role of astrocytes in these diseases and potentially uncover promising<br>drug targets with minim could provide insignment into the role of astrocytes in the role of an applemental provide interest promising<br>drug targets with minimal side effects, given their brain and cell type-specific expression. To achieve<br>this goa drug targets with minimal side effects, given their brain and cell type-specific expression. To defined<br>this goal, we generated snucRNAseq data from postmortem brain tissues of healthy individuals<br>using and adapted an iCel this goal, we generated with random primers, enabling us to<br>detect both polyA and non-polyA transcripts. We generated over 400 million reads per sample and<br>identified 17031 IncRNAs in total (Fig. 1B). Next, we identified a detect both polyA and non-polyA transcripts. We generated over 400 million reads per sample and<br>identified 17031 IncRNAs in total (Fig. 1B). Next, we identified astrocyte-enriched IncRNAs by<br>comparing the mean expression i identified 17031 IncRNAs in total (Fig. 1B). Next, we identified astrocyte-enriched IncRNAs by<br>comparing the mean expression in astrocytes with that in all other cell types, resulting in ten<br>IncRNAs with a ratio of >20 fol comparing the mean expression in astrocytes with that in all other cell types, resulting in ten<br>IncRNAs with a ratio of >20 fold enrichment (Fig. 1C). To facilitate functional analysis we decided to<br>initially focus on lncR IncRNAs with a ratio of >20 fold enrichment (Fig. 1C). To facilitate functional analysis we decided to initially focus on lncRNAs (marked in red) that have a mouse homolog, allowing for further studies in both murine and h initially focus on lncRNAs (marked in red) that have a mouse homolog, allowing for further studies in<br>both murine and human cells. LncRNAs were considered homologs when the genes were<br>syntenically located in the same genom interpretent murine and human cells. LncRNAs were considered homologs when the genes were<br>syntenically located in the same genomic locus, and the transcripts exhibited a sequence similarity of<br>at least 40%. The most enrich both murine and human cells. Little and the transcripts exhibited a sequence similarity of<br>at least 40%. The most enriched and conserved lncRNA in astrocytes was *PRDM16-DT* which was<br>almost exclusively expressed in astroc syntenically reaction in the same genomic located incRNA in astrocytes was *PRDM16-DT* which was almost exclusively expressed in astrocytes (Fig. 1C-D). Next, we reanalyzed an RNAseq dataset encompassing 45 human tissues [ almost exclusively expressed in astrocytes (Fig. 1C-D). Next, we reanalyzed an RNAseq dataset<br>encompassing 45 human tissues [21] and found that *PRDM16-DT* was significantly enriched in the<br>central nervous system compared almost exclusively expressed in astrocytes (Fig. 1C-D). Next, we reanalyzed an RNAseq dataset<br>encompassing 45 human tissues [21] and found that *PRDM16-DT* was significantly enriched in the<br>central nervous system compared

encompassing 45 human tissues [21] and found that PRDM16-DT was significantly ennemed in the<br>central nervous system compared to other human tissues (Fig. 1E).<br>In summary, these data suggest that *PRDM16-DT* is a brain-enr central nervous system compared to other human tissues (Fig. 1E).<br>In summary, these data suggest that *PRDM16-DT* is a brain-<br>expressed in astrocytes. To further validate this observation, *N*<br>*PRDM16-DT* in human iPSC-der expressed in astrocytes. To further validate this observation, we examined the expression of *PRDM16-DT* in human iPSC-derived cortical neurons, microglia, and astrocytes using qPCR.<br>Consistent with the snucRNA-seq data, expressed in astrocytes. To further validate this contraction, microglia, and astrocytes using qPCR.<br>Consistent with the snucRNA-seq data, *PRDM16-DT* showed significant enrichment in astrocytes (Fig.<br>1F). To assess whethe PRDM16-DT in human iPSC-derived cortical neurons, iniclogia, and astrocytes using qPCR.<br>Consistent with the snucRNA-seq data, *PRDM16-DT* showed significant enrichment in astrocytes (Fig.<br>1F). To assess whether this cell-t Consistem with the shuckwa-seq data, *PRDM16-DT* showed significant emficition astrocytes (Fig. 1F). To assess whether this cell-type specificity is conserved across species, we analyzed the expression of the *PRDM16-DT* m expression of the *PRDM16-DT* mouse homolog, *Prdm16os*, in primary astrocytes, neurons, and<br>microglia (Fig. 1G). Similar to human *PRDM16-DT*, mouse *Prdm16os* exhibited high enrichment in<br>astrocytes. Furthermore, we empl expression of the PRDM16-DP mouse homolog, Pram16os, in primary astrocytes, hearons, and<br>microglia (Fig. 1G). Similar to human PRDM16-DT, mouse Prdm16os exhibited high enrichment in<br>astrocytes. Furthermore, we employed MAC astrocytes. Furthermore, we employed MACS sorting to isolate astrocytes, oligodendrocytes,<br>microglia, and a neuron-enriched fraction from the adult mouse brain. Consistent with our previous<br>findings, *Prdm16os* was found t microglia, and a neuron-enriched fraction from the adult mouse brain. Consistent with our previous<br>findings, Prdm16os was found to be most highly expressed in astrocytes (Fig. 1H). microglia, and a neuron-enriched fraction from the adult model frame enriched fraction with enrique<br>findings, Prdm16os was found to be most highly expressed in astrocytes (Fig. 1H). findings, *Pram1*6os was found to be most highly expressed in astrocytes (Fig. 1H).



- 1<br>- 1<br>- 1<br>- 1 Figure 1: FIGM16-DT/FFIGM160S is an astrocyte-specific linctival emittied in the brain. A. Strategy<br>to identify astrocyte-specific lncRNAs in the brain and characterize their function. B. UMAP<br>clustering based on snucRNAse to identify astrocyte specific literatives in the brain and characterize their ranction. **B.** OMAT<br>clustering based on snucRNAseq (iCELL8 technology) from healthy human brains (prefrontal cortex,<br>BA9). **C.** Left panel: Bar BA9). C. Left panel: Bar graph showing the list of IncRNAs enriched in astrocytes when compared to all other cell types in the human brain. IncRNAs marked in red have a mouse homolog. Right panel: Schematic illustration sh all other cell types in the human brain. IncRNAs marked in red have a mouse homolog. Right panel:<br>Schematic illustration showing the genomic localization of human *PRMD16-DT* and mouse<br>*Prdm16os*. **D.** Left panel: Violin p Schematic illustration showing the genomic localization of human *PRMD16-DT* and mouse<br>*Prdm16os.* **D.** Left panel: Violin plot showing the expression of *PRDM16-DT* in different cell types of<br>the human prefrontal cortex a *Prdm16os.* **D.** Left panel: Violin plot showing the expression of *PRDM16-DT* in different cell types of the human prefrontal cortex along with corresponding cell type marker genes. .Right panel: UMAP clustering as depict Tram16os. D. Left panel: Violin plot showing the expression of TribW16 DT in different cell types of<br>the human prefrontal cortex along with corresponding cell type marker genes. .Right panel: UMAP<br>clustering as depicted i clustering as depicted in (B) showing the expression of *PRMD16-DT*. **E.** Left panel: Expression of PRDM16-DT in different human tissues (depicted are only tissues in which expression was detectable). One-way ANOVA reveal PRDM16-DT in different human tissues (depicted are only tissues in which expression or<br>PRDM16-DT in different human tissues (depicted are only tissues in which expression was<br>detectable). One-way ANOVA revealed a signific detectable). One-way ANOVA revealed a significant difference among the groups (p < 0.0001).  $***P < 0.0001$  for central nervous system vs. any of the other tissues (unpaired t-<br>Tests). Right panel: Violin plot showing the ex  $0.0001$ ). \*\*\*\*P < 0.0001 for central nervous system vs. any of the other tissues (unpaired t-<br>Tests). Right panel: Violin plot showing the expression of PRDM16-DT as counts per million<br>normalized to the samples size for 0.0001).  $***P < 0.0001$  for central nervous system vs. any of the other tissues (unpaired t-<br>Tests). Right panel: Violin plot showing the expression of PRDM16-DT as counts per million Tests). Right panel: Violin plot showing the expression of PRDM16-DT as counts per immorition-<br>normalized to the samples size for the central nervous system vs. the average expression across 44<br>other human tissues (only ti other human tissues (only tissues in which PRDM16-DT expression was detected are shown) \*\*\*\**P*<br>
< 0.0001; unpaired t-Test. **F.** Bar chart showing the expression of *PRDM16-DT* in human iPSC-<br>
derived astrocytes, neurons a  $< 0.0001$ ; unpaired t-Test. **F.** Bar chart showing the expression was detected are shown)  $\leq$  0.0001; unpaired t-Test. **F.** Bar chart showing the expression of *PRDM16-DT* in human iPSC-derived astrocytes, neurons and derived astrocytes, neurons and microglia (unpaired t test; \*\*\*P $\mathbb{R} \leq 0.0001$ , \*\*\*\*P $\mathbb{R} \leq 0.0001$ , ns = not significant). **G.** Prdm16os expression in mouse primary astrocytes, neurons and microglia (unpaired t t derived as provides as pression in mouse primary astrocytes, neurons and microglia (unpaired that; \*\*\*P $\mathbb{R}$ <br/> $\mathbb{R}$  and  $\mathbb{R}$  and  $\mathbb{R}$  and  $\mathbb{R}$  and  $\mathbb{R}$  are not significant). H. Promit for expressi significant). G. Pram160s expression in mouse primary astrocytes, neurons and microgia (unpaired t<br>test; \*\*\*P[20.001, \*\*\*\*P[2<[20.0001, ns = not significant]. H. *Prdm16os* expression in astrocytes,  $\frac{16 \times 60.001}{16 \times 60.001}$ ,  $\frac{16 \times 60.0001}{16 \times 60.0001}$ , ns = not significant). H. Pram1600s expression in astrocytes, test; \*\*\*P**E**<**EO.001**). Right panel: Bar charts showing qPCR data from astrocytes, oligodendrocytes, microglia and neuronal fraction isolated from the adult mouse brain for marker genes for astrocytes (Aldehyde Dehydrogen microglia and neuronal fraction isolated from the adult mouse brain for marker genes for astrocytes<br>(Aldehyde Dehydrogenase 1 Family Member L1, Aldh1l), oligodendrocytes (myelin basic protein,<br>Mbp), microglia (Integrin Sub (Aldehyde Dehydrogenase 1 Family Member L1, Aldh1l), oligodendrocytes (myelin basic protein,<br>Mbp), microglia (Integrin Subunit Alpha M, Itgam) and neurons (RNA Binding Fox-1 Homolog 3,<br>Rbfox3). Error bars indicate SD.<br>Prdm (Aldehyde Dehydrogenase 1 Family Member L1, Aldh11), oligodendrocytes (myellin basic protein, Mbp), microglia (Integrin Subunit Alpha M, Itgam) and neurons (RNA Binding Fox-1 Homolog 3, Rbfox3). Error bars indicate SD.<br>Prd

### Mbp), incroglia (*Integrin Subunit Alpha M)*, *Rgam*) and neurons (*RNA Binding Fox-1 Homolog 3*,<br>Rbfox3). Error bars indicate SD.<br>Prdm16os and PRDM16-DT are downregulated in response to pathological insults and in<br>Alzheim Prdm16os and PRDM16-DT<br>Alzheimer's disease<br>To investigate whether PRDM1 Prdm16os and PRDM16-DT are downregulated in response to pathological insults and in Alzheimer's disease

 To investigate whether Pholitic DT is dysregulated in heurodegenerative diseases, we analyzed its<br>expression in postmortem brain samples (prefrontal cortex, BA9) obtained from healthy controls and<br>patients with AD. Our ana patients with AD. Our analysis revealed a significant decrease in *PRDM16-DT* expression in AD<br>patients (Fig. 2A). This finding was further validated using data from the Agora database<br>(https://agora.adknowledgeportal.org/ patients with AD. Our analysis revealed a significant decrease in PRDM16-DT expression in AD<br>patients (Fig. 2A). This finding was further validated using data from the Agora database<br>(https://agora.adknowledgeportal.org/), (https://agora.adknowledgeportal.org/), which includes over 1000 postmortem brain samples from<br>both control individuals and AD patients. *PRDM16-DT* expression was found to be significantly<br>reduced in the frontal pole and both control individuals and AD patients. PRDM16-DT expression was found to be significantly

both control individuals and AD patients. *PRDM16-DT* expression was found to be significantly<br>reduced in the frontal pole and parahippocampal gyrus regions (Fig. 2B).<br>Subsequently, we analyzed *PRDM16-DT* expression in po reduced in the frontal pole and paramppocampal gyrus regions (Fig. 2B).<br>Subsequently, we analyzed *PRDM16-DT* expression in postmortem b<br>obtained from patients with frontotemporal dementia (FTD) carrying mu<br>*MAPT* genes, a Subsequently, we analyzed *PRDM16-DT* expression in positionem brain tissues (frontal lobe)<br>obtained from patients with frontotemporal dementia (FTD) carrying mutations in *C90RF72*, *GRN*, or<br>*MAPT* genes, as well as from obtained from patients with frontotemporal dementia (FTD) carrying mutations in C90M72, GMN, GP<br>
MAPT genes, as well as from control individuals that are available via the RiMOD database [22] [23].<br>
We did not observe any WAT P genes, as well as from control individuals that are available via the RiMOD database [22] [23].<br>We did not observe any significant difference in *PRDM16-DT* expression between these groups (Fig.<br>2C). Additionally, we **2C)**. Additionally, we examined *PRDM16-DT* expression in a transcriptome dataset derived from postmortem brains of individuals with schizophrenia, as compared to controls [24]. Similar to our findings in FTD patients, th 2C). Additionally, we examined PNDM16-DT expression in a transcriptome dataset derived from<br>postmortem brains of individuals with schizophrenia, as compared to controls [24]. Similar to our<br>findings in FTD patients, there findings in FTD patients, there was no discernible difference in *PRDM16-DT* expression between schizophrenia patients and controls (Fig. 2D). These results collectively suggest that *PRDM16-DT* expression is decreased spe schizophrenia patients and controls (Fig. 2D). These results collectively suggest that *PRDM16-DT* expression is decreased specifically in the brains of AD patients.<br>It is widely believed that the accumulation of amyloid b

schizophrenia patients and controls (Fig. 2D). These results collectively suggest that *TRDM16-DT*<br>expression is decreased specifically in the brains of AD patients.<br>It is widely believed that the accumulation of amyloid b expression is decreased specifically in the brains of a probability<br>is decreased that the accumulation of amyloid beta (<br>pathological changes in AD pathogenesis, occurring decades be<br>[26]. Reactive astrogliosis is also obs pathological changes in AD pathogenesis, occurring decades before clinical symptoms manifest [25]<br>[26]. Reactive astrogliosis is also observed very early in mouse models for amyloid pathology [27].<br>While Abeta42 can direct pathology [27].<br>
[26]. Reactive astrogliosis is also observed very early in mouse models for amyloid pathology [27].<br>
While Abeta42 can directly affect astrocytes, a prominent mechanism by which Abeta42 affects<br>
astrocyte While Abeta42 can directly affect astrocytes, a prominent mechanism by which Abeta42 affects<br>astrocytes is via the activation of microglia [28], which in turn release pro-inflammatory cytokines IL-<br>1α, TNF, and C1q that ar astrocytes is via the activation of microglia [28], which in turn release pro-inflammatory cytokines IL-<br>1 $\alpha$ , TNF, and C1q that are sufficient to induce reactive astrogliosis [3]. Based on these findings, we<br>wanted to t assessed the expression of *manizoos* in primary astrocytes sumalated with incrogia conditioned 1.4, The C1q that are summanded to test if the exposure of astrocytes to AB oligomers, media from activated microglia, or inflammatory cytokines would affect the expression of *PRDM16-DT* and *Prdm16os*. Firstly, we asses inflammatory cytokines would affect the expression of *PRDM16-DT* and *Prdm16os*. Firstly, we assessed the expression of *Prdm16os* in primary astrocytes stimulated with microglia-conditioned for a strong in the strong st inflammatory cytokines would affect the expression of PRDM16-DT and Pram160s. Firstly, we<br>assessed the expression of Prdm16os in primary astrocytes stimulated with microglia-conditioned<br>6 assessed the expression of Prdm16os in primary astrocytes stimulated with microglia-conditioned medium (media from microglia from microsity of the control mini-trep, emperod to control media (media<br>from microglia treated with PBS; MCM-PBS) (Fig. 2E). We observed a significant decrease in<br>*Prdm16os* levels in astrocyt *Prdm16os* levels in astrocytes following MCM-LPS treatment (Fig. 2F). Treatment of primary<br>astrocytes with the of II1 $\alpha$ , TNF, and C1q (3 cytokine cocktail) also resulted in a significant decrease<br>in *Prdm16os* levels, s astrocytes with the of II1 $\alpha$ , TNF, and C1q (3 cytokine cocktail) also resulted in a significant decrease<br>in *Prdm16os* levels, similar to the effect of microglia-conditioned media (Fig. 2E). Similarly, treating<br>primary in *Prdm16os* levels, similar to the effect of microglia-conditioned media (Fig. 2E). Similarly, treating<br>primary astrocytes with Abeta42 led to a significant reduction in *Prdm16os* levels (Fig. 2E).<br>Furthermore, stimulat primary astrocytes with Abeta42 led to a significant reduction in *Prdm16os* levels (Fig. 2E).<br>Furthermore, stimulation of human iPSC-derived astrocytes with the 3 cytokine cocktail or Abeta<br>resulted in a similar downregul primary astrocytes with Abeta42 led to a significant reduction in Pram16os levels (Fig. 2E).<br>Furthermore, stimulation of human iPSC-derived astrocytes with the 3 cytokine cocktail or Abeta<br>resulted in a similar downregulat

Furthermore, stimulation of them and the stimulation of the stimulation of the stimulation of the stimulation<br>In summary, our data demonstrate that *PRDM16-DT* is reduced in the brains of AD patients.<br>Additionally, exposur resulted in a similar downregulation of PhoM16-DT as observed in mouse astrocytes (Fig. 2F).<br>In summary, our data demonstrate that *PRDM16-DT* is reduced in the brains of AD pa<br>Additionally, exposure of both mouse and huma In summary, our data demonstrate that PRDM16-DT is reduced in the brains of AD patents.<br>Additionally, exposure of both mouse and human astrocytes to well-established AD risk factors<br>(MCM, 3 cytokine cocktail, Abeta42) resu (MCM, 3 cytokine cocktail, Abeta42) results in a similar decrease in *PRDM16-DT* and *Prdm16os*<br>levels. These findings provide a suitable experimental framework for studying the role of *PRDM16-DT*<br>*D* **R RDM16-DT PRD** 



Figure 2: Prdm16-DT is decreased in the brains of AD patients and in response to AD risk factors.<br>A. Bar chart showing qPCR data on the expression of PRDM16-DT in postmortem brain samples (prefrontal cortex, BA9) from control (n = 12) and AD patients (n = 13) (\*\*\*\*Pa<a0.0001, unpaired t-<br>Test) **B.** Log2 Fold changes of *PRDM16-DT* expression in different brain regions in AD patients<br>compared to controls ba (prefrontal cortex, BA9) from control (n = 12) and AD patients (n = 13) (\*\*\*\*\*\*\*\*\* Test) **B.** Log2 Fold changes of *PRDM16-DT* expression in different brain regions in AD patients compared to controls based on data from t Test) B. Log2 Fold changes of PRDM16-DT expression in different brain regions in AD patients<br>
compared to controls based on data from the Agora database<br>
(https://agora.adknowledgeportal.org/).(\*P < 0.05). C. Bar chart sh (https://agora.adknowledgeportal.org/).(\* $P < 0.05$ ). C. Bar chart showing the expression of PRDM16-<br>DT in postmortem tissue samples (frontal lobe) of FTD patients with *MAPT* (n = 10), *C9ORF72* (n = 8)<br>or *GRN* (n = 6) m (https://agora.adknowicdgeportal.org/).(\* x 0.05). C. Bar chart showing the expression of NDM16<br>DT in postmortem tissue samples (frontal lobe) of FTD patients with *MAPT* (n = 10), *C9ORF72* (n = 8)<br>or *GRN* (n = 6) mutat DT in postmortem tissue samples (frontal lobe) of FTD patients with MAPT (n = 10), C90/1/2 (n = 0)<br>or GRN (n = 6) mutations compared to non-demented controls (NDC, n = 13). **D.** Bar chart showing<br>the expression of PRDM16the expression of PRDM16-DT in postmortem brain tissue of controls (n = 279) compared to<br>schizophrenia patients (n = 258) obtained from a study by Wu et al., [24]. **E.** Left panel: Experimental<br>schizophrenia patients (n = schizophrenia patients ( $n = 258$ ) obtained from a study by Wu et al., [24]. E. Left panel: Experimental schizophrenia patients (n = 258) obtained from a study by Wu et al., [24]. E. Left panel: Experimental

design. Right panel: Bar plot showing Pram16os expression in mouse astrocytes after freatment with<br>LPS-activated microglia conditioned medium (MCM-LPS) compared to control (MCM-PBS), a 3<br>cytokine cocktail (3 Cyt) and AB-42 cytokine cocktail (3 Cyt) and Aß-42 treatment compared to the corresponding vehicle controls.<br>
\*\*\*\*Pa<a>20.00001, \*\*Pa<a>20.001 unpaired t-Test). F. Left panel: Experimental design. Right panel: Bar<br>
plot showing *PRMD16-* $***P\overline{2}$ < $\overline{2}0.00001$ ,  $**P\overline{2}$ < $\overline{2}0.001$  unpaired t-Test). F. Left panel: Experimental design. Right panel: Bar<br>plot showing *PRMD16-DT* expression in human iPSC-derived astrocytes after treatment with a 3<br>cy plot showing *PRMD16-DT* expression in human iPSC-derived astrocytes after treatment with a 3 cytokine cocktail (3 Cyt) and Aß-42 compared to the corresponding controls (\*\**P*<sub>I</sub>ZQD.01 unpaired t-Test). ACC: Anterior Cingu plot showing PRMD16-DP expression in human in 50 derived astrocytes after treatment with a 3<br>cytokine cocktail (3 Cyt) and Aß-42 compared to the corresponding controls (\*\*Pla<laboration Test). ACC: Anterior Cingulate Corte cytokine cocktail (3 Cyt) and AB-42 compared to the corresponding controls (\*\*PLESLO.01 unpaired t<br>Test). ACC: Anterior Cingulate Cortex, AD: Alzheimer's Disease, CBE: Cerebellum, 3 Cyt: 3 cytokine<br>cocktail, DLPFC: Dorsola cocktail, DLPFC: Dorsolateral Prefrontal Cortex, FTD: Frontotemporal Dementia, FP: Frontal pole; IFG: commy commy contribution is also contributed by the formulation pole content in the pole of the interior Fremporal Gyrus, STG: Superior<br>Temporal Gyrus, TCX: Temporal Cortex. Error bars represent SD.<br>**Prdm16os regulates gen** 

#### Prdm16os regulates genes involved in synaptic function, apoptosis and inflammatory processes

Inferior Frontal Gyrus, TCX: Temporal Cortex. Error bars represent SD.<br> **Prdm16os regulates genes involved in synaptic function, apoptosis and inflammatory processes**<br>
Given the lack of previous studies on *PRDM16-DT/Prdm1* Prdm16os regulates genes involved in synaptic function, apopto<br>Given the lack of previous studies on *PRDM16-DT/Prdm16os* in<br>role at the cellular and functional level. Since the function<br>subcellular localization [29], we i  $\begin{array}{c} \n\frac{1}{2} & \frac{1}{2} \\ \n\frac{1}{2} & \frac{1}{$ Figure 1.1 and the cellular and functional level. Since the function of lncRNAs is closely tied to their subcellular localization [29], we initially employed murine models to examine the localization of *Prdm16os*. Through subcellular localization [29], we initially employed murine models to examine the localization of *Prdm16os*. Through a combination of RNAscope and immunofluorescence staining for *Prdm16os* and the astrocyte marker Gfap, Prdm16os. Through a combination of RNAscope and immunofluorescence staining for Prdm16os and<br>the astrocyte marker Gfap, we determined that Prdm16os is predominantly located in the nucleus of<br>astrocytes in the adult mouse b Pram16os. Through a combination of RNAscope and immunionalities standing for *Pram16os* and<br>the astrocyte marker Gfap, we determined that *Prdm16os* is predominantly located in the nucleus of<br>astrocytes in the adult mouse astrocyte marker Grap, we determined that *Pram16os* is predominantly located in the nucleus of<br>astrocytes in the adult mouse brain (Fig. 3A). To validate this finding, we conducted nuclear and<br>cytoplasmic fractionation of extrocytes in the adult mouse brain (Fig. 3A). To validate this miding, we conducted nuclear and<br>cytoplasmic fractionation of primary mouse astrocytes and compared the levels of *Prdm16os* in each<br>compartment. Our analysis compartment. Our analysis revealed that *Prdm16os* primarily resides in the nuclear fraction (Fig. 3B), suggesting a potential role for *Prdm16os* in gene transcription regulation.<br>With this insight, we proceeded to perfor

suggesting a potential role for *Prdm16os* in gene transcription regulation.<br>With this insight, we proceeded to perform a knockdown (KD) of *Prdm16os* in primary astrocytes<br>using a *Prdm16os* GapmeR probe (Fig. 3C). We con suggesting a potential role for Pram16os in gene transcription regulation.<br>With this insight, we proceeded to perform a knockdown (KD) of *Prdm*<br>using a *Prdm16os* GapmeR probe (Fig. 3C). We confirmed that *Prdm16os*<br>effec with this insight, we proceeded to perform a knockdown (KD) of Pram16os in primary astrocytes<br>using a Prdm16os GapmeR probe (Fig. 3C). We confirmed that Prdm16os knockdown had no adverse<br>effects on cell viability after 48 using a Pram16os Gapmer probe (Fig. 3C). We committed that Pram16os knockdown had no adverse<br>effects on cell viability after 48 hours (Fig. 3D), prompting us to collect RNA samples for total RNA<br>sequencing at this time poi equencing at this time point. Subsequent differential expression analysis unveiled significant<br>changes, with 287 genes upregulated and 418 genes downregulated (log2FC > 1, FDR < 0.05,<br>basemean > 50) (Fig 3E, supplemental t changes, with 287 genes upregulated and 418 genes downregulated (log2FC > 1, FDR < 0.05, basemean > 50) (Fig 3E, supplemental table 1). Gene Ontology (GO) term analysis of the upregulated genes revealed pathways related t basemean > 50) (Fig 3E, supplemental table 1). Gene Ontology (GO) term analysis of the up-<br>regulated genes revealed pathways related to apoptosis such as "Intrinsic apoptotic signaling<br>pathway" and inflammatory processes s basemean > 50) (Fig 3E, supplemental table 1). Gene Ontology (GO) term analysis of the up-<br>regulated genes revealed pathways related to apoptosis such as "Intrinsic apoptotic signaling<br>pathway" and inflammatory processes s pathway" and inflammatory processes such as "Defense response to virus" (Fig. 3F, supplemental<br>table 2). The downregulated genes were linked to processes associated with synapse support<br>function such as "synapse organizati pathway" and inflammatory processes such as "Defense response to virus" (Fig. 3F, supplemental<br>table 2). The downregulated genes were linked to processes associated with synapse support<br>function such as "synapse organizati table 2). The downregulated genes were linked to processes associated with synapse support<br>function such as "synapse organization", "cognition", "learning and memory" and "regulation of<br>neurotransmitter levels" (Fig. 3F, s Function such as the set of the upregulated genes linked to inflammation and downregulated genes linked to synaptic function to confirm the RNAseq data via qPCR (Fig 3G). To test whether the increased expression of genes l genes linked to inflammation and downregulated genes linked to synaptic function to confirm the<br>RNAseq data via qPCR (Fig 3G). To test whether the increased expression of genes linked to<br>inflammation would result in elevat genes and the increased expression of genes linked to inflammation would result in elevated secretion of the corresponding cytokines, we also measured inflammation would result in elevated secretion of the corresponding cy inflammation would result in elevated secretion of the corresponding cytokines, we also measured inflammation would result in elevated secretion of the corresponding cytokines, we also measured



Figure 3: Prainfloos is localized to the nucleus and controls gene expression. A. Representative<br>images from the adult mouse hippocampus showing the RNAscope signal for *Prdm16os* and<br>immunofluorescence for Gfap. Nuclei a immunofluorescence for Gfap. Nuclei are stained with DAPI. The right panel shows a higher magnification from a different hippocampal region. **B.** Bar plot showing qPCR values for *Prdm16os* in nuclear and cytoplasmic frac magnification from a different hippocampal region. **B.** Bar plot showing qPCR values for *Prdm16os* in nuclear and cytoplasmic fractions prepared from primary astrocytes (\*\*\**P* < 0.001, unpaired tTest).<br>**C.** Bar plot sho magnification from a different imppocallipal region. B. Bar plot showing qr ex values for *Fram16os* in nuclear and cytoplasmic fractions prepared from primary astrocytes (\*\*\**P* < 0.001, unpaired tTest).<br>C. Bar plot show **C.** Bar plot showing qPCR results for *Prdm16os* after treating primary astrocytes with GapmeRs to knock down (KD) *Prdm16os*. RNA was collected 48 hours after the addition of *Prdm16os GapmeRs* or control oligonucleotid C. Bar plot showing qPCR results for *Fram16os* after treating primary astrocytes with GapmeRs to<br>knock down (KD) *Prdm16os.* RNA was collected 48 hours after the addition of *Prdm16os GapmeRs* or<br>control oligonucleotides control oligonucleotides (\*\*\*\*P < 0.0001, unpaired tTest) **D.** Bar plot showing cell viability of primary astrocytes 48h after *Prdm16os* knock down in comparison to the treatment with control oligomers. **E.** Volcano plot primary astrocytes 48h after *Prdm16os* knock down in comparison to the treatment with control<br>oligomers. **E.** Volcano plot shows the up- and down-regulated genes in primary astrocytes 48h after<br>*Prdm16os* knock down. **F.** primary astrocytes 48h after *Tramitoos* knock down in comparison to the treatment with control<br>oligomers. **E.** Volcano plot shows the up- and down-regulated genes in primary astrocytes 48h after<br>*Prdm16os* knock down. **F.** *Prdm16os* knock down. F. Plot showing the results of a GO term analysis for the up- and downregulated genes displayed in (E) (Analysis was done using clusterProfiler (v4.6.0) [30]. Two-<br>sided hypergeometric test was used downregulated genes displayed in (E) (Analysis was done using clusterProfiler (v4.6.0) [30]. Two-<br>sided hypergeometric test was used to calculate the importance of each term and the Benjamini-<br>Hochberg procedure was appli pricis and the solution of the solution of Profilm (Panalysis was denoted via ELISA (\*\*\*\*P < 0.0001, unpaired tTest). KD: knockdown, NC: negative control. Error bars indicate SEM.<br>unpaired tTest). KD: knockdown, NC: negati unpaired tTest). KD: knockdown, NC: negative control. Error bars indicate SEM. Hochberg procedure was applied for the P value correction). **G.** Bar plots showing the results of qPCR experiments for selected genes that were found to be deregulated upon *Prdm16os* knock down via RNAseq. Left panel: Se down via RNAseq. Left panel: Selected up-regulated genes. Right panel: Selected downregulated<br>genes \*\*\*\*P < 0.0001, \*\*\*P < 0.001; \*\*P < 0.01; unpaired tTest). H. Bar chart showing the effect of<br>Prdm16 knock down on IL-6 l genes \*\*\*\* $P < 0.0001$ , \*\*\* $P < 0.001$ ; \*\* $P < 0.01$ ; unpaired tTest). H. Bar chart showing the effect of Prdm16 knock down on IL-6 levels in the corresponding media measured via ELISA (\*\*\*\* $P < 0.0001$ , unpaired tTest). KD:

### Promito knock down on IL-6 levels in the corresponding media measured via ELISA (\*<br>unpaired tTest). KD: knockdown, NC: negative control. Error bars indicate SEM.<br>The knockdown of *Prdm16os* in a under the knockdown of *Prdm16os* in astrocytes leads to impaired glutamate upta<br>and affects neuronal activity and spine density  $\begin{bmatrix} 1 \\ 2 \end{bmatrix}$ The knockdown of Prdm16os in astrocytes leads to impaired glutamate uptake, lactate secretion and affects neuronal activity and spine density

The RNASeq data hinted at several cellular processes controlled by Fram16os. For instance, the<br>sequencing data suggested that the loss of *Prdm16os* may compromise the neuronal support<br>function of astrocytes and we decided sequencing data suggested that the loss of Pram16os may complemise the heatonal support<br>function of astrocytes and we decided to study this potential mechanics further at first. One crucial<br>role of astrocytes is to remove Function of astrocytes is to remove glutamate from the extracellular space. This process is primarily<br>mediated by two main glutamate transporters, Glt-1 and Glast. Both glutamate transporters showed<br>downregulation in the R mediated by two main glutamate transporters, Glt-1 and Glast. Both glutamate transporters showed<br>downregulation in the RNAseq dataset, a finding confirmed by qPCR analysis (Fig. 4A) and at the<br>protein level (Fig. 4B). Cons downregulation in the RNAseq dataset, a finding confirmed by qPCR analysis (Fig. 4A) and at the

In addition to glutamate uptake, astrocytes provide metabolic support in the CNS by secreting<br>lactate, which is an important energy substrate for neurons. Also in this case, there are two relevant protein lever (Fig. 4B). Consistently, the uptake of extracellular glutamate was impaired following the<br>knockdown (KD) of *Prdm16os* in primary astrocytes (Fig. 4C).<br>In addition to glutamate uptake, astrocytes provide meta knockdown (KD) of Pram16os in primary astrocytes (Fig. 4C).<br>In addition to glutamate uptake, astrocytes provide meta<br>lactate, which is an important energy substrate for neurons.<br>transporters expressed in astrocytes that ar In addition to generate uppeak) and opportune increasing uppper in the CNS by perturing<br>Iactate, which is an important energy substrate for neurons. Also in this case, there are two relevant<br>transporters expressed in astro transporters expressed in astrocytes that are essential for memory function, Monocarboxylate<br>transporter (Mct) 1 and Mct4 [31]. We confirmed via qPCR that the mRNA levels of *Mct4*, but not<br>*Mct1*, were decreased in respon transporter (Mct) 1 and Mct4 [31]. We confirmed via qPCR that the mRNA levels of *Mct4*, but not *Mct1*, were decreased in response to the KD of *Prdm16os* (Fig. 4D). Next, we directly addressed the question if loss of *Pr Mct1*, were decreased in response to the KD of *Prdm16os* (Fig. 4D). Next, we directly addressed the question if loss of *Prmd16os* in astrocytes would affect neuronal function. For this, we cultured neurons alone or in c Mct1, were decreased in response to the KD of Pram16os (Fig. 4D). Next, we directly addressed the<br>question if loss of *Prmd16os* in astrocytes would affect neuronal function. For this, we cultured<br>neurons alone or in co-cu question if loss of Primations in astrocytes would affect neuronal function. For this, we cultured<br>neurons alone or in co-culture with astrocytes treated before with either *Prmd16os* GapmerRs<br>(*Prdm16os* KD astrocytes) or (Prdm16os KD astrocytes) or control oligomers (control astrocytes). We observed that co-culturing (Prdm16os KD astrocytes) or control oligomers (control astrocytes). We observed that co-culturing primary neurons with control astrocytes was, as expected, able to enhance dendritic spine density,<br>while no such effect was observed when neurons were co-cultured with *Prdm16os* KD astrocytes<br>(Fig. 4F). We also performed (Fig. 4F). We also performed a knockdown of the human homologue of *Prmd16os - PRDM16-DT -* in human iPSC-derived astrocytes (Fig. 4G). Whereas in mouse astrocytes the transcripts encoding both glutamate transporters were (Fig. 4F). We also performed a knockdown of the human homologue of *Primations* - *PRDM16-DT* - in<br>human iPSC-derived astrocytes (Fig. 4G). Whereas in mouse astrocytes the transcripts encoding both<br>glutamate transporters w the levels of *GLT-1* remained unchanged (Fig. 4H). We could also confirm the down-regulation of synaptic plasticity genes *NRXN1* and *GRIN1* (Fig. 4H). The observed functional consequences were similar across species, as glutamate transporters were downregulated, in human astrocytes only GLAST was affected, while<br>the levels of *GLT-1* remained unchanged (Fig. 4H). We could also confirm the down-regulation of<br>synaptic plasticity genes *NRXN* synaptic plasticity genes *NRXN1* and *GRIN1* (Fig. 4H). The observed functional consequences were<br>similar across species, as also the human astrocytes showed reduced glutamate uptake after the KD<br>of *PRDM16-DT* (Fig. 4I). similar across species, as also the human astrocytes showed reduced glutamate uptake after the KD<br>of *PRDM16-DT* (Fig. 4I). Similarly, also the expression of the lactate transporter *MCT4* and the<br>secretion of L-lactate wa similar across of *PRDM16-DT* (Fig. 4I). Similarly, also the expression of the lactate transporter *MCT4* and the secretion of L-lactate was decreased (Fig. 4K-L). These data indicate that the general function of *Prdm16os* secretion of L-lactate was decreased (Fig. 4K-L). These data indicate that the general function of *Prdm16os* and *PRDM16-DT* as being important for neuronal support is conserved across species. secretion of L-lactate was decreased (Fig. 4K-L). These data indicate that the general function of<br>Prdm16os and PRDM16-DT as being important for neuronal support is conserved across species. Prdm16os and PRDM16-DT as being important for neuronal support is conserved across species.



Figure 4: The loss of *Prdm16os* in astrocytes affects glutamate uptake, lactate secretion and<br>neuronal function. A. qPCR showing the levels of the two glutamate transporters *Glt-1* and *Glast* after the KD of *Prdm16os* in primary astrocytes. **B.** Left panel: Representative Immunoblot images of Glt-1 and Glast after the KD of *Prdm16os* in astrocytes. Right panel: Quantification of (B) n = 3/group.<br>**C.** Glutama after the KD of *Pram16os* in primary astrocytes. **B.** Left panel: Representative immanoplet images of<br>Glt-1 and Glast after the KD of *Prdm16os* in astrocytes. **D.** qPCR showing the levels of the two<br>lactate transporters **C.** Glutamate uptake after the KD of *Prdm16os* in astrocytes. Right panel: Quantification of (B) n = 3/group.<br> **C.** Glutamate uptake after the KD of *Prdm16os* in astrocytes. **D.** qPCR showing the levels of the two<br>
lact C. Glutamate uptake after the KD of *Framsoos* in astrocytes. D. qr ex showing the levels of the two lactate transporters *Mct1* and *Mct4* after the KD of *Prdm16os* in astrocytes. E. Lactate secretion after the KD of *Pr* lactate transporters McL1 and McL4 after the KD of Fram16os in astrocytes. L. Lactate secretion after<br>the KD of Prdm16os in astrocytes. F. Left panel: Representative images of dendrite and spine labeling<br>of neurons culture the KD of Pramizoos in astrocytes. F. Left panel: Representative images of dentatie and spine labeling<br>of neurons cultured alone (Neurons) or with either control astrocytes (Neurons + control astrocytes)<br>or *Prdm16os* KD a or *Prdm16os* KD astrocytes (Neurons + Prdm16os KD astrocytes). Right panel. Quantification of spine<br>density. **G-K** shows data from human iPSC-derived astrocytes upon the KD of *PRDM16-DT*. **G.** KD of<br>*PRDM16-DT* in human density. **G-K** shows data from human iPSC-derived astrocytes upon the KD of *PRDM16-DT*. **G.** KD of *PRDM16-DT* in human iPSC-derived astrocytes. H. qPCR showing the levels of the two glutamate density. G-K shows data from human if se derived astrocytes apon the KD of *TRDM16-DT*. G. KD of<br>*PRDM16-DT* in human iPSC-derived astrocytes. H. qPCR showing the levels of the two glutamate<br>transporters *GLT-1* and *GLAST* PRDM16-DT in human iPSC derived astrocytes. H. qPCR showing the levels of the two glutamate<br>transporters GLT-1 and GLAST and two synapse plasticity genes NRXN1 and GRIN1 after the KD of transporters GLT-1 and GLAST and two synapse plasticity genes NRXN1 and GRIN1 after the KD of

FRDM16-DT. I. Glutamate uptake after the KD of PRDM16-DT. **K**. Lactate secretion after the KD of PRDM16-DT. KD: hockdown, NC: negative control. Statistical significance was assessed by a one-way ANOVA with Tukey's post hoc dectate transporters MCT1 and MCT4 after the KD of TRDM16-DT. **K.** Lactate secretion after the KD<br>of *PRDM16-DT.KD:* knockdown, NC: negative control. Statistical significance was assessed by a one-<br>way ANOVA with Tukey's p

of PRDM16-DP.KD: knockdown, NC: negative control: Statistical sigmicance was assessed by a one-<br>way ANOVA with Tukey's post hoc test for (F) or a Student's unpaired t test for the other graphs;<br>\*P $\mathbb{R}$ < $\mathbb{Z}0.05$ , \*\*P way and the test for the other test for (F) or a Student emperator for the other graphs;<br>\*P@<@0.05, \*\*P@<@0.01, \*\*\*P@<@0.001; \*\*\*\*P@<@0.0001, ns = not significant.<br>In addition to impaired neuronal support function, the RNA In addition to impaired neuronal support function, the RNAseq data s<br>Prdm16os induces an inflammatory response (see Fig. 3G, H). However<br>astrocytes exhibited an increased expression of pro-inflammatory senes could no  $\frac{1}{6}$ *Prdm16os* induces an inflammatory response (see Fig. 3G, H). However, while iPSC-derived<br>astrocytes exhibited an increased expression of pro-inflammatory cytokines upon stimulation with<br>the 3 cytokine cocktail, the induct Promisos induces an immunitiony response (see Fig. 3G, H). However, while it se derived<br>astrocytes exhibited an increased expression of pro-inflammatory cytokines upon stimulation with<br>the 3 cytokine cocktail, the inductio the 3 cytokine cocktail, the induction of pro-inflammatory genes could not be confirmed when<br>
Prdm16-DT was knocked-down in human iPSC-derived astrocytes (Fig. S1).<br> **A** souther that we with the construction and the const



 $\frac{1}{2}$ Fig S1.<br>Although the reason for this discrepancy is unclear at present, it may suggest that the control of<br>inflammatory processes is not conserved across species and we therefore we decided to not follow<br>up on the potentia  $\frac{1}{2}$ inflammatory processes is not conserved across species and we therefore we decided to not follow inflammatory processes is not conserved across species and its institute its decided to instance.<br>up on the potential role of *Prdm16os* and *Prdm16-DT* in inflammation.<br>Prdm16os interacts with Rest and Suz12 to modulate t

up on the potential role of Pram1605 and Pram16-BP in inhammation.<br>Prdm16os interacts with Rest and Suz12 to modulate the expression<br>organization and function  $\frac{1}{2}$ Prdm16os interacts with Rest and Suz12 to modulate the expression of genes critical for synapse organization and function

So far our analysis suggests that *Prdm16os* and *PRDM16-DT* affect astrocytic processes linked to neuronal support via gene-expression control. One mechanism by which lncRNAs could control gene expression is to orchestrat expression is to orchestrate the availability of transcription factors or chromatin regulators to bind<br>specific DNA regions, such as promoter regions. To explore this further, we revisited the RNAseq<br>data from astrocytes u expecific DNA regions, such as promoter regions. To explore this further, we revisited the RNAseq<br>data from astrocytes upon *Prdm16os* KD and focused on the downregulated genes since these were<br>mainly associated with neuro specific DNA regions, such as promoter regions. To explore this further, we revisited the RNAseq<br>data from astrocytes upon *Prdm16os* KD and focused on the downregulated genes since these were<br>mainly associated with neuron data from astrocytes upon Pram16os RD and focused on the downleganated genes since these were<br>mainly associated with neuronal support function. We conducted an enrichment analysis to identify<br>transcription factors potentia main, accounted with neuronal support function. The conducted an entertainment analysis to taxant, the sanalyses revealed Suz12, a subunit of the Polycomb Repressive Complex 2 (PRC2) that mediates gene-repression via histo analyses revealed Suz12, a subunit of the Polycomb Repressive Complex 2 (PRC2) that mediates<br>gene-repression via histone 3 lysine 27 tri-methylation (H3K27me3), followed by RE1-Silencing<br>Transcription factor (Rest), also k analysis repression via histone 3 lysine 27 tri-methylation (H3K27me3), followed by RE1-Silencing<br>Transcription factor (Rest), also known as Neuron-Restrictive Silencer Factor (Nrsf) (Fig. 5A). Previous<br>studies have shown generalism: Transcription factor (Rest), also known as Neuron-Restrictive Silencer Factor (Nrsf) (Fig. 5A). Previous<br>studies have shown that lncRNAs can recruit PRC2 to specific chromatin locations [32] [33] [34].<br>Moreover Transcription factor (Rest), also known as Neuron-Restrictive Shencer Factor (Nrsf) (Fig. 3A). Previous<br>studies have shown that lncRNAs can recruit PRC2 to specific chromatin locations [32] [33] [34].<br>Moreover, it has been Studies have shown that leads that particles interact to silence gene-expression [35] [36]<br>
[37], with Rest being a key transcriptional regulator that represses neuronal genes in non-neuronal<br>
cells [38]. At the same time [37], with Rest being a key transcriptional regulator that represses neuronal genes in non-neuronal<br>cells [38]. At the same time it is known that due to their neuronal support function and under<br>specific conditions, astroc cells [38]. At the same time it is known that due to their neuronal support function and under<br>specific conditions, astrocytes express genes typically associated with neurons such as ion channels<br>and genes associated with specific conditions, astrocytes express genes typically associated with neurons such as ion channels species associated with synaptic function including neurotransmitter receptors, cell adhesion<br>molecules, and other neuromodulatory genes, thus contributing to synaptic modulation [38] [39]<br>[40] [41]. Furthermore, astrocyte and genes associated with sympton including measurementation including molecules, and other neuromodulatory genes, thus contributing to synaptic modulation [38] [39] [40] [41]. Furthermore, astrocytes are capable of transf [40] [41]. Furthermore, astrocytes are capable of transferring RNA, proteins, and even mitochondria<br>to neurons, promoting synaptic plasticity [42] [43] [44] [45]. It is therefore tempting to speculate<br>that *Prdm16os* may to neurons, promoting synaptic plasticity [42] [43] [44] [45]. It is therefore tempting to speculate that *Prdm16os* may affect the function of PRC2 and Rest to fine tune the expression of genes linked to neuronal support that *Prdm16os* may affect the function of PRC2 and Rest to fine tune the expression of genes linked<br>to neuronal support function in astrocytes.<br>To test this hypothesis, we conducted RNA-immunoprecipitation (RNA-IP) for S

to neuronal support function in astrocytes.<br>To test this hypothesis, we conducted RNA-immunoprecipitation (RNA-IP) for Suz12 (Fig. 5B) and<br>Rest (Fig. 5C) and demonstrated their interaction with *Prdm16os*. To investigate w To test this hypothesis, we conducted RN<br>Rest (Fig. 5C) and demonstrated their inter<br>affects the interaction Rest with promoter To test this hypothesis, we conducted RNA-immunoprecipitation (RNA-IP) for Suziz (Fig. 5B) and<br>Rest (Fig. 5C) and demonstrated their interaction with *Prdm16os*. To investigate whether *Prdm16os*<br>affects the interaction Re affects the interaction Rest with promoter regions of potential target genes, we performed Suz12<br>chromatin immunoprecipitation followed by qPCR (ChIP-qPCR) in primary astrocytes that were<br>treated with a control oligonucleo chromatin immunoprecipitation followed by qPCR (ChIP-qPCR) in primary astrocytes that were<br>treated with a control oligonucleotides (control) or GapmeRs to mediate the knockdown of<br>*Prdm16os*. For qPCR, we selected genes fo chromatic intrinsic prosperiment following qpc in (chip-qpc), in primary sensorytes that were<br>treated with a control oligonucleotides (control) or GapmeRs to mediate the knockdown of<br>*Prdm16os*. For qPCR, we selected genes Prdm16os. For qPCR, we selected genes for which we had confirmed their decreased expression<br>upon *Prdm16os* and Prdm16-DT knockdown in primary astrocytes and iPSC-derived human<br>astrocytes. We found that the levels of Rest Promitious. For qPent, we selected genes for which we had committed then decreased expression<br>upon *Prdm16os* and Prdm16-DT knockdown in primary astrocytes and iPSC-derived human<br>astrocytes. We found that the levels of Res astrocytes. We found that the levels of Rest binding to the promoter regions of candidate mRNAs<br>linked to synaptic plasticity, namely *Glast, Nrxn1* and *Grin1* were increased after the KD of<br>*Prdm16os*. There were no sign astrocytes are complete that the levels of an among to the promoter regions of enhancement in a linked to synaptic plasticity, namely *Glast, Nrxn1* and *Grin1* were increased after the KD of *Prdm16os*. There were no sign *Prdm16os.* There were no significant changes in Rest binding to the promoter of glycerinaldehyd-3-<br>phosphat-dehydrogenase (*Gapdh*) that was used as a housekeeping control gene (**Fig. 5D**). Since<br>Suz12 is part of the PRC2 Promitious. There were no significant changes in Rest binding to the promoter of given inatelyd-3-<br>phosphat-dehydrogenase (*Gapdh*) that was used as a housekeeping control gene (**Fig. 5D**). Since<br>Suz12 is part of the PRC2 phosphat-dehydrogenase (Gapah) that was used as a housekeeping control gene (Fig. 5D). Since<br>Suz12 is part of the PRC2 complex regulating H3K27me3, we decided to analyze if the KD of<br>*Prdm16os* would affect H3K27me3, a mar Prdm16os would affect H3K27me3, a mark linked to gene repression. ChIP-qPCR analysis from<br>14 Prdm16os would affect H3K27me3, a mark linked to gene repression. ChIP-qPCR analysis from

astrocytes treated with control oligomers or GapmeRs targeting *Prdm16os* revealed significantly increased H3K27me3 levels at the promoter regions of Glast and Grin1 upon *Prdm16os* KD (Fig 5E).<br>For *Nrx1*, we detected a n For *Nrx1*, we detected a non-significant trend, and no effect was observed for Gapdh (Fig 5E). Finally, we could confirm for this experimental setting that the transcripts for *Glast*, *Nrxn1 and Grin1* were decreased in For *NYX1*, we detected a non-significant trend, and no effect was observed for Glapdh (Fig 5E). Thany, we could confirm for this experimental setting that the transcripts for *Glast*, *Nrxn1* and *Grin1* were decreased in we could commit for this experimental setting that the transcripts for Glast, NYXII and Grin1 were<br>decreased in astrocytes upon Prdm16os knock down (Fig 5D). These findings suggest that Prdm16os<br>might act as a decoy for Re might act as a decoy for Rest and Suz12 to enable and fine-tune the expression of astrocytic genes<br>that have important neuronal support functions. might act as a decoy for Rest and Suzzle and fine-tune the expression of astrocytic genes<br>that have important neuronal support functions. that have important neuronal support functions.



Figure 5: Prdm16os binds to Suz12 and Rest and affects Rest and H3K27me3 levels at the promoter on the downregulated genes after the KD of *Prdm16os*. **B.** *Prdm16os* interacts with Suz12 as shown<br>by RNA immunoprecipitation (RNA-IP). Gapdh mRNA was used as additional control. **C.** *Prdm16os*<br>interacts with Rest as sh by RNA immunoprecipitation (RNA-IP). Gapdh mRNA was used as additional control. **C.** *Prdm16os*<br>interacts with Rest as shown by RNA immunoprecipitation (RNA-IP) 18s RNA was used as additional<br>control. **D.** ChIP for Rest of by RNA immunoprecipitation (RNA-H). Gapan mRNA was used as additional control. C. Pram16os<br>interacts with Rest as shown by RNA immunoprecipitation (RNA-IP) 18s RNA was used as additional<br>control. **D.** ChIP for Rest of prim control. **D.** ChIP for Rest of primary astrocytes treated with control oligomers (control) or *Prdm16os* control. control. D. ChIP for Rest of primary astrocytes treated with control oligomers (control) or Prdm16os

GapmeRs (*Prdm16os* KD), followed by qPCR for promoter regions of genes important for neuronal support and synapse function (*Glast, Nrxn1, Grin1*), which were downregulated upon *Prdm16os* KD. The housekeeping gene Gapdh support and synapse function (Glast, *WAH*, G*HH*), which were downlegulated upon *Pram16os* KD.<br>The housekeeping gene Gapdh is used as a control. E. ChIP for H3K27me3 of primary astrocytes<br>treated with control oligomers ( The housekeeping gene Gapan is used as a control. **L.** Chin for H3K27me3 or primary astrocytes<br>treated with control oligomers (control) or *Prdm16os* GapmeRs (*Prdm16os* KD) followed by qPCR for<br>promoter regions of the gen promoter regions of the genes *Glast, Nrxn1* and *Grin1*. The housekeeping gene *Gapdh* is used as<br>control. F. Bar charts showing the expression of *Glast, Nrxn1* and *Grin1* in astrocytes upon *Prdm16os*<br>KD. **G.** Heat map promoter regions of the genes Glast, *WART* and Grin1. The housekeeping gene Gapan is used as<br>control. F. Bar charts showing the expression of *Glast*, *Nrxn1* and *Grin1* in astrocytes upon *Prdm16os*<br>KD. **G.** Heat map sh control. T. Bar charts showing the expression of Glast, Nrxn1 and Gm11 in astrocytes upon Pram16os<br>KD. **G.** Heat map showing the fold changes of Rest and H3K27me3 levels as well as the transcript<br>levels of *Glast, Nrxn1*, levels of *Glast, Nrxn1, Grin1* and *Gapdh* in astrocytes upon *Prdm16os* KD. Statistical significance was assessed by a one-way ANOVA with Tukey's post hoc test; \*P**R<RO.05, \*\*\*PR<RO.001;**<br>\*\*\*\*PR<RO\_0001. ns = not signif devels of Glast, Nrxn1, Grin1 and Gapan in astrocytes upon Pram16os KD. Statistical significance was<br>assessed by a one-way ANOVA with Tukey's post hoc test; \*PR<RO.05, \*\*\*PR<RO.001;<br>\*\*\*\*PR<RO.0001, ns = not significant.<br>CR

### x\*\*\*P $\mathbb{R}$ <20.0001, ns = not significant.<br>CRISPR-mediated restoration of *Prdm16os* expression can rescue cytokine-induced functional deficits in astrocytes \*\*\*\*PL<L0.0001, ns = not significant. CRISPR-mediated restoration of Prdm16os expression can rescue cytokine-induced functional deficits in astrocytes

 $\frac{1}{2}$ risk factors (see Fig. 2), our next objective was to investigate whether reinstating the expression of this lncRNA would be sufficient to ameliorate the observed functional defects. To achieve this, we decided to use the 3 risk factors (see Fig. 2), this lncRNA would be sufficient to ameliorate the observed functional defects. To achieve this, we decided to use the 3-cytokine cocktail exposure model (see Fig. 2E) and employed CRISPR activati the manner means are summarine to ameliance the successive interesting function (crising activation (CRISPRa) targeting the promoter region of *Prdm16os* via guide RNAs (gRNA). As a control, a gRNA with no target in the DN (CRISPRa) targeting the promoter region of *Prdm16os* via guide RNAs (gRNA). As a control, a gRNA with no target in the DNA was used (Scramble). We tested 3 different gRNAs under basal conditions and observed that gRNA-1, with no target in the DNA was used (Scramble). We tested 3 different gRNAs under basal conditions<br>and observed that gRNA-1, and 3 significantly increased the expression of *Prdm16os* with gRNA-3<br>leading to a 2-fold increas and observed that gRNA-1, and 3 significantly increased the expression of *Prdm16os* with gRNA-3<br>leading to a 2-fold increase (Fig. 6A). Considering the superior efficacy of gRNA-3, it was selected for<br>subsequent experimen leading to a 2-fold increase (Fig. 6A). Considering the superior efficacy of gRNA-3, it was selected for subsequent experiments. The plasmid to express CRISPRa also contained a Gfp signal allowing us to confirm successful leading to a 2-fold increase (Fig. 6A). Considering the superior efficacy of gRNA-3, it was selected for<br>subsequent experiments. The plasmid to express CRISPRa also contained a Gfp signal allowing us to<br>confirm successful confirm successful transfection of primary astrocytes (Fig. 6B). Next, astrocytes were transfected with CRISPRa and gRNA-3 (gRNA-3) upon concurrent stimulation with the 3 cytokine cocktail (3 Cyt), or vehicle as control (v with CRISPRa and gRNA-3 (gRNA-3) upon concurrent stimulation with the 3 cytokine cocktail (3 Cyt), or vehicle as control (vehicle). Cell transfected with CRISPRa and scramble control guide RNA were used as control (Scrambl or vehicle as control (vehicle). Cell transfected with CRISPRa and scramble control guide RNA were<br>used as control (Scramble) (Fig. 6C\_MODEL).<br>In line with our previous observations, treating cells with the 3 cytokine cock

or used as control (Scramble) (Fig. 6C\_MODEL).<br>In line with our previous observations, treating cells with the 3 cytokine cocktail reduced the levels<br>of *Prdm16os*, while CRISPRa treatment reinstated *Prdm16os* expression used as control (Scramble) (Fig. 0C\_MODEL).<br>In line with our previous observations, treatiof *Prdm16os*, while CRISPRa treatment reinst<br>**6D**). Encouraged by this observation, we wan<br>would also affect glutamate uptake and l In the CRISPRa treatment reinstated *Prdm16os* expression to physiological levels (Fig.<br> **GD)**. Encouraged by this observation, we wanted to test if the reinstatement of *Prdm16os* expression<br>
Would also affect glutamate u **6D)**. Encouraged by this observation, we wanted to test if the reinstatement of *Prdm16os* expression would also affect glutamate uptake and lactate release, which were both reduced upon *Prdm16os* KD and are compromised would also affect glutamate uptake and lactate release, which were both reduced upon *Prdm16os*<br>KD and are compromised in AD pathogenesis. In agreement with this, glutamate uptake (Fig. 6E) and<br>lactate release (Fig. 6F) wa Would also affect glutamate uptake and lactate release, which were both reduced upon Pram16os<br>KD and are compromised in AD pathogenesis. In agreement with this, glutamate uptake (Fig. 6E) and<br>lactate release (Fig. 6F) was lactate release (Fig. 6F) was significantly impaired upon cytokine treatment. However, when astrocytes were treated with cytokines when Prdm16os expression levels were increased via CRISPRa, this was sufficient to reinstate glutamate uptake (Fig. 6C) and lactate release (Fig 6D). CRISPRA, this was sufficient to refilstate glutamate uptake (Fig. 6C) and lactate release (Fig 6D).

Interestingly, glutamate uptake and lactate release was also increased when *Pramitoos* levels had<br>been elevated via CRIPRa in vehicle-treated cells (Fig. 6E,F) further suggesting a key role of<br>*Prdm16os* in the regulation *Prdm16os* in the regulation of these vital astrocyte functions. Using qPCR we confirmed that key genes linked to glutamate uptake (*Glast*) and lactate release (*Mct4*) that we had previously found to be affected by *Prdm* Pramitions in the regulation of these vital astrocyte functions. Osing qr CR we commited that key<br>genes linked to glutamate uptake (*Glast*) and lactate release (*Mct4*) that we had previously found to<br>be affected by *Prdm* genes linked to glutamate uptake (Glast) and lactate release (Mct4) that we had previously found to<br>be affected by *Prdm16os* and *PRDM16-DT* KD were significantly decreased upon 3 Cyt treatment (Fig<br>6G). CRISPR a-mediated **6G**). CRISPR a-mediated increased expression of *Prdm16os* was able to ameliorate this effect (Fig 6G).

6G).<br>6G).<br>In conclusion these data suggest that strategies to elevate *Prmd16-DT* could help to reinstate<br>important neuronal support functions of astrocytes that are compromised in response to stimuli 66).<br>In co<br>impo<br>asso  $\frac{1}{6}$ Important neuronal support functions of astrocytes that are compromised in response to stimuli associated with AD pathogenesis. important neuronal support functions of anti-space that are compromised in response to stimuliar<br>associated with AD pathogenesis. associated with AD pathogenesis.



Figure 6: CRISPRa-mediated overexpression of Prdm16os can rescue functional impairments induced by cytokine treatment. A. Bar Chart showing the expression of *Prdm16os* in astrocytes<br>transfected using CRRIPRa in combination with g1, 2 or 3 in comparison of cells treated with scrambled RNA  $(s)$ . B. Representative immunofluorescence images showing the transfection of primary astrocytes with CRISPRa plasmids containing a scramble or Prdm16os-targeting g3 RNA. C. primary astrocytes with CRISPRa plasmids containing a scramble or Pram16os-targeting g3 RNA. C.

Scheme of the experimental approach. D. qPCR showing the levels of *Prdm16os* in astrocytes treated with either the 3 cytokine cocktail (3 Cyt) ot vehicle (control) in the presence of a either scramble RNA or g3 RNA to me RNA or g3 RNA to mediate CRISPRa of *Prdm16os*. Note that CRISPRa with g3 increased *Prdm16os*<br>expression in the control group when compared to scramble RNA (one-way ANOVA followed by<br>tTest; \*\*P < 0.01) and is able to rei RNA or g3 RNA to mediate CRISTRa or *Frum16os*. Note that CRISTRa with g3 increased *Frum16os*<br>expression in the control group when compared to scramble RNA (one-way ANOVA followed by<br>tTest; \*\*P < 0.01) and is able to rein tTest;  $*P < 0.01$ ) and is able to reinstate  $Prdm16os$  expression to physiological levels upon cytokine<br>treatment (one-way ANOVA followed by tTest;  $***P < 0.001$ ). E. Bar charts showing the results from<br>the Glutamate uptake a treatment (one-way ANOVA followed by tTest; \*\*\*P < 0.001). E. Bar charts showing the results from<br>the Glutamate uptake assay of primary astrocytes after cytokine treatment and CRISPRa. F. Bar<br>charts showing the results fro the Glutamate uptake assay of primary astrocytes after cytokine treatment and CRISPRa. F. Bar charts showing the results from the Lactate release assay of primary astrocytes after cytokine treatment and CRISPRa. Note that the Glutamate uptake assay of primary astrocytes after cytokine treatment and chisi ha. 1, Bar<br>charts showing the results from the Lactate release assay of primary astrocytes after cytokine<br>treatment and CRISPRa. Note that charts and CRISPRa. Note that cytokine treatment impairs glutamate uptake and lactate release<br>which is reinstated upon CIRPRa mediated overexpression of *Prdm16os.* **G.** Bar charts showing qPCR<br>results for *Glast* (upper p which is reinstated upon CIRPRa mediated overexpression of *Prdm16os*. **G.** Bar charts showing qPCR<br>results for *Glast* (upper panel) and *Mct4* (lower panel) in the 4 experimental groups. Note that<br>CRIPRa mediated overexp which is reinstated upon ChY-Na mediated overexpression of *Prum16os*. **G.** Bar charts showing qPCN<br>results for *Glast* (upper panel) and *Mct4* (lower panel) in the 4 experimental groups. Note that<br>CRIPRa mediated overexp CRIPRa mediated overexpression can reinstate physiological expression of *Glast* and *Mct4* upon cytokine treatment. For A, D-G: Statistical significance was assessed by a one-way ANOVA with Tukey's post hoc test; \*P $\mathbb{$ cytokine treatment. For A, D-G: Statistical significance was assessed by a one-way ANOVA with<br>Tukey's post hoc test; \*PIXI20.05, \*\*PIXI20.01; \*\*\*\*PIXI20.0001 Tukey's post hoc test; \*P $\mathbb{R}$ < $\mathbb{R}$ 0.05, \*\*P $\mathbb{R}$ < $\mathbb{R}$ 0.01; \*\*\*\*P $\mathbb{R}$ < $\mathbb{R}$ 0.0001

#### **Discussion**

 $T_{\text{S}}$ <br> $\text{Dissussion}$ <br> $\text{In this study, we identified the human IncRNA BPM16, D1, and}$  $\begin{array}{c} \n\frac{1}{2} & \frac{1}{2} \\ \n\frac{1}{2} & \frac{1}{$ |
|
| In this study, we identified the human lnctwa PRDM16-DP and its mouse homologue, Pram160s, to<br>be enriched in the brain, where they are almost exclusively expressed in astrocytes when we cortical<br>tissues. This finding align be entitled in the brain, interesting, and antitate entities, impresent interespect in the brain of the brain<br>specific expression patterns [16]. We focused on *PRDM16-DT* in this study because it also has a<br>homolog in mice patients, while no change in expression was observed in schizophrenia patients or in the brains of specific expression patterns [16]. We focused on PRDM16-DP in this study because it also has a<br>homolog in mice, and conservation between species is an indicator of functionality [46]. Further<br>analysis revealed that *PRDM16* analysis revealed that *PRDM16-DT* levels are decreased in the brains of Alzheimer's disease (AD) patients, while no change in expression was observed in schizophrenia patients or in the brains of patients with frontotempo analysis revealed that *PRDM16-DT* levels are decreased in the brains of Alzheimer's disease (AD)<br>patients, while no change in expression was observed in schizophrenia patients or in the brains of<br>patients with frontotempo patients with frontotemporal dementia (FTD). Although data on lncRNAs in the brains of AD patients<br>are still rare compared to the analysis of the coding transcriptome [47] [48], previous studies have<br>reported lncRNAs dereg pare still rare compared to the analysis of the coding transcriptome [47] [48], previous studies have<br>reported lncRNAs deregulated in postmortem brains from AD patients. For example, *BACE1-AS*,<br>which controls the producti reported IncRNAs deregulated in postmortem brains from AD patients. For example, *BACE1-AS*, which controls the production of amyloid beta peptides, has been reported to be deregulated [49].<br>While analyzing human brain tis

which controls the production of amyloid beta peptides, has been reported to be deregulated [49].<br>While analyzing human brain tissue provides a unique opportunity to elucidate the processes<br>underlying AD pathogenesis, the While analyzing human brain tissue provides a unique opportunity to elucidate the processe<br>underlying AD pathogenesis, the data might be affected by factors such as post-mortem delay an<br>other variables [50] [51]. Therefore underlying AD pathogenesis, the data might be affected by factors such as post-mortem delay and<br>other variables [50] [51]. Therefore, it is important to note that *PRDM16-DT* and *Prdm16os*<br>expressions were also decreased other variables [50] [51]. Therefore, it is important to note that *PRDM16-DT* and *Prdm16os*<br>expressions were also decreased when human iPSC-derived or mouse astrocytes were exposed to expressions were also decreased when human iPSC-derived or mouse astrocytes were exposed to expressions were also decreased when human iPSC-derived or mouse astrocytes were exposed to

stimulti associated with a 3-cytokine cocktail shown to mediate microglia-induced reactive astrogliosis [3], and Abeta42.<br>In line with the observation that *Prdm16os* was mainly localized to the nucleus, we observed a subs a 3-cytokine cocktain that the observation that *Prdm16os* was mainly localized to the nucleus, we observ<br>substantial effect on gene expression upon knockdown of *Prdm16os*, with the expression of g<br>important for neuronal In line with the observation that *Fram16os* was mainly localized to the hacleas, we observed a<br>substantial effect on gene expression upon knockdown of *Prdm16os*, with the expression of genes<br>important for neuronal suppor substantial effect on gene expression upon knockdown of *Prum16os*, with the expression of genes<br>important for neuronal support being downregulated. Among these were the glutamate transporter<br>Glast and the lactate transpor Glast and the lactate transporter *Mct4*. Similarly, *GLAST* and *MCT4* were downregulated when<br>PRDM16-DT was knocked down in human iPSC-derived astrocytes. Consistently, KD of Prdm16os or<br>PRDM16-DT in mouse or human astro PRDM16-DT was knocked down in human iPSC-derived astrocytes. Consistently, KD of Prdm16os or<br>PRDM16-DT in mouse or human astrocytes impaired glutamate uptake and lactate release. Loss of<br>Prdm16os expression in astrocytes a PRDM16-DT in mouse or human astrocytes impaired glutamate uptake and lactate release. Loss of<br>Prdm16os expression in astrocytes also increased the expression of proinflammatory cytokines.<br>However, this effect was not obser Prdm16os expression in astrocytes also increased the expression of proinflammatory cytokines.<br>However, this effect was not observed in human iPSC-derived astrocytes, despite their confirmed<br>response to the 3-cytokine cockt Promisos expression in astrocytes also increased the expression of prominamiatory cytokines.<br>However, this effect was not observed in human iPSC-derived astrocytes, despite their confirmed<br>response to the 3-cytokine cockta response to the 3-cytokine cocktail stimulation, as evidenced by increased expression of cytokines<br>such as IL6 and IL1b.<br>While the molecular mechanisms underlying this discrepancy remain unclear, it is interesting to note<br>

such as IL6 and IL1b.<br>While the molecular mechanisms underlying this discrepancy remain unclear, it is interesting to note<br>that studies have reported that during AD pathogenesis, astrocytes exhibit decreased levels of the such as IL6 and IL1b.<br>While the molecular<br>that studies have rep<br>glutamate transporte That studies have reported that during AD pathogenesis, astrocytes exhibit decreased levels of the glutamate transporters *Glast* and genes involved in synapse organization, such as neuroligins and neurexins [52] [53]. The Blutamate transporters *Glast* and genes involved in synapse organization, such as neuroligins and<br>neurexins [52] [53]. These genes were all downregulated upon *Prdm16os* or *PRDM16-DT* knockdown<br>in our study. Moreover, sn glutamate transporters Glast and genes involved in synapse organization, such as neuroligins and<br>neurexins [52] [53]. These genes were all downregulated upon *Prdm16os* or *PRDM16-DT* knockdown<br>in our study. Moreover, snuc in our study. Moreover, snucRNAseq studies on the brains of AD patients suggest a modest increase<br>in proinflammatory genes in astrocytes and, more strikingly, a strong decrease in the expression of<br>homeostatic genes involv in proinflammatory genes in astrocytes and, more strikingly, a strong decrease in the expression of<br>homeostatic genes involved in synapse regulation [54]. This is in line with other data on familial AD<br>cases suggesting tha in the bomeostatic genes involved in synapse regulation [54]. This is in line with other data on familial AD<br>cases suggesting that astrocytes in AD brains are characterized by transcriptional underexpression of<br>key genes l cases suggesting that astrocytes in AD brains are characterized by transcriptional underexpression of<br>key genes linked to astrocytic function [54].<br>Nevertheless, we cannot exclude a role for *PRDM16-DT* in inflammatory pro

key genes linked to astrocytic function [54].<br>Nevertheless, we cannot exclude a role for *PRDM16-DT* in inflammatory processes in human<br>astrocytes during AD pathogenesis. Future research is necessary to elucidate this aspe key formulation of the set of the Nevertheless, we cannot exclude a role<br>astrocytes during AD pathogenesis. Future<br>detail. astrocytes during AD pathogenesis. Future research is necessary to elucidate this aspect in more<br>detail.<br>Taken together, the data from our analysis of postmortem human brains and human and mouse

detail.<br>Taken together, the data from our analysis of postmortem human brains and human and mouse<br>astrocytes consistently suggest a role for astrocytic *Prdm16os* and *PRDM16-DT* in neuronal support<br>functions. In line with Taken<br>astrocy<br>functio astrocytes consistently suggest a role for astrocytic *Prdm16os* and *PRDM16-DT* in neuronal support<br>functions. In line with this, we observed that astrocytes failed to enhance spine density in the<br>absence of *Prdm16os*. A durations. In line with this, we observed that astrocytes failed to enhance spine density in the absence of *Prdm16os*. Although there is no data for *PRDM16-DT* or *Prdm16os*, and limited data on the role of lncRNAs in as functions. In this case, the state of the many density in the absence of *Prdm16os*. Although there is no data for *PRDM16-DT* or *Prdm16os*, and limited data on the role of lncRNAs in astrocytes, our findings align with p absence of Pram16os. Although there is no data for PRDM16-DP of Pram16os, and infiled data on<br>the role of lncRNAs in astrocytes, our findings align with previous studies showing that lncRNAs can<br>regulate astrocyte function the regulate astrocyte function. For example, *Neat1*, a ubiquitously expressed lncRNA, is increased in<br>astrocytes in response to ischemia and AD models [55] [56]. Similarly, *MEG3* is a lncRNA upregulated<br>in human neurons regulate astrocyte function. For example, Weat1, a ubiquitously expressed lnckWA, is increased in<br>astrocytes in response to ischemia and AD models [55] [56]. Similarly, *MEG3* is a lncRNA upregulated<br>in human neurons xenog in human neurons xenografted in AD mouse models, and its inhibition ameliorated amyloid-induced in human neurons  $x_0$  models, and its inhibition amelion amplitude, and its induced amolitation amol

necroptosis [57]. This aligns with previous findings showing that *MEG3* inhibition could rescue<br>Abeta-induced phenotypes in a rat model of AD, including reactive astrogliosis [58]. Other lncRNAs<br>associated with neurodegen associated with neurodegenerative conditions and astrocyte function include NKILA and MALAT1

associated with neurodegenerative conditions and astrocyte function include *NKILA* and *MALAT1*<br>[59] [60].<br>However, unlike *PRDM16-DT*, all these lncRNAs are ubiquitously expressed, and therefore targeting<br>then therapeuti However,<br>However,<br>them the<br>the brain However, unlike *FRDM16-DT*, all these lickwas are ubiquitously expressed, and therefore targeting<br>them therapeutically may lead to unwanted side effects. Therefore, targeting lncRNAs exclusive to<br>the brain and specificall the brain and specifically to astrocytes offers the potential for more precise therapeutics. Along this<br>line, our data show that CRISPRa-mediated increase of *Prdm16os* expression in astrocytes can<br>ameliorate the phenotype the brain and show that CRISPRa-mediated increase of *Prdm16os* expression in astrocytes can<br>ameliorate the phenotypes induced by exposure to AD risk factors. These findings are significant<br>because targeting astrocyte func ameliorate the phenotypes induced by exposure to AD risk factors. These findings are significant<br>because targeting astrocyte function has been suggested as a suitable approach for addressing<br>specific early phases of AD pat because targeting astrocyte function has been suggested as a suitable approach for addressing

specific early phases of AD pathogenesis [7].<br>Our data suggest that *PRDM16-DT* and *Prdm16os* may mainly influence astrocyte function by<br>controlling gene expression. This aligns with previous studies indicating that a key Specific early phases of AD pathogenesis [7].<br>Specific data suggest that *PRDM16-DT* and *Pathontoning* gene expression. This aligns with<br>the regulation of gene expression in the i Controlling gene expression. This aligns with previous studies indicating that a key role of lncRNAs is<br>the regulation of gene expression in the nucleus, often through interactions with transcription<br>factors or chromatin-m controlling gene expression in the nucleus, often through interactions with transcription<br>factors or chromatin-modifying enzymes [61] [14]. We observed that *Prdm16os* interacts with Suz12,<br>a subunit of the PRC2 complex th the regulation of gene expression in the nucleus, often interact measurements with Suz12,<br>factors or chromatin-modifying enzymes [61] [14]. We observed that *Prdm16os* interacts with Suz12,<br>a subunit of the PRC2 complex th a subunit of the PRC2 complex that orchestrates gene silencing via H3K27-trimethylation [35] [36]<br>[37], and Rest, a key transcriptional regulator that represses neuronal genes in non-neuronal cells<br>[38]. This is interestin and Rest, a key transcriptional regulator that represses neuronal genes in non-neuronal cells<br>[38]. This is interesting since Rest and PRC2 were shown to interact to control gene expression [35].<br>While Rest orchestrates th

[38]. This is interesting since Rest and PRC2 were shown to interact to control gene expression [35].<br>While Rest orchestrates the repression of neuronal genes in non-neuronal cells, astrocytes are<br>known to express several [38]. This is interest were repression of neuronal genes in non-neuronal cells, astrocytes and While Rest orchestrates the repression of neuronal genes in non-neuronal cells, astrocytes and provincial such genes, including Whown to express several such genes, including synaptic cell adhesion molecules like neurexins and<br>neuroligins that mediate astrocyte-synapse interactions [62] and other synapse-associated genes<br>[63] [64] [65]. It is tempt Representing to the unceral several such the uncertainty of the uncertainty including to the promoter regions of these genes in astrocytes, thereby preventing complete gene silencing. Our data suggest that *Prdm16os* may r [63] [64] [65]. It is tempting to speculate that a mechanism exists to prevent Rest from binding to the promoter regions of these genes in astrocytes, thereby preventing complete gene silencing. Our data suggest that *Prd* promoter regions of these genes in astrocytes, thereby preventing complete gene silencing. Our data<br>suggest that *Prdm16os* may regulate the expression of these target genes by acting as a decoy for<br>Rest and Suz12. In this suggest that *Prdm16os* may regulate the expression of these target genes by acting as a decoy for<br>Rest and Suz12. In this scenario, the loss of *PRDM16-DT*, as observed in AD brains, would<br>compromise the fine-tuning of ge suggest that *Fram16os* may regulate the expression of these target genes by acting as a decoy for<br>Rest and Suz12. In this scenario, the loss of *PRDM16-DT*, as observed in AD brains, would<br>compromise the fine-tuning of ge Rest and Suziz. In this scenario, the loss of *PRDM16-DT*, as observed in AD brains, would<br>compromise the fine-tuning of gene expression pathways in astrocytes, leading to a loss of neuronal<br>support function and synaptic p support function and synaptic plasticity. This view is consistent with previous data suggesting that<br>impaired astrocyte function plays an important role in neurodegenerative processes [66] [28] [67]<br>[68]. impaired astrocyte function plays an important role in neurodegenerative processes [66] [28] [67]<br>[68].<br>It is important to acknowledge the limitations of our study. Increasing evidence suggests astrocyte<br>beterogoneity acro

[68].<br>It is important to acknowledge the limitations of our study. Increasing evidence suggests astrocyte<br>heterogeneity across the brain, revealing several distinct astrocyte subtypes between and within<br>health projects [6  $[t \text{ is } i]$ <br> $\text{heter}$ <br> $\text{brain}$ heterogeneity across the brain, revealing several distinct astrocyte subtypes between and within brain regions [69] [70]. Investigating the expression pattern of  $Prdm16os$  and  $PRDM16-DT$  in brain brain regions [69] [70]. Investigating the expression pattern of *Pram16os* and *PRDM16-DT* in brain

Function and in the course of AD. Another important question for future research is to elucidate the<br>regulation of PRDM16-DT expression to better understand the mechanisms underlying its reduced<br>levels in the brains of AD Function and *FRDM16-DT* expression to better understand the mechanisms underlying its reduced<br>levels in the brains of AD patients and in AD model systems. Although our data suggest that<br>*PRDM16-DT* is specifically decreas regulation of *PRDM16-DT* expression to better understand the mechanisms underlying its reduced<br>levels in the brains of AD patients and in AD model systems. Although our data suggest that<br>*PRDM16-DT* is specifically decrea PRDM16-DT is specifically decreased in the brains of AD patients while remaining unaffected in FTD<br>and schizophrenia patients, we cannot exclude the possibility that PRDM16-DT is affected in other<br>brain diseases. Additiona PRDM16-DT is specifically decreased in the brains of AD patients while remaining unaffected in the and schizophrenia patients, we cannot exclude the possibility that *PRDM16-DT* is affected in other brain diseases. Additio brain diseases. Additionally, it could be considered to test the role of *Prdm16os* in transgenic mouse<br>models. However, in line with the principles of the 3Rs (Replacement, Reduction, and Refinement),<br>in this study we dec models. However, in line with the principles of the 3Rs (Replacement, Reduction, and Refinement), in this study we decided to focus on cellular models and confirm key findings in human iPSC-derived cells rather than mouse cells rather than mouse models with the aim to reduce the reliance on animal experimentation,

In summary, our data suggest that PRDM16-DT could be a suitable drug target to ameliorate which is getting increasingly difficult in the European Union and in Germany in particular.<br>In summary, our data suggest that *PRDM16-DT* could be a suitable drug target to ameliorate<br>neurodegenerative processes associated In summary, our data suggest that *PRDM16-DT* could be a suitable drug target to<br>neurodegenerative processes associated with AD pathogenesis. The specific expression<br>*PRDM16-DT* in astrocytes of the human brain offers a un In summary, our data suggest that PRDM16-DT could be a suitable drug target to ameliorate<br>neurodegenerative processes associated with AD pathogenesis. The specific expression pattern of<br>PRDM16-DT in astrocytes of the human  $PRDM16-DT$  in astrocytes of the human brain offers a unique possibility to target distinct phases of AD pathogenesis with minimal unwanted side effects. PRDM16-DT in astrocytes of the human brain offers a unique possibility to target district phases of<br>AD pathogenesis with minimal unwanted side effects. AD pathogenesis with minimal unwanted side effects.

 $\overline{a}$  $\frac{1}{\sqrt{2}}$ Methods and Material

Human tissues

For snucRNAseq tissue samples (prefrontal cortex, BA9) were obtained with ethical approval from<br>the ethics committee of the University Medical Center Göttingen and upon informed consent from<br>the New York brain bank (femal years, PMD = 16,4  $\pm$  7,4 h; Braak & Braak stage IV) were obtained with ethical approval from the the New York branch bank (female: n = 1, age = 1, postmation delay, postmation = 1, age = 7,<br>postmortem delay = 5:24h). Brains (prefrontal cortex, BA9) from control (n = 4 females & 5 males;<br>age = 73,7 ± 9,5 years, PMD = age = 73,7 ± 9,5 years, PMD = 20,5 ± 4,1 h) and AD patients (n = 4 females & 6 males; age = 85,3 ± 7,3 years, PMD = 16,4 ± 7,4 h; Braak & Braak stage IV) were obtained with ethical approval from the ethics committee of th years, PMD = 16,4 ± 7,4 h; Braak & Braak stage IV) were obtained with ethical approval from the<br>ethics committee of the University Medical Center Göttingen and upon informed consent from the<br>Harvard Brain Tissue Resource C years, PMD = 19,4 ± 7,4 h; Braak & Branch stage IV) were cleaned with stated approximation in<br>ethics committee of the University Medical Center Göttingen and upon informed consent from the<br>Harvard Brain Tissue Resource Cen Harvard Brain Tissue Resource Center (Boston, USA).<br> **Sorting of neuronal and non-neuronal nuclei from human brain**<br>
The isolation protocol of single nuclei from frozen human brain was adapted from the previously

### Sorting of neuronal and non-neuronal nuclei from human brain

Sorting of neuronal and non-neuronal nuclei from hu<br>The isolation protocol of single nuclei from frozen l<br>published protocol with following modifications [71] published protocol with following modifications [71] [72]. In short, frozen tissues were homogenized<br>using a Dounce homogenizer in 500 µl EZ prep lysis buffer (Sigma NUC101) supplemented with 1:200<br>RNAse inhibitor (Promega pusing a Dounce homogenizer in 500 μl EZ prep lysis buffer (Sigma NUC101) supplemented with 1:200<br>RNAse inhibitor (Promega, N2615) for 60 times in a 1.5 ml Eppendorf tube using micro pestles. The<br>volume was adjusted to 20 RNAse inhibitor (Promega, N2615) for 60 times in a 1.5 ml Eppendorf tube using micro pestles. The volume was adjusted to 2000  $\mu$ l with lysis buffer and incubated on ice for 7 min. Cell lysates were centrifuged for 5 min When we are inhibited (Promaga, N2612), the contribution and incubated on ice for 7 min. Cell lysates were centrifuged for 5 min at 500 $\sqrt{2}$  at 4 °C and supernatants were discarded. The pellet was resuspended in 2000 µl centrifuged for 5 min at 500 $\sqrt{2}$  at 4 °C and supernatants were discarded. The pellet was resuspended in 2000 µl lysis buffer followed by an incubation on ice for 5 min. Lysates were centrifuged (500 $\sqrt{2}$  at 4 °C) and resuspended in 2000 µl lysis buffer followed by an incubation on ice for 5 min. Lysates were<br>centrifuged (500 $\overline{2}$ × $\overline{2}$ g at 4 °C) and the pellet was resuspended in 1500 µl nuclei suspension buffer<br>(NSB, 0.5% BSA, 1 centrifuged (500 $\mathbb{Z} \times \mathbb{Z}$ ) and the pellet was resuspended in 1500 µl nuclei suspension buffer<br>(NSB, 0.5% BSA, 1:100 Roche Protease inhibitor, 1:200 RNAse inhibitor in 1 $\mathbb{Z} \times \mathbb{Z}$ PBS) centrifuged again<br>for 5 m CHERA (NSB, 0.5% BSA, 1:100 Roche Protease inhibitor, 1:200 RNAse inhibitor in 1 $\mathbb{R} \times \mathbb{R}$ PBS) centrifuged again<br>for 5 min (500 $\mathbb{R} \times \mathbb{R}$ ) at 4 °C). The pellet was finally resuspended in 1000 µl NSB and staine for 5 min (500 $\overline{2}$  at 4 °C). The pellet was finally resuspended in 1000  $\mu$ l NSB and stained with anti-<br>NeuN-AlexaFluor®488 (MAB377X) for 1h at 4°C rotating followed by centrifugation for 5 min<br>(500 $\overline{2}$ × $\overline{2}$ g  $\frac{1}{2}$  means for  $\frac{1}{2}$  and  $\frac{1}{2}$  at  $\frac{1}{2}$  and  $\frac{1}{2}$   $\frac{1}{2}$  and  $\frac{1}{2}$   $\frac{1}{2}$  and  $\frac{1}{2}$  and (500 $\overline{a}$ X $\overline{a}$ g at 4 °C). The pellet was washed once with 500  $\mu$ L NSB and the pellet was resuspended in 300 $\mu$ L NSB and stained with 1:100 7AAD (Invitrogen, Cat: 00–6993-50). Single NeuN-positive and NeuN-negati (500 UL NSB and stained with 1:100 7AAD (Invitrogen, Cat: 00–6993-50). Single NeuN-positive and<br>
NeuN-negative nuclei were sorted using the BD FACS Aria III sorter. Sorted nuclei were counted in<br>
the Countess II FL Automat NeuN-negative nuclei were sorted using the BD FACS Aria III sorter. Sorted nuclei were counted in<br>the Countess II FL Automated Counter. Single-nuclei RNA-seq using the iCELL8 System was<br>performed at the NGS Integrative Gen the Countess II FL Automated Counter. Single-nuclei RNA-seq using the iCELL8 System was<br>performed at the NGS Integrative Genomics Core Unit in Göttingen, Germany. Approximately 1200<br>single nuclei per sample were sequenced. performed at the NGS Integrative Genomics Core Unit in Göttingen, Germany. Approximately 1200 performed at the NGS Integrative Genomics Core Unit in Göttingen at the NGS Integrative Genomics Core Unit in Göttingen, Genomics Core Unit in Göttingen, Germany. Approximately 12000 Single nucleus total RNA sequencing and

#### Single nucleus total RNA sequencing and Analysis

The Takara ICELL8 5,184 nano-well chip was used with the full-length SMART-Seq ICELL8 Reagent Kit !<br>|
| For single nuclei library performances as described in [73]. Briefly, nuclei suspensions were<br>fluorescent-labelled with live/dead stain, Hoechst 33,342 for 15 min prior to their dispensing into<br>the Takara ICELL8 5,184 nano fluorescent-labelled with live/dead stain, Hoechst 33,342 for 15 min prior to their dispensing into<br>the Takara ICELL8 5,184 nano-well chip. CellSelect Software (Takara Bio) was used to visualize and<br>24 the Takara ICELL8 5,184 nano-well chip. CellSelect Software (Takara Bio) was used to visualize and the Takara ICELL8 5,184 nano-well chip. Cells 5,184 nano-well chip. Cells to visualize and<br>24 select wells containing single nuclei. A total of four 5184 nano-wells chips were used for all samples<br>obtaining 1200 to 1400 nuclei/sample. Final libraries were amplified and pooled as they were<br>extracted from each of the sequenced on the HiSeq 4000 (Illumina) to obtain on average lof 0.3 to 0.4 Mio reads per nuclei (SE; extracted from each of the single nanotest input sites metallic the puller and size selection<br>using Agencourt AMPure XP magnetic beads (Beckman Coulter) to obtain an average library size of<br>500 bp. A typical yield for a li using Agenceura China in a magnetic beads (Elemental Centre), to clean in the energy means, year of 500 bp. A typical yield for a library comprised of  $21,300$  cells was  $215$  nM. Libraries were sequenced on the HiSeq 400 sequenced on the HiSeq 4000 (Illumina) to obtain on average<sup>r</sup> and was likely exact sequenced on the HiSeq 4000 (Illumina) to obtain on averager and to 0.4 Mio reads per nuclei (SE;<br>50 bp). Illumina's conversion software b so bp). Illumina's conversion software bcl2fastq (v2.20.2) was used for adapter trimming and converting the base calls in the per-cycle BCL files to the per-read FASTQ format from raw images.<br>For further processing, the Co 50 converting the base calls in the per-cycle BCL files to the per-read FASTQ format from raw images.<br>For further processing, the Cogent NGS Analysis pipeline (v1.5.1) was used to generate a gene-count<br>matrix. The demuxer For further processing, the Cogent NGS Analysis pipeline (v1.5.1) was used to generate a gene-count<br>matrix. The demuxer (cogent demux) was used to create demultiplexed FASTQ files from the<br>barcoded sequencing data. The res For Formulary of the Cogent demux) was used to create demultiplexed FASTQ files from the<br>barcoded sequencing data. The resulting output was then used as input for the analyzer (cogent<br>analyze) which performs trimming, mapp barcoded sequencing data. The resulting output was then used as input for the analyzer (cogent<br>analyze) which performs trimming, mapping and gene counting to create a gene counts matrix.<br>Quality control was done by evaluat analyze) which performs trimming, mapping and gene counting to create a gene counts matrix.<br>Quality control was done by evaluating the quality report provided by the Cogent analyzer.<br>The SCANPY package was used for pre-fil

Quality control was done by evaluating the quality report provided by the Cogent analyzer.<br>The SCANPY package was used for pre-filtering, normalization and clustering. Initially, cells that<br>reflected low-quality cells (eit The SCANPY package was used for pre-filtering, normalization and clustering. Initially,<br>reflected low-quality cells (either too many or too few reads, cells isolated almost exclusive<br>expressing less than 10% of house-keepi The SCANP of the expressing less than 10% of house-keeping genes) were excluded. Next, counts were scaled by the total library size multiplied by 10.000, and transformed expressing less than 10% of house-keeping genes) were excluded. Next, counts were scaled by the total library size multiplied by 10.000, and transformed to log space. Highly variable genes were identified based on dispersi total library size multiplied by 10.000, and transformed to log space. Highly variable genes were identified based on dispersion and mean, the technical influence of the total number of counts was regressed out, and the va identified based on dispersion and mean, the technical influence of the total number of counts was<br>regressed out, and the values were rescaled. Principal component analysis (PCA) was performed on<br>the variable genes, and UM regressed out, and the values were rescaled. Principal component analysis (PCA) was performed on<br>the variable genes, and UMAP was run on the top 50 principal components (PCs). The top 50 PCs<br>were used to build a k-nearestregressed out, and the variable interactions with parameterizations were values of the variable genes, and UMAP was run on the top 50 principal components (PCs). The top 50 PCs were used to build a k-nearest-neighbours cel were used to build a k-nearest-neighbours cell-cell graph with k= 50 neighbours. Subsequently,<br>spectral decomposition over the graph was performed with 50 components, and the Leiden graph-<br>clustering algorithm was applied spectral decomposition over the graph was performed with 50 components, and the Leiden graph-<br>clustering algorithm was applied to identify cell clusters. We confirmed that the number of PCs<br>captures almost all the variance expression on the graph and visual inspection. Violin plots for market genes were created using the clustering algorithm was applied to identify cell clusters. We confirmed that the number of PCs<br>captures almost all the variance of the data. For each cluster, we assigned a cell-type label using<br>manual evaluation of gene manual evaluation of gene expression for sets of known marker genes by plotting marker gene expression on the UMAP and visual inspection. Violin plots for marker genes were created using the<br>"stacked\_violin function" as implemented in SCANPY.<br>"stacked\_violin function" as implemented in SCANPY. expression on the UMAP and Visual inspection. Violin plots for marking genes were created using the<br>"stacked\_violin function" as implemented in SCANPY.<br>Primary astrocyte culture

#### Primary astrocyte culture

"stacked violin function" as in Scale<br>Primary mouse astrocyte cultures were prepared<br>Primary mouse astrocyte cultures were prepared  $\frac{1}{2}$ Primary mouse astrocyte cultures were prepared from postnatal day 1-2 mice as previously<br>described [74] with minor modifications. Briefly, pups were sacrificed by decapitation and the brains quickly removed. Cortices and hippocampi were dissected and dissociated using 0.05 % Trypsin-

Ethylenediaminetetraacetic acid (EDTA) (Gibco). Cells from 2-3 mice were plated into one T75 flask<br>coated with poly-D-lysine (PDL) and cultured with DMEM, 10% fetal bovine serum and 1%<br>penicillin/streptomycin (all Gibco) ( penicillin/streptomycin (all Gibco) (DMEM+) in a humidified incubator with 5% CO2 at 37°C for 7-8<br>days. Then, cells were placed on a shaker (Incu-Shaker Mini, Benchmark) and shaken at 160 rpm for<br>6 hours to loosen neurons penium, on epoco, pair (all Gibco) (DMEM+) in a humidimental members into 5% CO2 at 37°C.<br>days. Then, cells were placed on a shaker (Incu-Shaker Mini, Benchmark) and shaken at 160 rpm for<br>6 hours to loosen neurons and nondeals have placed on a shaker (Includent mini, Benchmark), and shaked at  $\frac{1}{2}$  rpm for  $\frac{1}{2}$  from  $\frac{1}{2}$  from  $\frac{1}{2}$  from  $\frac{1}{2}$  from a shaken at 160 rpm for a minutes at 37°C. DMEM+ was added to inactiva fincubated with 0.25% Trypsin-EDTA (Gibco) for 4 minutes at 37°C. DMEM+ was added to inactivate<br>the trypsin and the cell suspension was transferred to fresh tubes and centrifuged for 4 minutes at<br>400 g. The cells were res the trypsin and the cell suspension was transferred to fresh tubes and centrifuged for 4 minutes at 400 g. The cells were resuspended in Neurobasal<sup>TM</sup> Plus Medium containing 2% B-27<sup>TM</sup> Plus Supplement, 1% penicillin/str 400 g. The cells were resuspended in Neurobasal™ Plus Medium containing 2% B-27™ Plus<br>Supplement, 1% penicillin/streptomycin, 1x GlutaMAX™ Supplement (all from Gibco) (NB+) and 5<br>ng/mL Heparin-Binding EGF-Like Growth Fac Supplement, 1% penicillin/streptomycin, 1x GlutaMAX™ Supplement (all from Gibco) (NB+) and 5 ng/mL Heparin-Binding EGF-Like Growth Factor (HB-EGF) (Sigma-Aldrich) and plated at a density of 15,000 cells/cm<sup>2</sup> onto PDL- or Supplement, 1% percentional matrix of the set of the maintained in the incubator, and half the medium was changed two times a week.<br>
Primary cortical neuron ng/mL Heparin-Binding Let Land Fremm Factor (HL Liv) (Sigma-Aldrich) and plated at a density of<br>15,000 cells/cm<sup>2</sup> onto PDL- or 0.1% polyethylenimine (PEI)-coated cell culture dishes. Cultures were<br>then maintained in the i 15,000 cells/cm-<br>then maintained<br>**Primary cortical**<br>Pregnant CD-1 m then maintained in the incubator, and half the medium was changed two times a week.<br> **Primary cortical neuron culture**<br>
Pregnant CD-1 mice were sacrificed using pentobarbital. Embryonic day 17 embryos were taken out

### Primary cortical neuron culture

Then mary cortical neuron culture<br>Then may cortical neuron culture<br>Then maintained in the medium was changed and the meninges were removed and the brains quickly removed in ice-cold PBS. The meninges were removed and and the brains quickly removed in ice-cold PBS. The meninges were removed and the cortices dissected. Then, cell dissociation was performed using the Papain Dissociation System (Worthington) as described by the manufactur dissected. Then, cell dissociation was performed using the Papain Dissociation System (Worthington)<br>as described by the manufacturer's instructions. The cells were resuspended in NB+ and seeded at a<br>density of 120,000 cell as described by the manufacturer's instructions. The cells were resuspended in NB+ and seeded at a<br>density of 120,000 cells/cm<sup>2</sup> for glutamate treatment or 60,000 cells/cm<sup>2</sup> for spine density analysis.<br>For the co-cultur

density of 120,000 cells/cm<sup>2</sup> for glutamate treatment or 60,000 cells/cm<sup>2</sup> for spine density analysis.<br>For the co-cultures, inserts containing astrocytes were added to cortical neurons on day in vitr<br>(DIV) 14 and the exp density of 120,000 cells/cm<sup>-</sup> for glutamate treatment or 60,000 cells/cm<sup>-</sup> for spine density analysis.<br>For the co-cultures, inserts containing astrocytes were added to cortical neurons on day in vitr<br>(DIV) 14 and the exp

Human iPSC-derived astrocytes<br>Human iPSC-derived astrocytes (Ncyte Astrocytes) were obtained from Ncardia. Cells were thawed ()<br>(Dividion in the experience of the Human iPSC-derived astrocytes<br>(Dividion divided astrocytes (Neyte Astrocytes) were and cultured according to the manufacturer's instruction Human iPSC-derived astrocytes<br>Human iPSC-derived astrocytes<br>and cultured according to the m<br>culture for 1-2 weeks before per and cultured according to the manufacturer's instructions. After thawing, the astrocytes were left in<br>culture for 1-2 weeks before performing experiments.<br>Stimulation of primary astrocytes and culture for 1-2 weeks before performing experiments.<br>Culture for 1-2 weeks before performing experiments.<br>Thimary microglia were cultured as previously described [75]. On DIV 10, they were stimulated with

#### Stimulation of primary astrocytes

Stimulation of primary astrocytes<br>Primary microglia were cultured as previously describe<br>100 ng/mL Lipopolysaccharide (LPS) for 4 hours. Ther 100 ng/mL Lipopolysaccharide (LPS) for 4 hours. Then, the medium was changed to NB+, collected<br>after 6 hours, filtered using a 0.22 µm filter and stored at -80°C until use. On DIV13 of astrocyte<br>culture, it was added as a after 6 hours, filtered using a 0.22  $\mu$ m filter and stored at -80°C until use. On DIV13 of astrocyte culture, it was added as a 1:1 mixture and RNA was isolated 24 hours later. culture, it was added as a 1:1 mixture and RNA was isolated 24 hours later.<br>
26 culture, it was added as a 1:1 mixture and RNA was isolated 24 hours later.<br>The culture and RNA was in the culture and RNA was in the culture and RNA was in the culture and RNA was in th<br>The culture and RNA was in the cult

To mimic the activation of astrocytes by microglia, primary astrocytes were treated with a cytokine<br>mixture consisting of II-1 $\alpha$  (3 $\overline{2}$ ng mI-1, Sigma, 13901), TNF (30 $\overline{2}$ ng mI-1, Cell Signaling Technology,<br>8902S

mixture consisting of Il-1, MyBioSource, MBS143105) for 24 hours, as described previously [3].<br>In another approach, Amyloid beta 1-42 (Cayman Chemicals) was incubated at 37°C for 1 hour to<br>form protofibrils and then added 89029291, Minoring minoring minority interactions, Matematics, Matematics, Premission, Laples<br>1. In another approach, Amyloid beta 1-42 (Cayman Chemicals) was incubated at 37°C for 1 hour to<br>1. Antisense LNA Gapmers In an entertainty approach, Amylois at the actor of 1-42 (Cayman Chemicals) was included at 37°C for 24 hours.<br> **Antisense LNA Gapmers**<br>
Antisense LNA Gapmers targeting *Prdm16os* and *PRDM16-DT* and negative controls (NC)

#### Antisense LNA Gapmers

Form prototions and then added to an object and the concentration of 1 µM on DIV13 for 24 hours.<br>Antisense LNA Gapmers<br>designed and purchased from Qiagen having the following sequences: Antisense ENA Gapmers targeting Pram16os and PRDM16-DP and negative controls (NC) were<br>designed and purchased from Qiagen having the following sequences:<br>NC: GCTCCCTTCAATCCAA<br>Prdm16os: TGCGACGTCTCAAGATG

designed and purchased and purchased in the following sequence<br>Prom Liagen and purchases:<br>The following sequences: The fol NC: CONTROLLING<br>Prdm16os: TGCGACGTCTA<br>PRDM16-DT: TACAGAACT<br>Transfection

PRDM16-DT: TACAGAACTGGTCAT<br>PRDM16-DT: TACAGAACTGGTCAT<br>Transfection was performed us Transfection was performed using Lipofectamine RNAiMAX (ThermoFisher) according to the manufacturer's instructions. Cells were treated on DIV12 and all experiments were performed on DIV14. .<br>.<br>. manufacturer's instructions. Cells were treated on DIV12 and all experiments were performed on DIV14.<br>Prdm16os overexpression using CRISPRa<br>Plasmids containing the guide RNA (gRNA), a dCas9-VP64 and a tGFP reporter gene were obtained

#### Prdm16os overexpression using CRISPRa

Pramat<br>Prdm1t<br>Plasmic<br>from O from Origene (GE100074). The gRNAs for targeting Prdm16os were also designed by Origene and had the following sequences:<br>a gRNA-1: AGACGGTCACCTCGCCTCCA from Original Central Asiation original the following sequences:<br>FRNA-1: AGACGGTCACCTCGCCTCCA<br>FRNA-2: GATAGTTGGGACACGGGTCC<br>FRNA-3: GAGCCCGAAGCTGCAGCCAC

gRNA-1: AGACGGTCACCTCGC<br>gRNA-2: GATAGTTGGGACACG<br>gRNA-3: GAGCCCGAAGCTGCA<br>As a negative control, a sc gRNA-2: GATAGTTGGGACACGGGTCC<br>gRNA-3: GAGCCCGAAGCTGCAGCCAC<br>As a negative control, a scramble<br>(GE100077). To increase gene expres gRNA-3: GAGCCCGAAGCTGCAGCCAC<br>As a negative control, a scramble<br>(GE100077). To increase gene express<br>The cells were transfected before

S<br>As a negative control, a scramble<br>(GE100077). To increase gene express<br>The cells were transfected before :<br>(ThermoFisher). 100,000 cells were (GE100077). To increase gene expression, an enhancer vector (GE100056) was used in all cases.<br>The cells were transfected before seeding on DIV7 using the Neon Nxt electroporation device<br>(ThermoFisher). 100,000 cells were e The cells were transfected before seeding on DIV7 using the Neon Nxt electroporation de<br>(ThermoFisher). 100,000 cells were electroporated using 0.5 µg CRISPRa plasmid and 0.162<br>enhancer plasmid (1300 V, 20 ms, 2 pulses). C ThermoFisher). 100,000 cells were electroporated using 0.5 µg CRISPRa plasmid and 0.1625 µg<br>enhancer plasmid (1300 V, 20 ms, 2 pulses). Cells were seeded in 24 well plates and cultured for two<br>days before treatment with th enhancer plasmid (1300 V, 20 ms, 2 pulses). Cells were seeded in 24 well plates and cultured for two<br>days before treatment with the cytokine cocktail or PBS. RNA isolation and functional experiments<br>were performed 24 hours days before treatment with the cytokine cocktail or PBS. RNA isolation and functional experiments<br>were performed 24 hours later. days beformed 24 hours later.<br>Were performed 24 hours later.<br>27 were performed 24 hours later.

#### Glutamate uptake

with 100 µM glutamate in HBSS for 1 hour at 37°C. Afterwards, the supernatant was collected and<br>the amounts of remaining glutamate in the HBSS were measured using the Glutamate-Glo<sup>rm</sup> Assay<br>(Promega) according to the man the amounts of remaining glutamate in the HBSS were measured using the Glutamate-Glo™ Assay<br>(Promega) according to the manufacturer's instructions. Luminescence was recorded with a<br>FLUOstar® Omega (BMG). (Promega) according to the manufacturer's instructions. Luminescence was recorded with a<br>FLUOstar® Omega (BMG). (Promega) according to the manufacturer's intributions conditions into the recorded with a<br>FLUOstar® Omega (BMG).<br>To estimate lactate secretion, the complete medium was changed one hour before performing

#### Lactate secretion

actate secretion<br>To estimate lactate secre<br>Transfection. After 48 h Transfection. After 48 hours, the medium was collected and diluted 1:40 in PBS. Lactate<br>concentrations were measured using the Lactate- Glo™ Assay (Promega) according to the<br>manufacturer's protocol.<br>Cutonlocmic and nuclea transferred and the mediation. After the mediation. After and distributed and distributed and the mediation.<br>
Cytoplasmic and nuclear fractionation<br>
Cytoplasmic and nuclear fractionation concentrations were manufacturer's protocol.<br>Cytoplasmic and nuclear fractionation<br>Primary astrocytes cultured in 6 well plates were dissociated on DIV14 with 0.25% trypsin-EDTA.

#### Cytoplasmic and nuclear fractionation

manufacturer protocol.<br>Cytoplasmic and nuclear<br>Primary astrocytes cultur<br>After centrifuging at 400 After centrifuging at 400 g for five minutes and washing the cells with PBS, 500 µL EZ prep lysis<br>buffer (Sigma-Aldrich) supplemented with RNase inhibitor (Promega) was added to each sample and<br>incubated on ice for seven m buffer (Sigma-Aldrich) supplemented with RNase inhibitor (Promega) was added to each sample and<br>incubated on ice for seven minutes. The samples were centrifuged and the supernatant was<br>aspirated and collected, containing t incubated on ice for seven minutes. The samples were centrifuged and the supernatant was<br>aspirated and collected, containing the cytoplasmic fraction. The nuclear pellet was washed with 2<br>mL EZ prep lysis buffer, incubated aspirated and collected, containing the cytoplasmic fraction. The nuclear pellet was washed with 2<br>mL EZ prep lysis buffer, incubated on ice for five minutes and centrifuged again. The supernatant<br>was aspirated and the pel as a spirated and the pellet resuspended in 1.5 mL phosphate buffered saline (PBS) supplemented with 0.5% bovine serum albumin (BSA), RNase inhibitor and protease inhibitor (Roche). The samples were centrifuged and the su was aspirated and the pellet resuspended in 1.5 mL phosphate buffered saline (PBS) supplemented<br>with 0.5% bovine serum albumin (BSA), RNase inhibitor and protease inhibitor (Roche). The samples<br>were centrifuged and the sup with 0.5% bovine serum albumin (BSA), RNase inhibitor and protease inhibitor (Roche). The samples<br>were centrifuged and the supernatant aspirated, leaving 250 µL of liquid in the tube. TRIzol<sup>na</sup> LS<br>Reagent (ThermoFisher) w were centrifuged and the supernatant aspirated, leaving 250  $\mu$ L of liquid in the tube. TRIzol<sup>TM</sup> LS<br>Reagent (ThermoFisher) was added to both the cytoplasmic and nuclear fraction and the samples<br>were stored at -20°C unt Reagent (ThermoFisher) was added to both the cytoplasmic and nuclear fraction and the samples<br>were stored at -20°C until RNA was extracted.<br>Glutamate treatment of primary neurons were stored at -20°C until RNA was extracted.<br>Glutamate treatment of primary neurons<br>Neuronal and neuron-astrocyte co-cultures were incubated on DIV 17 with 100 µM glutamate for 15

#### Glutamate treatment of primary neurons

Were stored at 20°C until Andrew states in<br>Glutamate treatment of primary neurons<br>Neuronal and neuron-astrocyte co-cultures we<br>minutes at 37°C. Then, the medium containing Neuronal and neuron-astropyce of cultures were incubated on DIV 17 with 10 parameters co-cultures at 37°C. Then, the medium containing glutamate was removed and replaced with fresh NB+<br>for 3 hours. Cell viability was measu minutes at 37°C. The measured at 37°C. The more measured as a set of the more measured was replaced the more shared with Spine density analysis.<br>Spine density analysis

# for 3 hours. Cell via bility, was measured using Prestors (ThermoFisher).<br>Spine density analysis Spine density analysis

Dendritic spines were labelled using the dye 1,1<sup>0</sup>-dioctadecyl-3,3,3<sup>0</sup>,3<sup>0</sup>tetramethylindocarbocyanine perchlorate (Dil) (Life Technologies) as described previously [76], using<br>2% paraformaldehyde (PFA) for fixation. Spine density and dendrite length were measured with<br>ImageJ software.

#### $\blacksquare$ MACS-sorting of oligodendrocytes, astrocytes and microglia

Cells were isolated from the cortex of three months old male C57B/6J mice using the adult brain Image Software<br>MACS-sorting of<br>Cells were isolate<br>dissociation kit (c dissociation kit (cat. no. 130-107-677, Miltenyi) according to the manufacturer's protocol with minor<br>modifications. Briefly, mice were sacrificed using pentobarbital and the brains were quickly<br>removed. To remove major pa modifications. Briefly, mice were sacrificed using pentobarbital and the brains were quickly<br>removed. To remove major parts of the meninges, the brains were rolled over Whatman paper and<br>then the cortices were dissected an moved. To remove major parts of the meninges, the brains were rolled over Whatman paper and<br>then the cortices were dissected and placed into the enzyme mixes. The tissue was incubated at<br>37°C for 30 minutes in a water bath remove may remove may part of the meninges, the mention are removed to rolled at<br>then the cortices were dissected and placed into the enzyme mixes. The tissue was incubated at<br>37°C for 30 minutes in a water bath and tritur 37°C for 30 minutes in a water bath and triturated gently three times during this period. Then, the samples were applied to 40  $\mu$ m cell strainers and the protocol was followed for debris and red blood cell removal. Olig  $\frac{3}{2}$  for 30 minutes were applied to 40 µm cell strainers and the protocol was followed for debris and red blood<br>cell removal. Oligodendrocytes were isolated using Anti-O4 microbeads (1:40, cat. no. 130-094-543),<br>astr samples were isolated using Anti-O4 microbeads (1:40, cat. no. 130-094-543), astrocytes using Anti-ACSA2 microbeads (1:10, cat. no. 130-097-678) and microglia with Anti-Cd11b microbeads (1:10, cat. no. 130-093-634). Purity extracted engels into the tremoval and games is matter and (2003) and microglia with Anti-Cd11b<br>microbeads (1:10, cat. no. 130-093-634). Purity of the cell type populations was determined by<br>qPCR. astrocytes unig Anti-Actor Entreprendict (2:25) and microscopy and microbeads (1:10, cat. no. 130-093-634). Purity of the cell type populations was determined by qPCR.<br>QPCR.<br>In situ hybridization combined with immunofluore qPCR.<br>In situ hybridization combined with immunofluorescence<br>We performed RNAscope Fluorescent Multiplex assays (ACD Bio) combined with

#### In situ hybridization combined with immunofluorescence

qPCR.<br>
In situ hybridization combined with immunofluorescence<br>
We performed RNAscope Fluorescent Multiplex assays (ACD Bio) combined with<br>
immunofluorescence according to the manufacturer's instructions for fresh frozen ti We performed RNAscope Fluorescent Multiplex assays (ACD Bio) combined with<br>immunofluorescence according to the manufacturer's instructions for fresh frozen tissue sections.<br>Briefly, 18 µm sections were fixed with 10% neutr Briefly, 18 µm sections were fixed with 10% neutral buffered formalin (NBF) and dehydrated with<br>ethanol. Hydrogen peroxide was applied to the sections, followed by the incubation with anti-Gfap<br>(rabbit, Abcam; 1:250) at 4° ethanol. Hydrogen peroxide was applied to the sections, followed by the incubation with anti-Gfap<br>(rabbit, Abcam; 1:250) at 4°C overnight. On the next day, after a post-primary fixation step with 10%<br>NBF, the protocol for entricted to the RNA scope® Multiplex Fluorescent Reagent Kit v2 (Acd Bio) was followed,<br>NBF, the protocol for the RNA scope® Multiplex Fluorescent Reagent Kit v2 (Acd Bio) was followed,<br>using probes designed against *Prd* NBF, the protocol for the RNAscope® Multiplex Fluorescent Reagent Kit v2 (Acd Bio) was followed,<br>using probes designed against *Prdm16os* (Acdbio) and TSA Plus Cyanine 5 (Akoya Biosciences; 1:750)<br>for detection of the prob using probes designed against *Prdm16os* (Acdbio) and TSA Plus Cyanine 5 (Akoya Biosciences; 1:750)<br>for detection of the probes. Afterwards, the slides were incubated with Alexa Fluor<sup>m</sup> 555 goat anti-<br>rabbit secondary an for detection of the probes. Afterwards, the slides were incubated with Alexa Fluor<sup>m</sup> 555 goat antirabbit secondary and the motions of the more interesting.<br>
In a week after staining.<br>
In a step of the mounted with a teical damages were easy of the mounted using the mounted using the second of<br>
All images were taken wit

#### Imaging

Prolong Gold Antifade Reagent (Thermo Reage and Prolong Antifade at the Lemmarc Coming).<br>Prolonging<br>Antifanges were taken with a Leica dmi8 microscope fitted with a STEDycon STED/Confoci<br>(Abberior) in the confocal mode, wi (Abberior) in the confocal mode, with a 63X or 100X oil immersion objective.<br>29 (Abberior) in the confocal mode, with a 63X or 100X oil immersion objective.

#### RNA extraction

Concentrator-5 kit (Zymo Research) according to the manufacturer's instructions.<br>
RNA concentrations were determined by Nanodrop (ThermoFisher) or Qubit using the RNA HS Assay<br>
kit (ThermoFisher). The quality of the sample RNA concentrations were determined by Nanodrop (ThermoFisher) or Qubit using<br>kit (ThermoFisher). The quality of the samples used for sequencing was assessed<br>(Agilent Technologies). kit (ThermoFisher). The quality of the samples used for sequencing was assessed with a Bioanalyzer<br>(Agilent Technologies). kit (Thermorfisher). The quality of the samples used for sequencing the sectors using a Bioanalyzer<br>(Agilent Technologies).<br>Libraries were prepared using the SMARTer Stranded Total RNA Sample Prep Kit - HI Mammalian kit

#### Library preparation and total RNA sequencing

(Agilent Technology)<br>1991 - Libraries were prepared<br>1991 - Takara Bio) using 300 (Takara Bio) using 300 ng RNA as starting material. Libraries were amplified for 13 cycles and the quality of the preparations determined on the Bioanalyzer. The multiplexed libraries were sequenced in a NextSeq 2000 (Illu (Takara Bio) and good material. Library and the Bioanalyzer. The multiplexed libraries were sequenced in a NextSeq 2000 (Illumina) with a 50 bp single-read configuration.<br>Bioinformatic analysis equenced in a NextSeq 2000 (Illumina) with a 50 bp single-read configuration.<br> **Bioinformatic analysis**<br>
Processing and demultiplexing of raw reads was performed using bcl2fastq (v2.20.2). For quality

#### Bioinformatic analysis

sequences in a NextSeq 2000 (Indianally 1999 of the single-read configuration.<br>Bioinformatic analysis<br>Processing and demultiplexing of raw reads was performed using bcl2fastq<br>control of raw sequencing data, FastQC (v0.11.5 Frontrol of raw sequencing data, FastQC (v0.11.5) was used. Reads were aligned to the mouse<br>(mm10) genome with the STAR aligner (v2.5.2b), and read counts were generated using feature<br>Counts (v1.5.1). Differential gene ex (mm10) genome with the STAR aligner (v2.5.2b), and read counts were generated using feature<br>Counts (v1.5.1). Differential gene expression was performed with DESeq2 (v1.38.3) [77] using<br>normalized read counts and correctin (mm2) generate minimum stand angular (reading), and read counts (v1.38.3) [77] using<br>Counts (v1.5.1). Differential gene expression was performed with DESeq2 (v1.38.3) [77] using<br>normalized read counts and correcting for un Counts and correcting for unwanted variation detected by RUVSeq (v1.32.0) [78].<br>GO term analysis was performed with clusterProfiler (v4.6.0) [30]. Analysis for the enrichment of transcription factors targeting downregulate GO term analysis was performed with clusterProfiler (v4.6.0) [30]. Analysis for the enrichment of<br>transcription factors targeting downregulated genes was performed using ENRICHR<br>(https://maayanlab.cloud/Enrichr/). Franscription factors targeting downregulated genes was performed using ENRICHR<br>(https://maayanlab.cloud/Enrichr/).<br>CDNA, qPCR

#### cDNA, qPCR

cDNA was prepared with the Transcriptor cDNA first strand Synthesis Kit (Roche) using 20-800 ng RNA as starting material and random hexamer primers.

Synthesized cDNA was diluted up to ten-fold with nuclease-free water. qPCR reactions were<br>prepared with Light Cycler 480 SYBR Master Mix (Roche) and run in duplicates in a Light Cycler 480 Runthesized cDNA was diluted up to ten-fold with<br>prepared with Light Cycler 480 SYBR Master Mix (Roch<br>(Roche). Primer sequences can be found in supplemen Synthesized contributed up to ten-fold with nuclease the control of the control with nuclease-<br>prepared with Light Cycler 480 SYBR Master Mix (Roche) and run in duplicates in a Light Cycler 480<br>(Roche). Primer sequences ca prepared with Light Cycler 480 Syard (Roche). Primer sequences can be found in supplemental table 3. Analysis was done using the 2^-<br>DDCt method [79].<br>Western Blot (Roches). Primer sequences can be found in supplemental table 3. Analysis was done using the 20---

# De et method [79].<br>Western Blot Western Blot

Cells were lysed in RIPA buffer (ThermoFisher) containing protease inhibitor. Protein concentration was determined using the Pierce BCA Protein Assay Kit (ThermoFisher), and 20 µg were used per well of a 4–20% Mini-PROTEA well of a 4–20% Mini-PROTEAN® TGX™ Precast Protein Gel (Bio-Rad). Protein denaturation was done<br>using 8 M urea in Laemmli Sample Buffer (Bio-Rad) at 40°C for 60 minutes.. The gels were run at 90 V<br>for 15 minutes, followed well of a 4–20% Mini-Precast Precast Precast Precast Precast Precast Bellington Gel (Bio-Rad).<br>
For 15 minutes, followed by 50 minutes at 120 V. For the transfer, low fluorescence PVDF<br>
membranes and the Trans-Blot Turbo T membranes and the Trans-Blot Turbo Transfer System (both from Bio-Rad) were used.<br>Membranes were blocked with 5% BSA in PBS + 0.1% Tween-20 (PBS-T) for 1 hour at 4°C . Primary

for 15 minutes, following by 50 minutes at 120 V. Following financies, the transference PV.<br>
Membranes were blocked with 5% BSA in PBS + 0.1% Tween-20 (PBS-T) for 1 hour at 4°C. Primary<br>
antibodies were incubated ON at 4°C Membranes were blocked with 5% BSA in PBS + 0.1% Tween-20 (PBS-T) for 1 hour at<br>antibodies were incubated ON at 4°C in 1% milk in TBS-T or 5% BSA in PBS-T. The follo<br>antibodies were used: anti-Eaat1 (rabbit, Abcam; 1:2500) antibodies were incubated ON at 4°C in 1% milk in TBS-T or 5% BSA in PBS-T. The following primary<br>antibodies were used: anti-Eaat1 (rabbit, Abcam; 1:2500), anti-Eaat2 (rabbit, Abcam; 1:800) and<br>anti-Gapdh (mouse, ThermoFis antibodies were used: anti-Eaat1 (rabbit, Abcam; 1:2500), anti-Eaat2 (rabbit, Abcam; 1:800) and<br>anti-Gapdh (mouse, ThermoFisher; 1:4000). On the next day, the membranes were washed with<br>PBS-T and incubated with the respect anti-Gapdh (mouse, ThermoFisher; 1:4000). On the next day, the membranes were washed with<br>PBS-T and incubated with the respective fluorescent secondary antibodies (IRDye, LI-COR). After<br>washing the membranes again, blots w PBS-T and incubated with the respective fluorescent secondary antibodies (IRDye, LI-COR). After washing the membranes again, blots were imaged with an Odyssey DLx (LI-COR) and the images were analyzed with ImageJ software. Washing the membranes again, blots were imaged with an Odyssey DLx (LI-COR) and the images<br>were analyzed with ImageJ software. Information on antibodies can be found in supplemental table<br>4. were analyzed with ImageJ software. Information on antibodies can be found in supplemental table<br>4.<br>RNA immunoprecipitation 4.<br>RNA immunoprecipitation<br>For RNA immunoprecipitation (RNA-IP), cells were cultured in T75 flasks. On DIV14, cells were

#### RNA immunoprecipitation

n<br>**RN**<br>Fo<br>dis dissociated with 0.25% trypsin-EDTA, washed with PBS and resuspended in fractionation buffer (10 mM Tris-HCl, 10 mM NaCl, 3 mM MgCl<sub>2</sub>, 0.5% Nonidet P-40 (NP-40), 1mM DTT, 100 units/mL RNase inhibitor, 1x protease inhibito resuspended again to lyse the cells. After centrifuging for five minutes at 1000 g and 4°C, the<br>supernatant, containing the cytoplasmic fraction, was transferred into fresh tubes. The nuclear inhibitor, 1x protease inhibitor). The samples were incubated on ice for 10 minutes and then resuspended again to lyse the cells. After centrifuging for five minutes at 1000 g and 4°C, the supernatant, containing the cyto inhibitor, 1x protects inhibitor, 1x protects interfering for five minutes at 1000 g and 4°C, the<br>supernatant, containing the cytoplasmic fraction, was transferred into fresh tubes. The nuclear<br>fraction was rinsed with TSE supernatant, containing the cytoplasmic fraction, was transferred into fresh tubes. The nuclear<br>fraction was rinsed with TSE buffer (10 mM Tris, 300 mM sucrose, 1 mM EDTA, 0.1% NP-40, 1mM<br>DTT, 100 units/mL RNase inhibitor, fraction was rinsed with TSE buffer (10 mM Tris, 300 mM sucrose, 1 mM EDTA, 0.1% NP-40, 1mM<br>DTT, 100 units/mL RNase inhibitor, 1x protease inhibitor) and centrifuged at 1000 g for five minutes.<br>Then, the pellet was resuspe fraction was rinsed minimized with the with the with the term of year. Then, 100 units/mL RNase inhibitor, 1x protease inhibitor) and centrifuged at 1000 g for five minutes.<br>Then, the pellet was resuspended in fresh TSE bu Then, the pellet was resuspended in fresh TSE buffer, transferred to bioruptor tubes and sonicated<br>in a Bioruptor Plus (both Diagenode) for ten cycles (30s on, 30s off). The samples were incubated on<br>ice for 20 minutes wit In a Bioruptor Plus (both Diagenode) for ten cycles (30s on, 30s off). The samples were incubated on<br>ice for 20 minutes with occasional vortexing and centrifuged at 14500 g for ten minutes. The<br>supernatant was transferred ice for 20 minutes with occasional vortexing and centrifuged at 14500 g for ten minutes. The<br>supernatant was transferred to a fresh tube and both fractions were flash-frozen and stored at -<br>80°C.<br>The nuclear lystes were th supernatant was transferred to a fresh tube and both fractions were flash-frozen and stored at -<br>80°C.<br>The nuclear lysates were thawed and the protein concentration determined using a BCA assay. 500<br>us lysate per sample we

soot.<br>The nuclear lysates were thawed and the protein concentration determined using a BCA assay. 500<br>ug lysate per sample were pre-cleared using 25 µL Pierce<sup>rm</sup> Protein A/G magnetic beads<br>(ThermeEisber) for 1 hour at 4° The n<br>The n<br>US |<br>Therm  $\mu$ g lysate per sample were pre-cleared using 25  $\mu$ L Pierce<sup>TM</sup> Protein A/G magnetic beads<br>(ThermoFisher) for 1 hour at 4°C to reduce unspecific binding. 1.5  $\mu$ g per sample of anti-Rest (rabbit,<br>ThermoFisher) antibod ThermoFisher) for 1 hour at 4°C to reduce unspecific binding. 1.5 μg per sample of anti-Rest (rabbit,<br>ThermoFisher) antibody or the IgG isotype control (Abcam) was incubated with 50 μL Protein A/G<br>31 ThermoFisher) antibody or the IgG isotype control (Abcam) was incubated with 50  $\mu$ L Protein A/G  $T_{\text{max}}$  and  $T_{\text{max}}$  or the IgG isotype control (Abcam) was included with 50  $\mu$ L Protein A/G  $\geq$  magnetic beads for two hours at RT and washed with RNA-IP buffer (50 mM Tris-HCl, 100 mM NaCl,<br>32 mM NaF, 0.5% NP-40). Then, the samples, after taking 10% as input, were added to the beads and incubated overnight at 4°C with mixing. On the next day, the beads were washed five times with<br>RNA-IP buffer, resuspended in proteinase K in proteinase K buffer (Qiagen) and incubated for one<br>hour at 37°C. The supernatant RNA-IP buffer, resuspended in proteinase K in proteinase K buffer (Qiagen) and incubated for one hour at 37°C. The supernatant was collected and RNA extracted using the RNA clean & concentrator-

#### Chromatin immunoprecipitation (ChIP)

Primary astrocytes were grown in 15 cm dishes and treated with NC or KD ASOs as described above. 5 km (2) are according to the manufacturer emits and the manufacturer.<br> **Chromatin immunoprecipitation (ChIP)**<br>
Primary astrocytes were grown in 15 cm dishes and treated with NO<br>
On DIV14, cells were dissociated with 0.25% Primary 2012 On DIV14, cells were dissociated with 0.25% trypsin-EDTA, washed with PBS and cross-linked using<br>1% PFA for 10 minutes. The reaction was quenched with 125 mM glycine for 5 minutes. The pellets<br>18 were washed w The DIV14, cells were the substitute with the transformation of the US and SPFA for 10 minutes. The reaction was quenched with 125 mM glycine for 5 minutes. The pellets were washed with PBS and, after removing all the liqu 18 PFA for 10 minutes. The reaction was quenched with 115 min gryptic true inhibited with pentures.<br>
180°C. For chromatin shearing, the weight of each pellet was measured and the pellets were<br>
resuspended in a 10x volume o were was the meaning of the weight of each pellet was measured and the pellets were<br>resuspended in a 10x volume of RIPA buffer supplemented with 0.8% SDS and 1x protease inhibitor.<br>The samples were sonicated for 20 cycles resuspended in a 10x volume of RIPA buffer supplemented with 0.8% SDS and 1x protease inhibitor.<br>The samples were sonicated for 20 cycles in a Bioruptor plus (30s on, 45s off). Chromatin shearing<br>was checked by taking a sm The samples were sonicated for 20 cycles in a Bioruptor plus (30s on, 45s off). Chromatin shearing<br>was checked by taking a small aliquot and decrosslinking the DNA by RNase A and Proteinase K<br>treatment for 1 hour at 65°C. The samples were sented for 20 cycles in a Biorpher plus (30s on) for 20, 1 cm shearing was checked by taking a small aliquot and decrosslinking the DNA by RNase A and Proteinase K treatment for 1 hour at 65°C. DNA was iso treatment for 1 hour at 65°C. DNA was isolated using the ZYMO ChIP Clean and Concentrator Kit.<br>Sheared chromatin size was determined using Bioanalyzer 2100 (DNA high sensitivity kit) and the<br>concentration was measured usin Sheared chromatin size was determined using Bioanalyzer 2100 (DNA high sensitivity kit) and the concentration was measured using Qubit 2.0 fluorometer (DNA high sensitivity kit).10 µg of chromatin was used along with 3 µg Sheared chromation was measured using Qubit 2.0 fluorometer (DNA high sensitivity kit).10 µg of chromatin was used along with 3 µg of antibody to do ChIP for Rest (rabbit, ThermoFisher) or the IgG isotype control (Abcam). chromatin was used along with 3 µg of antibody to do ChIP for Rest (rabbit, ThermoFisher) or the IgG<br>isotype control (Abcam). For the H3K27me3 mark, 800 ng of chromatin and 2 µg of H3K27me3<br>antibody (rabbit, MerckMillipore chromatin and 2 µg of H3K27me3<br>antibody (rabbit, MerckMillipore) was used. ChIP was performed as described previously [80] with<br>minor modifications. After pre-clearing the samples and overnight incubation with the respecti antibody (rabbit, MerckMillipore) was used. ChIP was performed as described previously [80] with<br>minor modifications. After pre-clearing the samples and overnight incubation with the respective<br>antibodies, 25 µL of BSA-blo minor modifications. After pre-clearing the samples and overnight incubation with the respective<br>antibodies, 25  $\mu$ L of BSA-blocked protein A magnetic beads were added to each sample and the<br>mixture was incubated on a ro antibodies, 25 µL of BSA-blocked protein A magnetic beads were added to each sample and the mixture was incubated on a rotator at 4 °C for 2 h. The complexes were washed with Low Salt Wash buffer (20 mM Tris-HCl at pH 8, antibodies, 25 μL of Bothemann protein A magnetic beads were vashed with Low Salt Wash<br>buffer (20 mM Tris-HCl at pH 8, 150 mM NaCl, 1% Triton X-100, 2 mM EDTA, 0.1% SDS, Roche<br>Complete protease inhibitors), High Salt Wash ministed on a rotation at 4 °C for 2 m and 2 complete and the complete protease inhibitors), High Salt Wash buffer (20 mM Tris-HCl at pH 8, 500 mM NaCl, 1%<br>Triton X-100, 2 mM EDTA, 0.1% SDS, Roche Complete protease inhibit Complete protease inhibitors), High Salt Wash buffer (20 mM Tris-HCl at pH 8, 500 mM NaCl, 1%<br>Triton X-100, 2 mM EDTA, 0.1% SDS, Roche Complete protease inhibitors), lithium chloride (LiCl)<br>Wash buffer (10 mM Tris-HCl at p Triton X-100, 2 mM EDTA, 0.1% SDS, Roche Complete protease inhibitors), lithium chloride (LiCl)<br>Wash buffer (10 mM Tris-HCl at pH 8, 1% NP-40, 1% sodium deoxycholate, 1 mM EDTA, 250 mM Tries is the set of the USA of the Complete proteins inhibitorly in the theory (Nash buffer (10 mM Tris-HCl at pH 8, 1% NP-40, 1% sodium deoxycholate, 1 mM EDTA, 250 mM LiCl) and 1x Tris-EDTA (TE) buffer. The DNA was elute LiCl) and 1x Tris-EDTA (TE) buffer. The DNA was eluted from the beads and purified using the ZYMO<br>ChIP Clean and Concentrator Kit. The DNA was eluted with 50 µL elution buffer and 1 µL was used<br>for the qPCR. ChIP Clean and Concentrator Kit. The DNA was eluted with 50  $\mu$ L elution buffer and 1  $\mu$ L was used for the qPCR. For the qPCR.<br>Statistical analysis

# for the quality of the quality of the quality of the quality of the quality  $\frac{1}{2}$ Statistical analysis

Statistical analysis was done using GraphPad Prism version 9. All graphs are shown as mean  $\pm$  SEM unless stated otherwise. For data analysis, either a two-tailed unpaired t-test or a one-way ANOVA with Tukey's post hoc with Tukey's post hoc test were applied. Enriched gene ontology and pathway analysis was with Tukey's post hoc test were applied. Entertaing gene ontology and pathway analysis was<br>performed using Fisher's exact test followed by a Benjamini-Hochberg correction.<br>Acknowledgements

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RNA-sequencing data will be available via GEO and EGA.

#### Data availability

Sananbenesi, A Schutz). AF and ID are supported by NIH RF1AG078299.<br>Data availability<br>RNA-sequencing data will be available via GEO and EGA.

#### Conflict of interest

Conflict of interest<br>The authors declare no conflict of interest

# $\blacksquare$ <br> $\blacksquare$ <br> $\blacksquare$  interature:<br> $\blacksquare$ Literature:

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