



PRDM16-DT is a novel lncRNA that regulates astrocyte function in Alzheimer's disease

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Abstract

Astrocytes provide crucial support for neurons, contributing to synaptogenesis, synaptic maintenance, and neurotransmitter recycling. Under pathological conditions, deregulation of astrocytes contributes to neurodegenerative diseases such as Alzheimer's disease (AD). While most research in this field has focused on protein-coding genes, non-coding RNAs, particularly long non-coding RNAs (lncRNAs), have emerged as significant regulatory molecules. In this study, we 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 patients and in AD models. In line with this, knockdown of *PRDM16-DT* and *Prdm16os* revealed its critical role in maintaining astrocyte homeostasis and supporting neuronal function by regulating genes essential for glutamate uptake, lactate release, and neuronal spine density through interactions with the RE1-Silencing Transcription factor (Rest) and Polycomb Repressive Complex 2 (PRC2). Notably, CRISPR-mediated overexpression of *Prdm16os* mitigated functional deficits in astrocytes induced by stimuli linked to AD pathogenesis. These findings underscore the importance of *PRDM16-DT* in astrocyte function and its potential as a novel therapeutic target for neurodegenerative disorders characterized by astrocyte dysfunction.

Keywords Long-non-coding RNA · Brain · Postmortem human brain · Astrocyte · Alzheimer's disease

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Introduction

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 synaptic pruning. Additionally, they play roles in neurotransmitter recycling and maintaining the blood-brain barrier, ion, pH, and fluid homeostasis [12, 41, 65]. In response to CNS damage, such as injury or disease, astrocytes can undergo morphological, molecular, and functional changes, a process often referred to as 'reactive astrogliosis' [20]. Depending on various factors, including the pathological context, affected astrocyte subpopulations, and environmental conditions, the adopted states can be either detrimental, where astrocytes contribute to neuroinflammatory processes while neuronal support function is compromised, or beneficial, acting in a neuroprotective manner [20].

There is increasing awareness of the role of astrocytes in neurodegenerative diseases such as 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 AD models [1, 35, 55, 57, 74]. Recent studies suggest a scenario in which activated microglia release interleukin 1 α (IL1 α), tumor necrosis factor α (Tnf α), and complement component 1q (C1q), driving astrocytes to a neurotoxic phenotype that contributes to synaptic dysfunction and neuronal cell death [41]. Similar results have been observed when astrocytes were exposed to amyloid beta 42 (A β 42) [39, 40]. However, the molecular mechanisms underlying the formation of neurotoxic astrocytic states are only beginning to emerge. A better understanding of these processes could facilitate the development of therapeutic approaches towards neurodegenerative diseases, especially since the deregulation of astrocytes may indicate specific stages of pathogenesis, allowing for stratified interventions [1].

In this context, basic and translational research in the past decades has mainly focused on the coding part of the genome, hence genes that are translated into proteins. However, only 1.5% of the human genome encodes for proteins while most of the transcriptome represents non-coding RNAs (ncRNAs), of which the majority is classified as long-non-coding RNAs (lncRNAs) [76].

lncRNAs are a heterogeneous group of RNA molecules with a length of more than 300 nucleotides that lack the potential to code for proteins [36, 49]. Initially, many of these non-coding RNAs have 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 biological processes [49, 52].

For instance, lncRNAs that are exported to the cytoplasm can control processes such as mRNA stability and translation, while lncRNAs that are retained in the nucleus often interact with transcription factors and epigenetic regulators to orchestrate gene expression programs [47, 49]. This is of particular interest since a significant proportion of lncRNAs exhibit tissue and cell type-specific expression patterns, with approximately 40% of human lncRNAs reported to be brain-specific, implying their critical involvement in brain development and function [15], but most likely also in the establishment and progression of brain-related disorders [7]. These characteristics make them promising drug targets [78], especially considering the growing recognition of RNA therapeutics as a promising avenue for treating various diseases, including brain disorders [13, 66]. However, the knowledge about lncRNAs in the brain is still limited. Especially for astrocytes, there is only very little information about functional involvements of lncRNAs in the context of reactive astrogliosis and neurodegenerative diseases. Taking into account the

increasing evidence that astrocytes may provide suitable targets for therapeutic intervention at distinct disease stages, we decided to study the role of astrocytic lncRNAs in the context of neurodegenerative diseases.

More specifically, we aimed to identify long non-coding RNAs (lncRNAs) that are specifically enriched in astrocytes and deregulated in neurodegenerative diseases. We hypothesized that such lncRNAs could serve as bona fide candidates for targeted drug interventions. By combining single nucleus RNA sequencing (snRNAseq) of the human brain with additional transcriptome data from human tissues we identify *PRDM16-DT* as the most highly enriched lncRNA in astrocytes with a murine homolog named *Prdm16os*. *PRDM16-DT* is downregulated in reactive astrocytes and in the brains of individuals with AD. Knockdown of *Prdm16os* followed by total RNA sequencing and functional assays revealed its important role in maintaining astrocyte homeostasis and supporting neuronal function. Furthermore, we find that *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 Repressive Complex 2 (PRC2). In line with these observations, reduced *Prdm16os* and *PRDM16-DT* levels decrease 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 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 characterized by astrocyte dysfunction.

Materials and methods

Human tissues

For snRNAseq tissue samples from control brains (Brains from individuals that did not suffer from any brain disease; prefrontal cortex, BA9) were obtained with ethical approval from the ethics committee of the University Medical Center Göttingen and upon informed consent from the New York brain bank that serves as the pathology core for the Columbia University Alzheimer's Disease Research Center funded by NIH/NIA (female: $n = 1$, age = 54, post-mortem delay: 6:41h; male: $n = 1$; age = 62, post-mortem delay = 5:24h). For the analysis of *PRDM16-DT* expression by qPCR, brains (prefrontal cortex, BA9) from control ($n = 4$ females & 5 males; 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 the University Medical Center Göttingen and upon informed consent from the Harvard Brain Tissue Resource Center (Boston, USA).

Sorting of neuronal and non-neuronal nuclei from human brain

The isolation protocol of single nuclei from frozen human brains was adapted from the previously published methods with the following modifications [62], 53. Frozen tissues were homogenized in 500 μ l of EZ Prep lysis buffer (Sigma), supplemented with a 1:200 dilution of RNase inhibitor (Promega), using a Dounce homogenizer for 60 strokes in a 1.5 ml Eppendorf tube with micro pestles. The volume was then adjusted to 2000 μ l with lysis buffer and incubated on ice for 7 minutes. Cell lysates were centrifuged for 5 min at $500 \times g$ at 4 °C and supernatants were discarded. The pellet was resuspended in 2000 μ l lysis buffer followed by an incubation on ice for 5 min. Lysates were centrifuged ($500 \times g$ at 4 °C) and the pellet was resuspended in 1500 μ l nuclei suspension buffer (NSB, 0.5% BSA, 1:100 Roche Protease inhibitor, 1:200 RNase inhibitor in 1 \times PBS) centrifuged again for 5 min ($500 \times g$ at 4 °C). The pellet was finally resuspended in 1000 μ l NSB and stained with anti-NeuN-AlexaFluor®488 (MAB377X) for 1h at 4°C rotating followed by centrifugation for 5 min ($500 \times g$ at 4 °C). The pellet was washed once with 500 μ l NSB and the pellet was resuspended in 300 μ l NSB and stained with 1:100 7AAD (Invitrogen, Cat: 00–6993-50). Single NeuN-positive and NeuN-negative nuclei were sorted using the BD FACS Aria III sorter. Sorted nuclei were counted in the Countess II FL Automated Counter. Single-nuclei RNA-seq using the iCELL8 System was performed at the NGS Integrative Genomics Core Unit in Göttingen, Germany. Approximately 1200 single nuclei per sample were sequenced.

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 for single nuclei library performances as described in [17]. Briefly, nuclei suspensions were fluorescent-labelled with live/dead stain, Hoechst 33,342 for 15 min prior to their dispensing into the Takara ICELL8 5184 nano-well chip. CellSelect Software (Takara Bio) was used to visualize and select wells containing single nuclei. A total of four 5184 nano-wells chips were used for all samples obtaining 1200–1400 nuclei/sample. Final libraries were amplified and pooled as they were extracted from each of the single nanowell chip. Pooled libraries were purified and size selected using Agencourt AMPure XP magnetic beads (Beckman Coulter)

to obtain an average library size of 500 bp. A typical yield for a library comprised of ~1300 cells was ~15 nM. Libraries were sequenced on the HiSeq 4000 (Illumina) to obtain on average of 0.3–0.4 Mio reads per nuclei (SE; 50 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. For further processing, the Cogent NGS Analysis pipeline (v1.5.1) was used to generate a gene-count matrix. The demuxer (cogent demux) was used to create demultiplexed FASTQ files from the barcoded sequencing data. The resulting output was then used as input for the analyzer (cogent analyze) which performs trimming, mapping and gene counting to create a gene counts matrix. Quality control was done by evaluating the quality report provided by the Cogent analyzer.

The SCANPY package was used for pre-filtering, normalization and clustering. Initially, cells that reflected low-quality cells (either too many or too few reads, cells isolated almost exclusively, cells expressing less than 10% of house-keeping genes) were excluded. Next, counts were scaled by the total library size multiplied by 10000, 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 values were rescaled. Principal component analysis (PCA) was performed on 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 cell–cell graph with $k = 50$ neighbours. Subsequently, spectral decomposition over the graph was performed with 50 components, and the Leiden graph-clustering algorithm was applied to identify cell clusters. We confirmed that the number of PCs captures almost all the variance of the data. For each cluster, we assigned a cell-type label using 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 “stacked_violin function” as implemented in SCANPY.

Primary astrocyte culture

Primary mouse astrocyte cultures were prepared from postnatal day 1–2 mice as previously described [79] 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 coated with poly-D-lysine (PDL) and cultured with DMEM, 10% fetal bovine serum and 1% penicillin/streptomycin (all Gibco) (DMEM+) in a humidified incubator with 5% CO₂ at

37°C for 7–8 days. Then, cells were placed on a shaker (Incubator Shaker Mini, Benchmark) and shaken at 160 rpm for 6 hours to loosen neurons and non-astrocytic glia. The medium was removed and the astrocytes incubated with 0.25% Trypsin–EDTA (Gibco) for 4 minutes at 37°C. DMEM+ was added to inactivate 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™ Plus Medium containing 2% B-27™ Plus 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/cm² onto PDL- or 0.1% polyethylenimine (PEI)-coated cell culture dishes. Cultures were then maintained in the incubator, and half the medium was changed two times a week.

Primary cortical neuron culture

Pregnant CD-1 mice were sacrificed using pentobarbital. Embryonic day 17 embryos were taken out 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 manufacturer's instructions. The cells were resuspended in NB+ and seeded at a density of 120,000 cells/cm² for glutamate treatment or 60,000 cells/cm² for spine density analysis.

For the co-cultures, inserts containing astrocytes were added to cortical neurons on day in vitro (DIV) 14 and the experiments were performed on DIV17.

Human iPSC-derived astrocytes

Human iPSC-derived astrocytes (Ncyte Astrocytes) were obtained from Ncardia. Cells were thawed and cultured according to the manufacturer's instructions. After thawing, the astrocytes were left in culture for 1–2 weeks before performing experiments.

Stimulation of primary astrocytes

Primary microglia were cultured as previously described [30]. On DIV 10, they were stimulated with 100 ng/mL Lipopolysaccharide (LPS) for 4 hours. Then, the medium was changed to NB+, collected after 6 hours, filtered using a 0.22 µm 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.

To mimic the activation of astrocytes by microglia, primary astrocytes were treated with a cytokine mixture consisting of Il-1α (3 ng ml⁻¹, Sigma, I3901), TNF (30 ng ml⁻¹, Cell Signaling Technology, 8902SF) and C1q

(400 ng ml⁻¹, MyBioSource, MBS143105) for 24 hours, as described previously [41].

In another approach, Amyloid beta 1–42 (Cayman Chemicals) was incubated at 37°C for 1 hour to form protofibrils and then added to astrocytes at a concentration of 1 µM on DIV13 for 24 hours.

Human tau fibrils were generated as previously described [10] (PMID: 34244499) and stored at 37°C. The fibrils were sonicated for 2 minutes prior to use. Then, primary astrocytes were treated with tau fibrils at a concentration of 1 µM on DIV13 for 24 hours. As a control, the buffer used for the generation of tau fibrils (25mM HEPES, 10mM KCl, 5mM MgCl₂, 0.01% NaN₃, pH 7.2) was added.

Antisense LNA Gapmers

Antisense LNA Gapmers targeting *Prdm16os* and *PRDM16-DT* and negative controls (NC) were designed and purchased from Qiagen having the following sequences:

NC: GCTCCCTTCAATCCAA

Prdm16os: TGCGACGTCTAAGATG

PRDM16-DT: TACAGAACTGGTCATT

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.

Prdm16os overexpression using CRISPRa

Plasmids containing the guide RNA (gRNA), a dCas9-VP64 and a tGFP reporter gene were obtained from Origene (GE100074). The gRNAs for targeting *Prdm16os* were also designed by Origene and had the following sequences:

gRNA-1: AGACGGTCACCTCGCCTCCA

gRNA-2: GATAGTTGGGACACGGGTCC

gRNA-3: GAGCCCGAAGCTGCAGCCAC

As a negative control, a scramble gRNA (Scramble) with no target in the genome was used (GE100077). To increase gene expression, an enhancer vector (GE100056) was used in all cases.

Primary astrocytes were transfected before seeding on DIV7 using the Neon Nxt electroporation device (ThermoFisher). 100,000 cells were electroporated using 0.5 µg CRISPRa plasmid and 0.1625 µg enhancer plasmid (1300 V, 20 ms, 2 pulses). Cells were seeded in 24 well plates and cultured for two days before treatment with the cytokine cocktail or PBS. RNA isolation and functional experiments were performed 24 hours later.

Glutamate uptake

On DIV14, primary astrocytes were washed with HBSS for 10 min at 37°C. Then, they were incubated with 100

μM glutamate in HBSS for 1 hour at 37°C . Afterwards, the supernatant was collected and the amounts of remaining glutamate in the HBSS were measured using the Glutamate-Glo™ Assay (Promega) according to the manufacturer's instructions. Luminescence was recorded with a FLUOstar® Omega (BMG).

Lactate secretion

To estimate lactate secretion, the complete medium was changed one hour before performing transfection. After 48 hours, the medium was collected and diluted 1:40 in PBS. Lactate concentrations were measured using the Lactate-Glo™ Assay (Promega) according to the manufacturer's protocol.

Cytoplasmic and nuclear fractionation

Primary astrocytes cultured in 6 well plates were dissociated on DIV14 with 0.25% trypsin-EDTA. After centrifuging at 400 g for five minutes and washing the cells with PBS, 500 μL EZ prep lysis buffer (Sigma–Aldrich) supplemented with RNase inhibitor (Promega) was added to each sample and incubated on ice for seven minutes. The samples were centrifuged and the supernatant was aspirated and collected, containing the cytoplasmic fraction. The nuclear pellet was washed with 2 mL EZ prep lysis buffer, incubated on ice for five minutes and centrifuged again. The supernatant was aspirated 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 supernatant aspirated, leaving 250 μL of liquid in the tube. TRIzol™ LS Reagent (ThermoFisher) was added to both the cytoplasmic and nuclear fraction and the samples were stored at -20°C until RNA was extracted.

Glutamate treatment of primary neurons

Neuronal and neuron-astrocyte co-cultures were incubated on DIV 17 with 100 μM glutamate for 15 minutes at 37°C . Then, the medium containing glutamate was removed and replaced with fresh NB+ for 3 hours. Cell viability was measured using PrestoBlue (ThermoFisher).

Spine density analysis

Dendritic spines were labelled using the dye 1,1'-diiododecyl-3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) (Life Technologies) as described previously [11], using 2% paraformaldehyde (PFA) for fixation. Spine density and dendrite length were measured with ImageJ software.

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 dissociation kit (cat. no. 130-107-677, Miltenyi) according to the manufacturer's protocol with minor modifications. Briefly, mice were sacrificed using pentobarbital and the brains were quickly removed. To remove major parts of the meninges, the brains were rolled over Whatman paper and then the cortices were dissected and placed into the enzyme mixes. The tissue was incubated at 37°C for 30 minutes in a water bath and triturated gently three times during this period. Then, the samples were applied to 40 μm cell strainers and the protocol was followed for debris and red blood cell removal. Oligodendrocytes 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 of the cell type populations was determined by qPCR.

In situ hybridization combined with immunofluorescence

We performed RNAscope Fluorescent Multiplex assays (ACD Bio) combined with immunofluorescence according to the manufacturer's instructions for fresh frozen tissue sections. Briefly, 18 μm sections were fixed with 10% neutral buffered formalin (NBF) and dehydrated with ethanol. Hydrogen peroxide was applied to the sections, followed by the incubation with anti-Gfap (rabbit, Abcam; 1:250) at 4°C overnight. On the next day, after a post-primary fixation step with 10% NBF, the protocol for the RNAscope® Multiplex Fluorescent Reagent Kit v2 (Acd Bio) was followed, using probes designed against *Prdm16os* (Acdbio) and TSA Plus Cyanine 5 (Akoya Biosciences; 1:750) for detection of the probes. Afterwards, the slides were incubated with Alexa Fluor™ 555 goat anti-rabbit secondary antibody (1:1000, ThermoFisher) and DAPI (Sigma–Aldrich) and mounted using Prolong Gold Antifade Reagent (ThermoFisher). Images were acquired within a week after staining.

Imaging

All images were taken with a Leica dmi8 microscope fitted with a STEDycon STED/Confocal (Abberior) in the confocal mode, with a 63X or 100X oil immersion objective.

RNA extraction

Cells were lysed using TRI Reagent (Sigma–Aldrich) and total RNA was isolated using the RNA clean & concentrator-5 kit (Zymo Research) according to the manufacturer's instructions.

RNA concentrations were determined by Nanodrop (ThermoFisher) or Qubit using the RNA HS Assay kit (ThermoFisher). The quality of the samples used for sequencing was assessed with a Bioanalyzer (Agilent Technologies).

Library preparation and total RNA sequencing

Libraries were prepared using the SMARTer Stranded Total RNA Sample Prep Kit-HI Mammalian kit (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 (Illumina) with a 50 bp single-read configuration.

Bioinformatic analysis

Processing and demultiplexing of raw reads was performed using bcl2fastq (v2.20.2). For quality control of raw sequencing data, FastQC (v0.11.5) was used. Reads were aligned to the mouse (mm10) genome with the STAR aligner (v2.5.2b), and read counts were generated using feature Counts (v1.5.1). Differential gene expression was performed with DESeq2 (v1.38.3) [45] using normalized read counts and correcting for unwanted variation detected by RUVSeq (v1.32.0) [58]. GO term analysis was performed with clusterProfiler (v4.6.0) [83]. Analysis for the enrichment of transcription factors targeting downregulated genes was performed using ENRICH (https://maayanlab.cloud/Enrichr/).

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 prepared with Light Cycler 480 SYBR Master Mix (Roche) and run in duplicates in a Light Cycler 480 (Roche). 18S was used as a housekeeping gene to normalize the expression of *Prdm16os* and *PRDM16-DT* and protein-coding gene expression was normalized to glyceraldehyde 3-phosphate dehydrogenase (Gapdh). Primer sequences can be found

in supplemental table 3. Analysis was done using the 2^{−ΔΔCt} method [43].

Western Blot

Primary astrocytes 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-PROTEAN® TGX™ Precast Protein Gel (Bio-Rad). Protein denaturation was done using 8 M urea in Laemmli Sample Buffer (Bio-Rad) at 40°C for 60 minutes. The gels were run at 90 V for 15 minutes, followed by 50 minutes at 120 V. For the transfer, low fluorescence PVDF membranes and the Trans-Blot Turbo Transfer System (both from Bio-Rad) were used.

Membranes were blocked with 5% BSA in PBS + 0.1% Tween-20 (PBS-T) for 1 hour at 4°C. Primary antibodies were incubated overnight at 4°C in 1% milk in TBS-T or 5% BSA in PBS-T. The following primary antibodies were used: anti-Eaat1 (rabbit, Abcam; 1:2500), anti-Eaat2 (rabbit, Abcam; 1:800) and anti-Gapdh (mouse, ThermoFisher; 1:4000). On the next day, the membranes were washed with 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. Information on antibodies can be found in supplemental table 4.

RNA immunoprecipitation

For RNA immunoprecipitation (RNA-IP), primary astrocytes were cultured in T75 flasks. On DIV14, cells were dissociated with 0.25% trypsin-EDTA, washed with PBS and resuspended in fractionation buffer (10 mM Tris-HCl, 10 mM NaCl, 3 mM MgCl₂, 0.5% Nonidet P-40 (NP-40), 1mM DTT, 100 units/mL RNase 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 cytoplasmic fraction, was transferred into fresh tubes. The nuclear fraction was rinsed with TSE buffer (10 mM Tris, 300 mM sucrose, 1 mM EDTA, 0.1% NP-40, 1mM DTT, 100 units/mL RNase inhibitor, 1x protease inhibitor) and centrifuged at 1000 g for five minutes. Then, the pellet was resuspended in fresh TSE buffer, transferred to bioruptor tubes and sonicated in a Bioruptor Plus (both Diagenode) for ten cycles (30s on, 30s off). The samples were incubated on ice for 20 minutes with occasional vortexing and centrifuged at 14500 g for ten minutes. The supernatant was transferred to a fresh tube and both fractions were flash-frozen and stored at −80°C.

The nuclear lysates were thawed and the protein concentration determined using a BCA assay. 500 µg lysate per sample were pre-cleared using 25 µL Pierce™ Protein A/G magnetic beads (ThermoFisher) for 1 hour at 4°C to reduce unspecific binding. 1.5 µg per sample of anti-Rest (rabbit, ThermoFisher) antibody or the IgG isotype control (Abcam) was incubated with 50 µL Protein A/G magnetic beads for two hours at RT and washed with RNA-IP buffer (50 mM Tris–HCl, 100 mM NaCl, 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 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-5 kit (Zymo Research) according to the manufacturer's instructions.

Chromatin immunoprecipitation (ChIP)

Primary astrocytes were grown in 15 cm dishes and treated with NC or KD ASOs as described above. On DIV14, cells were dissociated with 0.25% trypsin-EDTA, washed with PBS and cross-linked using 1% PFA 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 liquid, flash-frozen in liquid-nitrogen and stored at – 80°C. For chromatin shearing, the weight of each pellet was measured and the pellets were resuspended in a 10x volume of RIPA buffer supplemented with 0.8% SDS and 1x protease inhibitor. The samples were sonicated for 20 cycles in a Bioruptor plus (30s on, 45s off). Chromatin 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 isolated using the ZYMO ChIP Clean and Concentrator Kit. 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 of antibody to do ChIP for Rest (rabbit, ThermoFisher) or the IgG isotype control (Abcam). For the H3K27me3 mark, 800 ng of chromatin and 2 µg of H3K27me3 antibody (rabbit, MerckMillipore) was used. ChIP was performed as described previously [22] with minor modifications. After pre-clearing the samples and overnight incubation with the respective 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, 150 mM NaCl, 1% Triton X-100, 2 mM EDTA, 0.1% SDS, Roche Complete protease inhibitors), High Salt Wash buffer (20 mM Tris–HCl at pH 8, 500 mM NaCl, 1% Triton X-100, 2 mM EDTA, 0.1%

SDS, Roche Complete protease inhibitors), lithium chloride (LiCl) Wash 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 eluted from the beads and purified using the ZYMO ChIP Clean and Concentrator Kit. The DNA was eluted with 50 µL elution buffer and 1 µL was used for the qPCR.

Statistical analysis

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

Data availability

RNA-sequencing data will be available via GEO for murine data (GSE271196; token: gfofgumwplkpxql) and EGA for human data.

Results

Single Nucleus RNA Sequencing identifies *PRDM16-DT* as an astrocyte-specific lncRNA in the human brain

Our objective was to identify astrocyte-specific lncRNAs enriched in the brain and dysregulated in neurodegenerative disorders (Fig. 1a). We hypothesized that studying such lncRNAs functionally could provide insights into the role of astrocytes in these diseases and potentially uncover promising drug targets with minimal side effects, given their brain and cell type-specific expression. To achieve this goal, we generated snucRNAseq data from postmortem brain tissues of healthy individuals using an adapted an iCell8 snucRNAseq protocol (Takara) with random primers, enabling us to detect both polyA and non-polyA transcripts. We generated over 400 million reads per sample and identified 17031 lncRNAs in total (Fig. 1b). Next, we identified astrocyte-enriched lncRNAs by comparing the mean expression in astrocytes with that in all other cell types, resulting in ten lncRNAs with a ratio of >20-fold enrichment (Fig. 1c, Fig. S1). 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 human cells. lncRNAs were considered homologs when the genes were syntenically located in the same genomic locus, and the

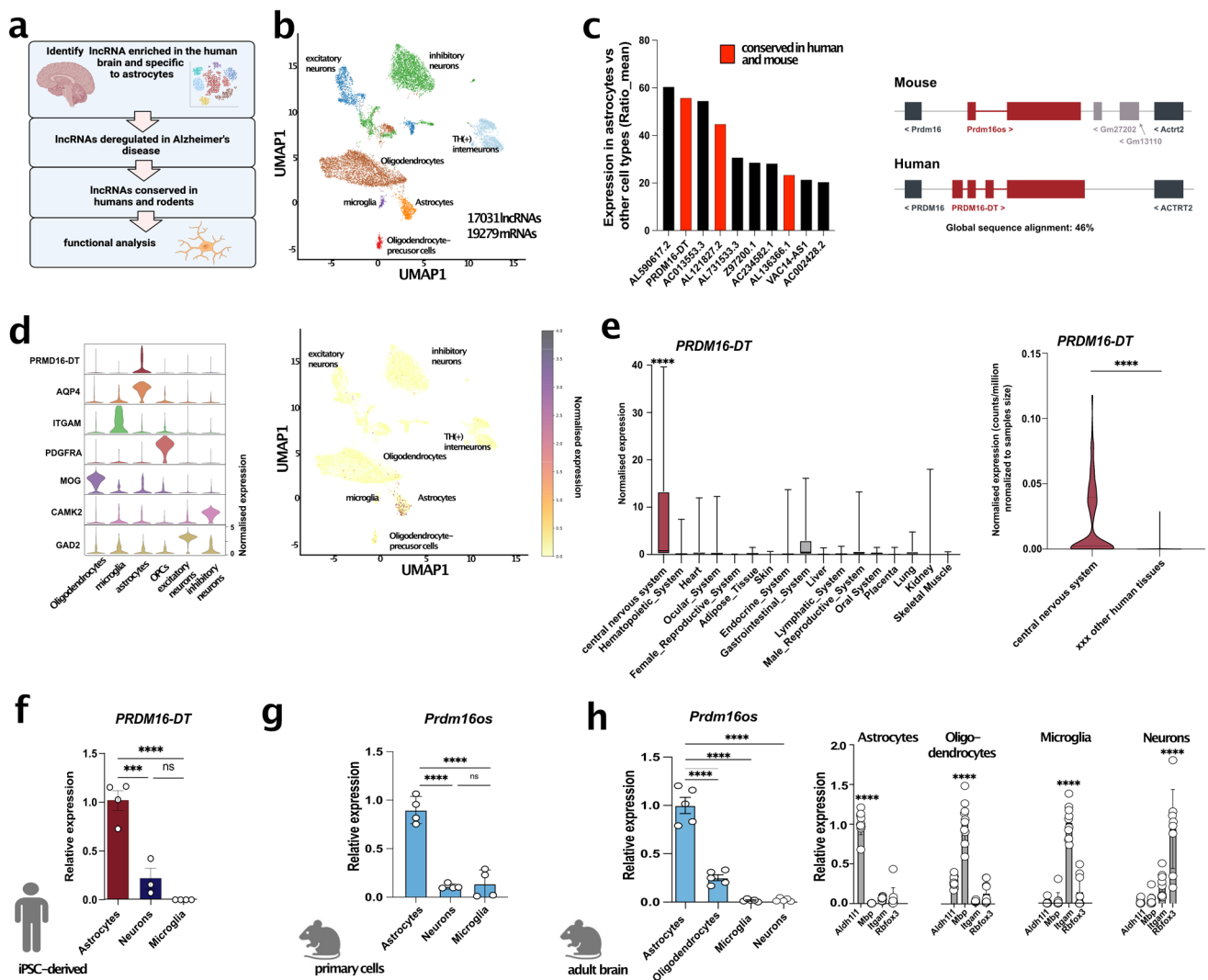


Fig. 1 *PRDM16-DT/Prdm16os* is an astrocyte-specific lncRNA enriched in the brain. **a** Strategy to identify astrocyte-specific lncRNAs in the brain and characterize their function. **b** UMAP clustering based on snucRNAseq (iCELL8 technology) from healthy human brains (prefrontal cortex, BA9). **c** Left panel: Bar graph showing the list of lncRNAs enriched in astrocytes when compared to all other cell types in the human brain. lncRNAs marked in red have a mouse homolog. Right panel: Schematic illustration showing the genomic localization of human *PRDM16-DT* and mouse *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 depicted in (b) showing the expression of *PRDM16-DT*. **e** Left panel: Expression of *PRDM16-DT* in different human tissues (depicted are only tissues in which expression was detectable) obtained from [44]. 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 -Tests). Right panel: Violin plot showing the expression of *PRDM16-DT* as counts per million normalized to the samples size for the central nervous system vs. the average expression across 44 other human tissues (only tissues in which *PRDM16-DT* expression was detectable are shown) **** $P < 0.0001$; unpaired t -Test. **f** Bar chart showing the expression of *PRDM16-DT* in human iPSC-derived astrocytes, neurons and microglia (unpaired t test; *** $P < 0.001$, **** $P < 0.0001$, ns = not significant). **g** *Prdm16os* expression in mouse primary astrocytes, neurons and microglia (unpaired t test; *** $P < 0.001$, **** $P < 0.0001$, ns not significant). **h** *Prdm16os* expression in astrocytes, oligodendrocytes, microglia and neuronal fraction isolated from the adult mouse brain (unpaired t test; *** $P < 0.001$). Right panel: Bar charts showing qPCR data from the adult mouse brain for marker genes for astrocytes (*Aldehyde Dehydrogenase 1 Family Member L1*, *Aldh1l1*), oligodendrocytes (*myelin basic protein*, *Mbp*), microglia (*Integrin Subunit Alpha M*, *Itgam*) and neurons (*RNA Binding Fox-1 Homolog 3*, *Rbfox3*). Genes were normalized to *18S* for *Prdm16os* and *PRDM16-DT* or *Gapdh* for protein-coding genes. Error bars indicate SEM

transcripts exhibited a sequence similarity of at least 40%. The most enriched and conserved lncRNA 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 [44] and found that *PRDM16-DT* was significantly enriched in the central nervous system compared to other human tissues (Fig. 1e).

In summary, these data suggest that *PRDM16-DT* is a brain-enriched lncRNA predominantly 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. Consistent with the snucRNA-seq data, *PRDM16-DT* showed significant enrichment in astrocytes (Fig. 1f). To assess whether this cell-type specificity is conserved across species, we analyzed the expression of the *PRDM16-DT* mouse homolog, *Prdm16os*, in primary astrocytes, neurons, and microglia (Fig. 1g). Similar to human *PRDM16-DT*, mouse *Prdm16os* exhibited high enrichment in astrocytes. Furthermore, we employed MACS sorting to isolate astrocytes, oligodendrocytes, microglia, and a neuron-enriched fraction from the

adult mouse brain. Consistent with our previous findings, *Prdm16os* was found to be most highly expressed in astrocytes (Fig. 1h).

Prdm16os and *PRDM16-DT* are downregulated in response to pathological insults and in Alzheimer's disease

To investigate whether *PRDM16-DT* is dysregulated in neurodegenerative diseases, we analyzed its expression in postmortem brain samples (prefrontal cortex, BA9) obtained from healthy controls and patients with AD. Our analysis revealed a significant decrease in *PRDM16-DT* expression in AD patients (Fig. 2a). This finding was further validated

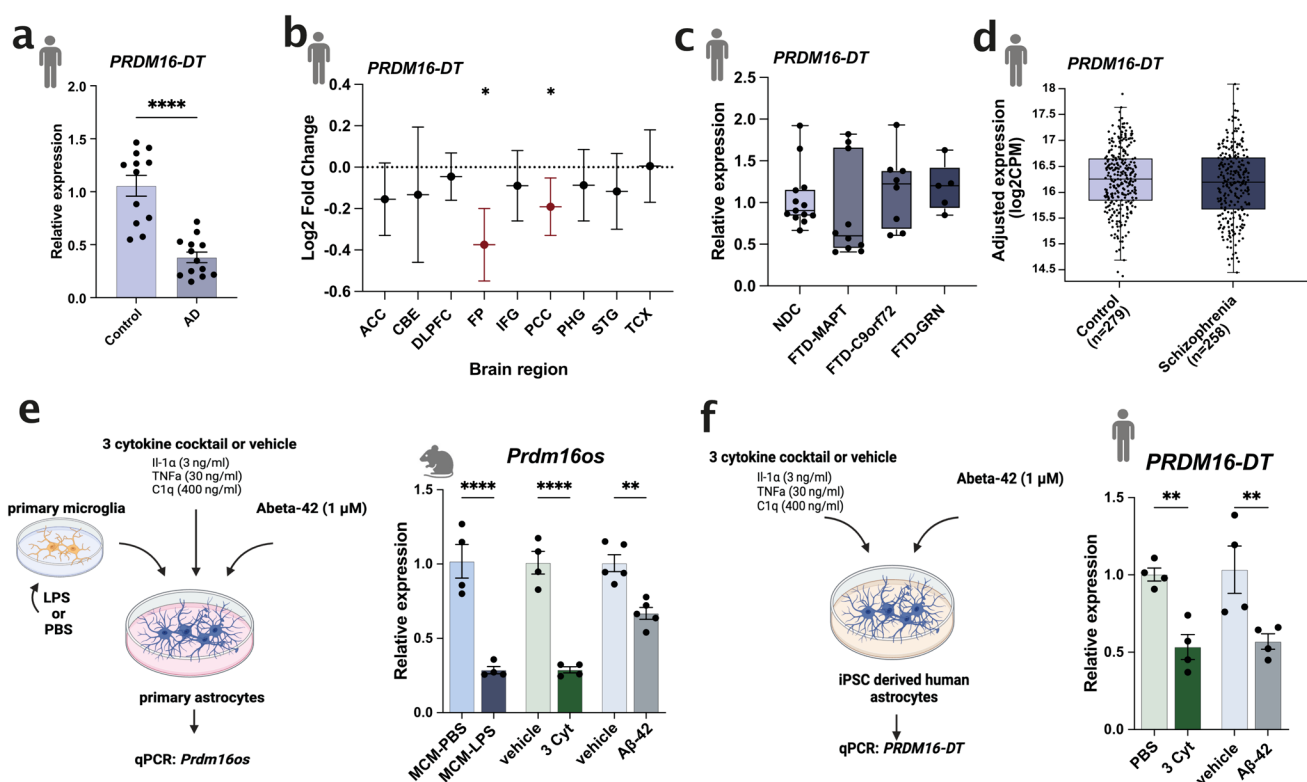


Fig. 2 *Prdm16-DT* is decreased in the brains of AD patients and in response to AD risk factors. **a** Bar chart showing qPCR data on the expression of *PRDM16-DT* in postmortem brain samples (prefrontal cortex, BA9) from control ($n = 12$) and AD patients ($n = 13$) ($****P < 0.0001$, unpaired t -Test). **b** Log2 Fold changes of *PRDM16-DT* expression in different brain regions in AD patients compared to controls based on data from the Agora database (<https://agora.adknowledgeportal.org/>). ($*P < 0.05$). **c** Bar chart showing the expression of *PRDM16-DT* in postmortem tissue samples (frontal lobe) of FTD patients with *MAPT* ($n = 10$), *C9ORF72* ($n = 8$) or *GRN* ($n = 6$) mutations compared to non-demented controls (NDC, $n = 13$). Data were obtained from the RiMOD database [37, 50]. **d** Bar chart showing the expression of *PRDM16-DT* in postmortem brain tissue of controls ($n = 279$) compared to schizophrenia patients ($n = 258$) obtained from a study by Wu et al., [81]. **e** Left panel: Experimental design. Right panel: Bar plot showing *Prdm16os* expression in mouse

astrocytes after treatment with LPS-activated microglia conditioned medium (MCM-LPS) compared to control (MCM-PBS), a 3 cytokine cocktail (3 Cyt) and A β -42 treatment compared to the corresponding vehicle controls ($****P < 0.00001$, $**P < 0.001$ unpaired t -Test). **f** Left panel: Experimental design. Right panel: Bar plot showing *PRDM16-DT* expression in human iPSC-derived astrocytes after treatment with a 3 cytokine cocktail (3 Cyt) and A β -42 compared to the corresponding controls ($**P < 0.01$ unpaired t test). ACC anterior cingulate cortex, AD Alzheimer's disease, CBE cerebellum, 3 Cyt 3 cytokine cocktail, DLPFC dorsolateral prefrontal cortex, FTD frontotemporal dementia, FP frontal pole; IFG inferior frontal gyrus, PCC posterior cingulate Cortex, PHG parahippocampal gyrus, STG superior temporal Gyrus, TCX temporal cortex. Gene expression was normalized to *18S*. Error bars represent SEM

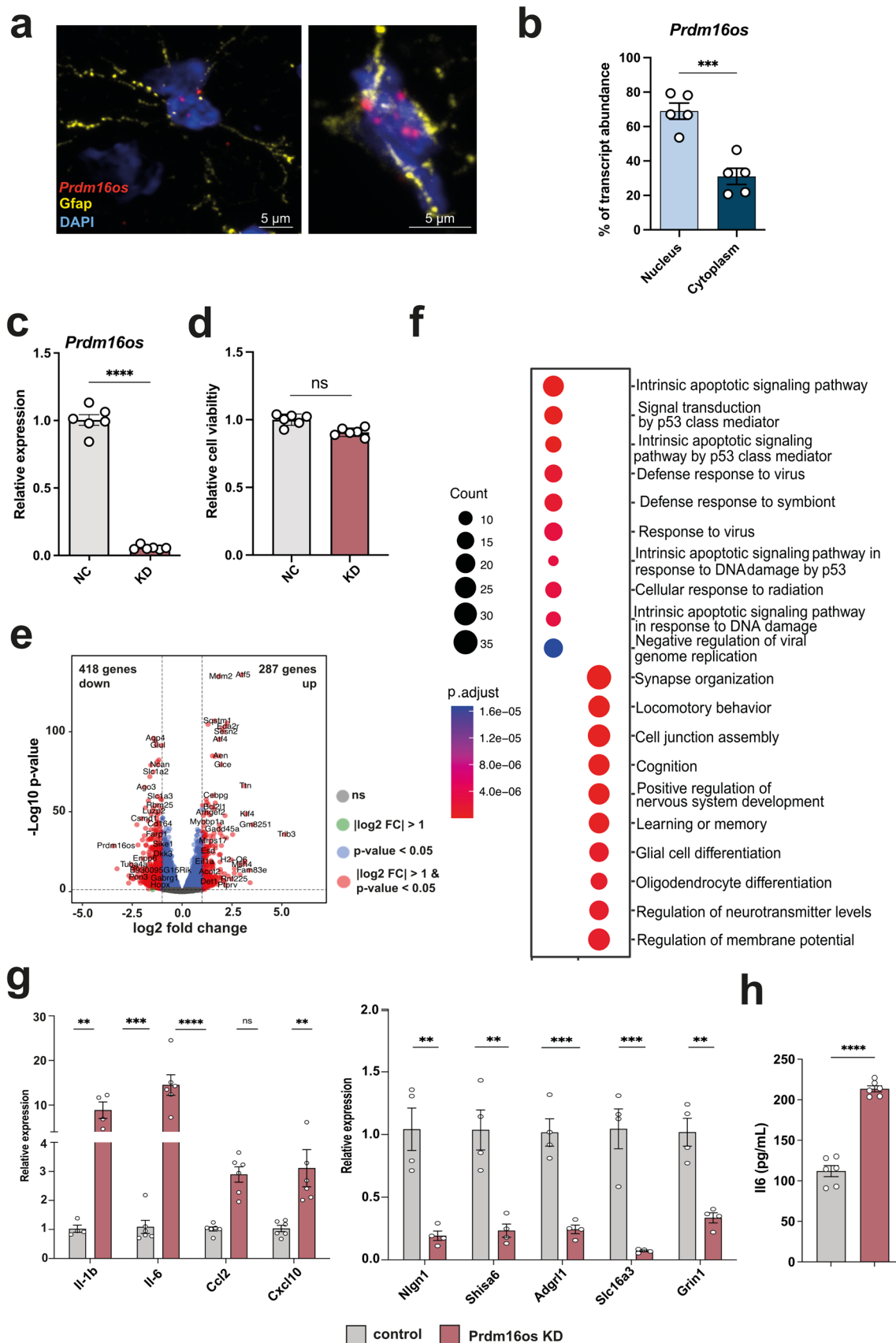


Fig. 3 *Prdm16os* is localized to the nucleus and controls gene expression. **a** Representative images from the adult mouse hippocampus showing the RNAscope signal for *Prdm16os* and immunofluorescence for Gfap. Nuclei are stained with DAPI. The right panel shows a higher magnification from a different hippocampal region. **b** Bar plot showing qPCR values for *Prdm16os* in nuclear and cytoplasmic fractions prepared from primary astrocytes (*** $P < 0.001$, unpaired t test). **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 oligonucleotides (**** $P < 0.0001$, unpaired t test). **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 shows the up- and down-regulated genes in primary astrocytes 48h after *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) [83]. Two-sided hypergeometric test was used to calculate the importance of each term and the Benjamini–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: Selected up-regulated genes. Right panel: Selected downregulated genes **** $P < 0.0001$, *** $P < 0.001$; ** $P < 0.01$; unpaired t test). **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 t test). KD knockdown, NC negative control. Gene expression was normalized to *18S* for *Prdm16os* or *Gapdh* for protein-coding genes. Error bars indicate SEM

using data from the Agora database (<https://agora.adknwledgeportal.org/>), which includes over 1000 postmortem brain samples from both control individuals and AD patients. *PRDM16-DT* expression was found to be significantly reduced in the frontal pole and parahippocampal gyrus regions (Fig. 2b).

Subsequently, we analyzed *PRDM16-DT* expression in postmortem brain tissues (frontal lobe) obtained from patients with frontotemporal dementia (FTD) carrying mutations in *C9ORF72*, *GRN*, or *MAPT* genes, as well as from control individuals that are available via the RiMOD database [37, 50]. We did not observe any significant difference in *PRDM16-DT* expression between these groups (Fig. 2c). Additionally, we examined *PRDM16-DT* expression in a transcriptome dataset derived from postmortem brains of individuals with schizophrenia, as compared to controls [81]. Similar to our 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 specifically in the brains of AD patients.

It is widely believed that the accumulation of amyloid beta (A β) oligomers is among the earliest pathological changes in AD pathogenesis, occurring decades before clinical symptoms manifest [32, 70]. Reactive astrogliosis is also observed very early in mouse models for amyloid pathology [27]. While Abeta42 can directly affect astrocytes,

a prominent mechanism by which Abeta42 affects astrocytes is via the activation of microglia [14], which in turn release pro-inflammatory cytokines IL-1 α , TNF, and C1q that are sufficient to induce reactive astrogliosis [41]. Based on these findings, we wanted to test if the exposure of astrocytes to A β oligomers, media from activated microglia, or inflammatory cytokines would affect the expression of *PRDM16-DT* and *Prdm16os*. First, we assessed the expression of *Prdm16os* in primary astrocytes stimulated with microglia-conditioned medium (media from microglia treated with LPS; MCM-LPS), compared to control media (media from microglia treated with PBS; MCM-PBS) (Fig. 2e). We observed a significant decrease in *Prdm16os* levels in astrocytes following MCM-LPS treatment (Fig. 2f). Treatment of primary astrocytes with the of IL1 α , TNF, and C1q (3 cytokine cocktail) also resulted in a significant decrease in *Prdm16os* levels, similar to the effect of microglia-conditioned media (Fig. 2e). Similarly, treating primary astrocytes with Abeta42 led to a significant reduction in *Prdm16os* levels (Fig. 2e). Furthermore, stimulation of human iPSC-derived astrocytes with the 3 cytokine cocktail or Abeta resulted in a similar downregulation of *PRDM16-DT* as observed in mouse astrocytes (Fig. 2f). Interestingly, the treatment of primary astrocytes with Tau fibrils did not affect *Prdm16os* expression, which is consistent with the findings in FTD brains (Fig. S2).

In summary, our data demonstrate that *PRDM16-DT* is reduced in the brains of AD patients. Additionally, exposure of both mouse and human astrocytes to well-established AD risk factors (MCM, 3 cytokine cocktail, Abeta42) results in a similar decrease in *PRDM16-DT* and *Prdm16os* levels. These findings provide a suitable experimental framework for studying the role of *PRDM16-DT/Prdm16os* in the context of AD.

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

Given the lack of previous studies on *PRDM16-DT/Prdm16os* in the CNS, we aimed to investigate its role at the cellular and functional level. Since the function of lncRNAs is closely tied to their subcellular localization [31], 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, we determined that *Prdm16os* is predominantly located in the nucleus of astrocytes in the adult mouse brain (Fig. 3a). To validate this finding, we conducted nuclear and cytoplasmic fractionation of primary mouse astrocytes and compared the levels of *Prdm16os* in each compartment. Our analysis revealed that *Prdm16os* primarily resides in

Fig. 4 The loss of *Prdm16os* in astrocytes affects glutamate uptake, lactate secretion and 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) $n = 3/\text{group}$. **c** Glutamate uptake after the KD of *Prdm16os* in astrocytes. **d** qPCR 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 *Prdm16os* in astrocytes. **f** Left panel: Representative images of dendrite and spine labeling of neurons cultured alone (Neurons) or with either control astrocytes (Neurons + control astrocytes) or *Prdm16os* KD astrocytes (Neurons + *Prdm16os* KD astrocytes). Right panel: Quantification of spine density. **g–k** 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 transporters *GLT-1* and *GLAST* and two synapse plasticity genes *NRXN1* and *GRIN1* after the KD of *PRDM16-DT*. **i** Glutamate uptake after the KD of *PRDM16-DT*. **j** qPCR showing the levels of the two lactate transporters *MCT1* and *MCT4* after the KD of *PRDM16-DT*. **k** Lactate secretion after the KD of *PRDM16-DT*. KD: knockdown, NC: negative control. Statistical significance was assessed by a one-way ANOVA with Tukey's post hoc test for (f) or a Student's unpaired *t* test for the other graphs; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; **** $P < 0.0001$, *ns* not significant. Gene expression was normalized to 18S for *Prdm16os* and *PRDM16-DT* or *Gapdh* for protein-coding genes. Error bars indicate SEM

apoptotic signaling pathway" and inflammatory processes such as "Defense response to virus" (Fig. 3f, supplemental table 2). The downregulated genes were linked to processes associated with synapse support function such as "synapse organization", "cognition", "learning and memory" and "regulation of neurotransmitter levels" (Fig. 3F, supplemental table 2). We selected several 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 linked to inflammation would result in elevated secretion of the corresponding cytokines, we also measured IL-6 levels in the media of primary astrocytes treated with *Prdm16os* GapmerRs or control using ELISA. IL-6 levels were significantly increased (Fig. 3h).

Prdm16os is located near the *Prdm16* gene, which encodes a transcription factor involved in neural stem cell homeostasis, development, and neuronal differentiation [51, 71]. It is therefore possible that *Prdm16os* may affect *Prdm16* expression. Indeed, in our RNAseq dataset *Prdm16* expression was decreased upon *PRDM16os* knock-down. Its expression was also decreased when primary astrocytes were subjected to the 3 cytokine mixture treatment, while no change in expression was observed when primary astrocytes were treated with Aβeta. Moreover, *Prdm16* expressed was also not altered in the brains of AD and schizophrenia patients but increased in FTD patients (Fig. S3). These findings suggest that *Prdm16os* and *Prdm16-DT* may affect

Prdm16 expression, although this might not be relevant in AD.

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 *Prdm16os*. For instance, the sequencing data suggested that the loss of *Prdm16os* may compromise the neuronal support function of astrocytes and we decided to study this potential mechanics further at first. One crucial role of astrocytes is to remove glutamate from the extracellular space. This process is primarily mediated by two main glutamate transporters, Glt-1 and Glast. Both glutamate transporters showed downregulation in the RNAseq dataset, a finding confirmed by qPCR analysis (Fig. 4a) and at the protein level (Fig. 4b). Consistently, the uptake of extracellular glutamate was impaired following the knockdown (KD) of *Prdm16os* in primary astrocytes (Fig. 4c).

In addition to glutamate uptake, astrocytes provide metabolic support in the CNS by secreting lactate, which is an important energy substrate for neurons. Also in this case, there are two relevant transporters expressed in astrocytes that are essential for memory function, Monocarboxylate transporter (Mct) 1 and Mct4 [67]. We confirmed via qPCR that the mRNA levels of *Mct4*, but not *Mct1*, were decreased in response to the KD of *Prdm16os* (Fig. 4d). In line with this, the release of lactate into the extracellular space was reduced following the KD of *Prdm16os* (Fig. 4e). Next, we directly addressed the question if loss of *Prdm16os* in astrocytes would affect neuronal function. For this, we cultured neurons alone or in co-culture with astrocytes treated before with either *Prdm16os* GapmerRs (*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, while no such effect was observed when neurons were co-cultured with *Prdm16os* KD astrocytes (Fig. 4f). We also performed a knockdown of the human homologue of *Prdm16os*—*PRDM16-DT*—in human iPSC-derived astrocytes (Fig. 4g). Whereas in mouse astrocytes the transcripts encoding both glutamate transporters were downregulated, in human astrocytes only *GLAST* was affected, while 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 also the human astrocytes showed reduced glutamate uptake after the KD of *PRDM16-DT* (Fig. 4i). Similarly, also the expression of the lactate transporter *MCT4* and the secretion of lactate was decreased (Fig. 4j–k). These data indicate that the general function of *Prdm16os*

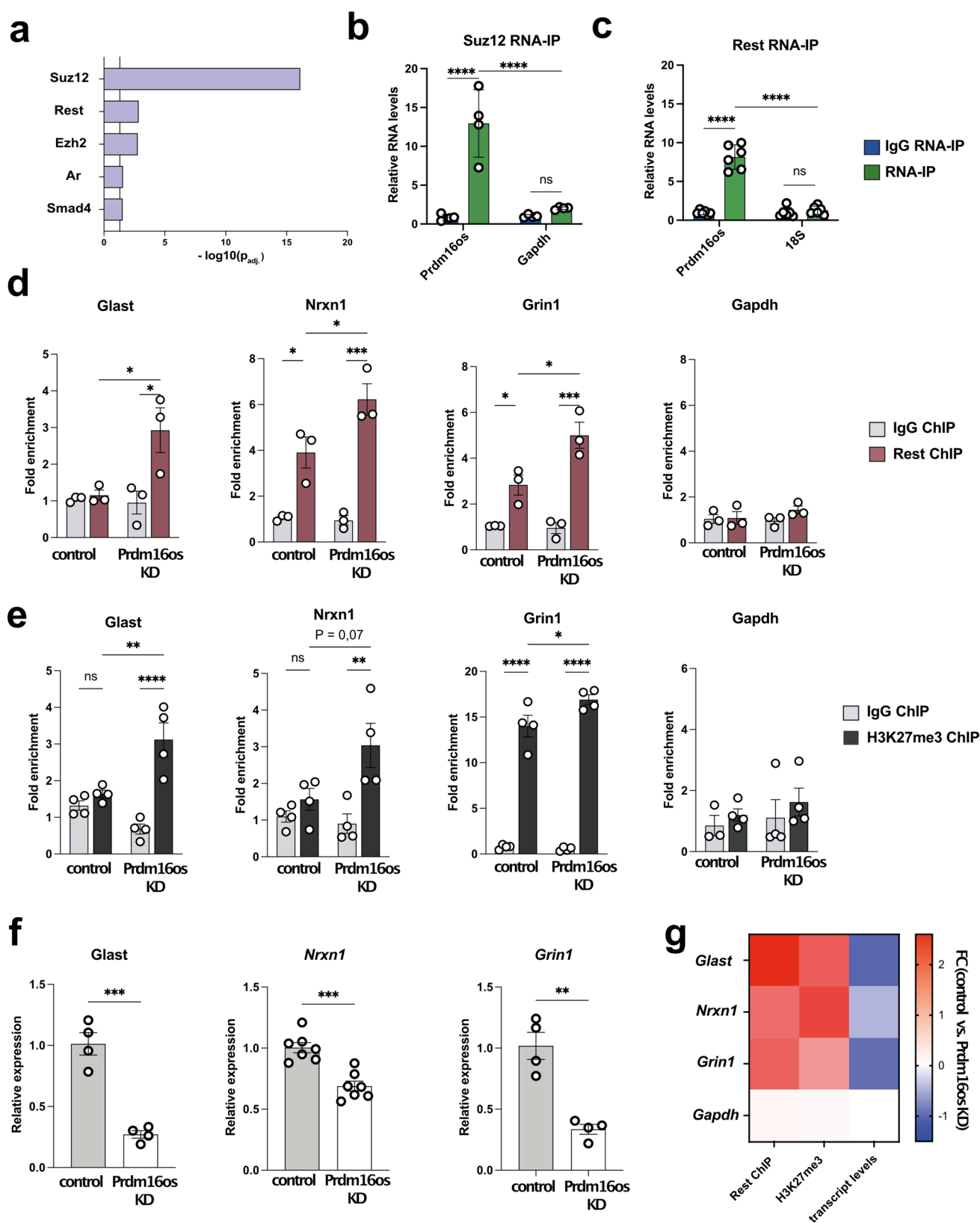


Fig. 5 *Prdm16os* binds to Suz12 and Rest and affects Rest and H3K27me3 levels at the promoter of genes important for neuronal function. **a** Enrichment analysis for transcription factors based on the downregulated genes after the KD of *Prdm16os*. **b** *Prdm16os* interacts with Suz12 as shown by RNA immunoprecipitation (RNA-IP). *Gapdh* mRNA was used as additional control. **c** *Prdm16os* interacts with Rest as shown by RNA immunoprecipitation (RNA-IP) 18S RNA was used as additional 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* is used as a control. **e** ChIP for H3K27me3 of primary astrocytes treated with control oligomers (control) or *Prdm16os* GapmeRs (*Prdm16os* KD) followed by qPCR for promoter regions of the genes *Glast*, *Nrxn1* and *Grin1*. The housekeeping gene *Gapdh* is used as control. **f** Bar charts showing the expression of *Glast*, *Nrxn1* and *Grin1* in astrocytes upon *Prdm16os* KD. **g** Heat map showing the fold changes of Rest and H3K27me3 levels as well as the transcript 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; * $P < 0.05$, *** $P < 0.001$; **** $P < 0.0001$, ns not significant. Gene expression was normalized to *Gapdh*. Error bars indicate SEM

and *PRDM16-DT* as being important for neuronal support is conserved across species.

In addition to impaired neuronal support function, the RNAseq data suggested that loss of *Prdm16os* induces an inflammatory response (see Fig. 3g, h). However, while iPSC-derived astrocytes exhibited an increased expression of pro-inflammatory cytokines upon stimulation with the 3 cytokine cocktail, the induction of pro-inflammatory genes could not be confirmed when *Prdm16-DT* was knocked-down in human iPSC-derived astrocytes (Fig. S4). Although the reason for this discrepancy is unclear at present, it may suggest that the control of inflammatory processes is not conserved across species and we therefore we decided to not follow up on the potential role of *Prdm16os* and *Prdm16-DT* in inflammation.

***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 orchestrate the availability of transcription factors or chromatin regulators to bind specific DNA regions, such as promoter regions. To explore this further, we revisited the RNAseq data from astrocytes upon *Prdm16os* KD and focused on the downregulated genes since these were mainly associated with neuronal support function. We conducted an enrichment analysis to identify transcription factors potentially controlling the expression of these downregulated genes. This analysis revealed

Suz12, a subunit of the Polycomb Repressive Complex 2 (PRC2) that mediates gene-repression via histone 3 lysine 27 tri-methylation (H3K27me3), followed by RE1-Silencing Transcription factor (Rest), also known as Neuron-Restrictive Silencer Factor (Nrsf) (Fig. 5a). Previous studies have shown that lncRNAs can recruit PRC2 to specific chromatin locations [33, 48, 75]. Moreover, it has been described that PRC2 and Rest interact to silence gene-expression [16, 68, 80], with Rest being a key transcriptional regulator that represses neuronal genes in non-neuronal cells [28]. At the same time, it is known that due to their neuronal support function and under specific conditions, astrocytes express genes typically associated with neurons such as ion channels and genes associated with synaptic function including neurotransmitter receptors, cell adhesion molecules, and other neuromodulatory genes, thus contributing to synaptic modulation [28, 56, 59, 61]. Furthermore, astrocytes are capable of transferring RNA, proteins, and even mitochondria to neurons, promoting synaptic plasticity [19, 24, 72, 73]. 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 function in astrocytes.

To test this hypothesis, we conducted RNA-immunoprecipitation (RNA-IP) for Suz12 (Fig. 5b) and Rest (Fig. 5c) and demonstrated their interaction with *Prdm16os*. To investigate whether *Prdm16os* affects the interaction of Rest with promoter regions of potential target genes, we performed Rest chromatin immunoprecipitation followed by qPCR (ChIP-qPCR) in primary astrocytes that were treated with a control oligonucleotides (control) or GapmeRs to mediate the knockdown of *Prdm16os*. For qPCR, we selected genes for which we had confirmed their decreased expression upon *Prdm16os* and *Prdm16-DT* knockdown in primary astrocytes and iPSC-derived human astrocytes. We found that the levels of Rest binding to the promoter regions of candidate mRNAs linked to synaptic plasticity, namely *Glast*, *Nrxn1* and *Grin1* were increased after the KD of *Prdm16os*. There were no significant changes in Rest binding to the promoter of glyceraldehyde-3-phosphate-dehydrogenase (*Gapdh*) that was used as a housekeeping control gene (Fig. 5d). Since Suz12 is part of the PRC2 complex regulating H3K27me3, we decided to analyze if the KD of *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). For *Nrxn1*, 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 astrocytes upon *Prdm16os* knock down (Fig. 5f) and the increased binding of Rest and H3K27me3 levels at the promoter regions of

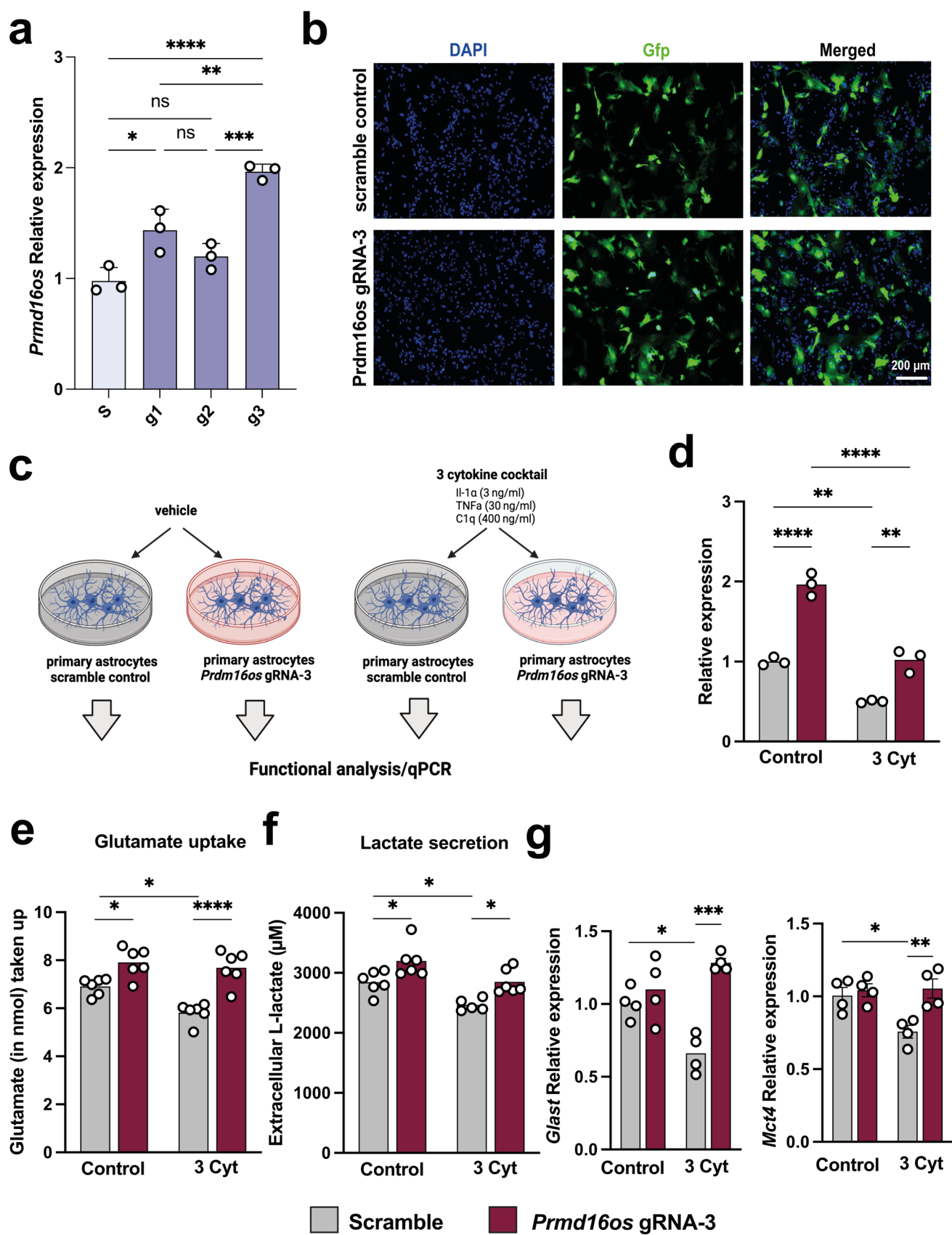


Fig. 6 CRISPRa-mediated overexpression of *Prdm16os* can rescue functional impairments induced by cytokine treatment. **a** Bar Chart showing the expression of *Prdm16os* in astrocytes transfected using CRISPRa in combination with g1, 2 or 3 in comparison of cells treated with scrambled RNA (s). **b** Representative immunofluorescence images showing the transfection of primary astrocytes with CRISPRa plasmids containing a scramble or *Prdm16os*-targeting g3 RNA. **c** Scheme of the experimental approach. **d** qPCR showing the levels of *Prdm16os* in astrocytes treated with either the 3 cytokine cocktail (3 Cyt) or vehicle (control) in the presence of either a scramble RNA or g3 RNA to mediate CRISPRa of *Prdm16os*. Note that CRISPRa with g3 increased *Prdm16os* expression in the control group when compared to scramble RNA (one-way ANOVA followed by *t* test; $**P < 0.01$) and was able to reinstate *Prdm16os* expression to physiological levels upon cytokine treatment (one-way ANOVA followed by *t* test; $***P < 0.001$). **e** Bar charts showing the results from 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 cytokine treatment impairs glutamate uptake and lactate release which is reinstated upon CRISPRa-mediated overexpression of *Prdm16os*. **g** Bar charts showing qPCR results for *Glast* (upper panel) and *Mct4* (lower panel) in the 4 experimental groups. Note that CRISPRa 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; $*P < 0.05$, $**P < 0.01$; $****P < 0.0001$. Gene expression was normalized to *18S* for *Prdm16os* or *Gapdh* for protein-coding genes. Error bars indicate SEM

target genes correlate with the decrease of mRNA levels (Fig 5g). These findings suggest that *Prdm16os* might act as a decoy for Rest and Suz12 to enable and fine-tune the expression of astrocytic genes that have important neuronal support functions.

CRISPR-mediated restoration of *Prdm16os* expression can rescue cytokine-induced functional deficits in astrocytes

Given the downregulation of *Prdm16os* in the brains of AD patients and in astrocytes exposed to AD 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-cytokine cocktail exposure model (see Fig 2E) and employed CRISPR activation (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, and 3 significantly increased the expression of *Prdm16os* with gRNA-3 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 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 (vehicle). Cells transfected with CRISPRa and scramble control guide RNA were used as control (Scramble) (Fig. 6c).

In line with our previous observations, treating cells with the 3 cytokine cocktail reduced the levels of *Prdm16os*, while CRISPRa treatment reinstated *Prdm16os* expression to physiological levels (Fig. 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 in AD pathogenesis. In agreement with this, glutamate uptake (Fig. 6e) and 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). Interestingly, glutamate uptake and lactate release was also increased when *Prdm16os* levels had been elevated via CRISPRa in vehicle-treated cells (Fig. 6e, f) further suggesting a key role of *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 *Prdm16os* and *PRDM16-DT* KD were significantly decreased upon 3 Cyt treatment (Fig 6g). CRISPRa-mediated increased expression of *Prdm16os* was able to ameliorate this effect (Fig 6g).

In conclusion, these data suggest that strategies to elevate *PRDM16-DT* could help to reinstate important neuronal support functions of astrocytes that are compromised in response to stimuli associated with AD pathogenesis.

Discussion

In this study, we identified the human lncRNA *PRDM16-DT* and its mouse homologue, *Prdm16os*, to be enriched in the brain, where they are almost exclusively expressed in astrocytes. This finding aligns with previous studies demonstrating that lncRNAs can exhibit tissue-specific expression patterns [15]. We focused on *PRDM16-DT* in this study because it also has a homolog in mice, and conservation between species is an indicator of functionality [34]. Further 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 FTD. Although data on lncRNAs in the brains of AD patients are still rare compared to the analysis of the coding transcriptome [46], 86, previous studies have reported lncRNAs 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 [21].

While analyzing human brain tissue provides a unique opportunity to elucidate the processes underlying AD pathogenesis, the data might be affected by factors such as post-mortem delay and other variables [18, 77]. Therefore, it is important to note that *PRDM16-DT* and *Prdm16os* expressions were also decreased when human iPSC-derived or mouse astrocytes were exposed to stimuli associated with AD. These stimuli included microglia-conditioned media after LPS treatment, a 3-cytokine cocktail shown to mediate microglia-induced reactive astrogliosis [41], and Aβ₄₂.

In line with the observation that *Prdm16os* was mainly localized to the nucleus, we observed a substantial effect on gene expression upon knockdown of *Prdm16os*, with the expression of genes important for neuronal support being downregulated. Among these were the glutamate transporter *Glast* and the lactate transporter *Mct4*. Similarly, *GLAST* and *MCT4* were downregulated when *PRDM16-DT* was knocked down in human iPSC-derived astrocytes. Consistently, KD of *Prdm16os* or *PRDM16-DT* in mouse or human astrocytes impaired glutamate uptake and lactate release. Loss of *Prdm16os* expression in astrocytes also increased the expression of proinflammatory cytokines. However, this effect was not observed in human iPSC-derived astrocytes, despite their confirmed response to the 3-cytokine cocktail stimulation, as evidenced by increased expression of cytokines such as *IL6* and *IL1b*.

While the molecular mechanisms underlying this discrepancy remain unclear, it is interesting to note 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 [26, 54]. These genes were all downregulated upon *Prdm16os* or *PRDM16-DT* knockdown in our study. Moreover, snucRNAseq studies on the brains of AD patients suggest a modest increase in proinflammatory genes in astrocytes and, more strikingly, a strong decrease in the expression of homeostatic genes involved in synapse regulation [6]. This is in line with other data on familial AD cases suggesting that astrocytes in AD brains are characterized by transcriptional underexpression of key genes linked to astrocytic function [6].

Nevertheless, we cannot exclude a role for *PRDM16-DT* in inflammatory processes in human astrocytes during AD pathogenesis. Future research is necessary to elucidate this aspect in more detail.

Taken together, the data from our analysis of postmortem human brains and human and mouse astrocytes consistently suggest a role for astrocytic *Prdm16os* and *PRDM16-DT* in neuronal support functions. In line with this, we observed that astrocytes failed to enhance spine density in the absence

of *Prdm16os*. Although there are no data for *PRDM16-DT* or *Prdm16os*, and limited data on the role of lncRNAs in astrocytes, our findings align with previous studies showing that lncRNAs can regulate astrocyte function. For example, *Neat1*, a ubiquitously expressed lncRNA, is increased in astrocytes in response to ischemia and AD models [29, 85]. Similarly, *MEG3* is a lncRNA upregulated in human neurons xenografted in AD mouse models, and its inhibition ameliorated amyloid-induced necroptosis [2]. This aligns with previous findings showing that *MEG3* inhibition could rescue Aβ₄₂-induced phenotypes in a rat model of AD, including reactive astrogliosis [82]. Other lncRNAs associated with neurodegenerative conditions and astrocyte function include *NKILA* and *MALAT1* [25, 84].

However, unlike *PRDM16-DT*, all these lncRNAs are ubiquitously expressed, and therefore, targeting them therapeutically may lead to unwanted side effects. Therefore, targeting lncRNAs exclusive to the brain and specifically to astrocytes offers the potential for more precise therapeutics. Along this line, our data show that CRISPR-mediated increase of *Prdm16os* expression in astrocytes can ameliorate the phenotypes induced by exposure to AD risk factors. These findings are significant because targeting astrocyte function has been suggested as a suitable approach for addressing specific early phases of AD pathogenesis [1].

Our data suggest that *PRDM16-DT* and *Prdm16os* may mainly influence astrocyte function by controlling gene expression. This aligns with previous studies indicating that a key role of lncRNAs is the regulation of gene expression in the nucleus, often through interactions with transcription factors or chromatin-modifying enzymes [49, 64]. We observed that *Prdm16os* interacts with Suz12, a subunit of the PRC2 complex that orchestrates gene silencing via H3K27-trimethylation [16, 68, 80], and Rest, a key transcriptional regulator that represses neuronal genes in non-neuronal cells [28]. This is interesting since Rest and PRC2 were shown to interact to control gene expression [16].

While Rest orchestrates the repression of neuronal genes in non-neuronal cells, astrocytes are known to express several such genes, including synaptic cell adhesion molecules like neurexins and neuroligins that mediate astrocyte-synapse interactions [69] and other synapse-associated genes [9, 38, 63]. 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 *Prdm16os* may regulate the expression of these target genes by acting as a decoy for Rest and Suz12. In this scenario, the loss of *PRDM16-DT*, as observed in AD brains, would compromise the fine-tuning of gene expression pathways in astrocytes, leading to a loss of neuronal support function and synaptic plasticity. This view is consistent with previous

data suggesting that impaired astrocyte function plays an important role in neurodegenerative processes [5, 14, 42, 60].

It is important to acknowledge the limitations of our study. Increasing evidence suggests astrocyte heterogeneity across the brain, revealing several distinct astrocyte subtypes between and within brain regions [3, 23]. Investigating the expression pattern of *Prdm16os* and *PRDM16-DT* in brain regions other than the cortex might provide further insights into their role in supporting neuronal function and in the course of AD. Another important question for future research is to elucidate the regulation of *PRDM16-DT* expression to better understand the mechanisms underlying its reduced levels in the brains of AD patients and in AD model systems. Although our data suggest that *PRDM16-DT* is specifically decreased in the brains of AD patients while remaining unaffected in FTD and schizophrenia patients, we cannot exclude the possibility that *PRDM16-DT* is affected in other brain diseases. It will therefore also be interesting to investigate if changes in *PRDM16-DT* expression are restricted to regions with AD pathology, especially since we did not observe differential expression in postmortem brains from FTD patients, despite the presence of neuroinflammation in FTD. Along the same lines, *PRDM16-DT* expression may be specific to AD-related amyloid pathology, including specific inflammatory processes that may differ between AD and FTD [4, 8]. At present, we are unable to conclusively answer this question, but it is noteworthy that the treatment of primary astrocytes with Tau fibrils did not affect *Prdm16os* expression.

Additionally, it could be considered to test the role of *Prdm16os* in transgenic mouse 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 models with the aim to reduce the reliance on animal experimentation, which is anyhow getting increasingly difficult in the European Union and in Germany in particular.

Finally, our data snucRNAseq data should be explored for other lncRNAs of interest. For example, lncRNAs AL590617.2 and VAC14-AS1 were highly expressed and enriched within astrocytes making these molecules interesting targets for future studies. In addition, our data should be explored for other cell type specific lncRNAs linked to brain diseases.

In summary, our data suggest that *PRDM16-DT* could be a suitable drug target to ameliorate neurodegenerative processes associated with AD pathogenesis. The specific expression pattern of *PRDM16-DT* in astrocytes of the human brain offers a unique possibility to target distinct phases of AD pathogenesis with minimal unwanted side effects.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s00401-024-02787-x>.

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Author contributions SS planned and conducted the majority of the experiments and wrote the manuscript. UF helped with experiments performed in cultured astrocytes. VG performed FANS. TP helped with statistical analysis. DMK performed analysis of snucRNAseq data. NH helped with the analysis of tissue and brain-specific lncRNAs. SB, ALS, and FS coordinated and performed RNAseq experiments. GS helped establish the snucRNAseq using the iCELL8 protocol. ID helped with the analysis of postmortem human brain tissue. AF conceived the project, supervised progress, and wrote the manuscript.

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Data availability RNA-sequencing is available via GEO database for murine data (GSE271196) and the European Genome-Phenome archive (EGA) for human data.

Declarations

Conflict of interest The authors declare no conflict of interest.

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