YAP and TAZ regulate Cc2d1b and Purb in Schwann cells

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ABSTRACT

Schwann cells are exquisitely sensitive to the elasticity of their environment and their differentiation and capacity to myelinate depend on the transduction of mechanical stimuli by YAP and TAZ. YAP/TAZ, in concert with other transcription factors, regulate several pathways including lipid and sterol biosynthesis as well as extracellular matrix receptor expression such as integrins and G-proteins. Yet, the characterization of the signaling downstream YAP/TAZ in Schwann cells is incomplete. Myelin sheath production by Schwann cell coincides with rapid up-regulation of numerous transcription factors. Here we show that ablation of YAP/TAZ alters the expression of transcription regulators known to regulate Schwann cell myelin gene transcription and differentiation. Furthermore, we link YAP/TAZ to two DNA binding proteins, Cc2d1b and Purb, which have no described roles in myelinating glial cells. We demonstrate that silencing of either Cc2d1b or Purb limits the formation of myelin segments. These data provide a deeper insight into the myelin gene transcriptional network and the role of YAP/TAZ in myelinating glial cells.

INTRODUCTION

The function of the nervous system relies on the ability of peripheral nerve fibers to transmit information to and from the target tissues. The speed of propagation of action potentials in these fibers is regulated by myelin, a multilamellar structure produced by Schwann cells (SCs) [1]. Damage to SC or peripheral myelin can be caused by numerous factors, including genetic mutations, toxic agents, inflammation, viral infections, metabolic alterations, hypoxia or physical trauma and results in severe peripheral neuropathies. SC integrate biochemical signaling pathways and mechanical stimuli coming from the extracellular matrix or from the axon [2-5]. These signals regulate an intricate network of transcription factors that control differentiation of SCs and myelination (e.g.: EGR2, YY1, ZEB2) [6-11]. The identification and characterization of the complete repertoire of transcription factors that modulate myelination is still incomplete [12,13].

The identification of transcription factors responding to a specific signal is one of the first steps in dissecting the underlying regulatory networks. We showed that in Yap fl/+ ; Taz fl/fl ; Mpz-Cre (Yap cHet ; Taz cKO) sciatic nerves, SCs lacking YAP/TAZ are unable to myelinate and experience a global dysregulation of transcription [14]. YAP/TAZ are two transcriptional activators of the HIPPO pathway, and play important roles in
controlling organ growth, cell differentiation, proliferation and survival. Mechanical stimulation can regulate YAP/TAZ through signals involving FAK, Src, PI3K and JNK pathways, or the formation of actomyosin filaments and accumulation of F-actin. In addition, YAP/TAZ in SCs can be activated through Crb/Amot proteins and laminin/G-protein signaling. YAP/TAZ regulate gene expression by binding to other DNA-binding transcription factors, especially TEAD transcription factors, but also p73, ERBB4, EGR-1 SMADs RUNXs and TBX5. TEADs role in myelination is unknown, but TEAD1 binding to transcriptional enhancers is induced during myelination. Furthermore, genes encoding for essential myelin proteins (i.e.: Mpz, Pmp22, Mbp and Mag) harbor TEAD elements and are downregulated in Yap cHet; Taz cKO sciatic nerves. Finally, it was suggested that YAP/TAZ and TEAD1 regulate myelin wrapping in cooperation with master myelin regulators EGR2 and SOX10.

RESULTS

YAP and TAZ regulate DNA-binding proteins in Schwann cells. To examine the function of YAP and TAZ at the whole-genome level we analyzed RNA-seq transcriptome profiling of Yap cHet; Taz cKO sciatic nerves at 3 days of age (NCBI GEO: GSE79115). We identified 2071 misregulated transcripts Fig.1. We narrowed our analysis to DNA-binding proteins and identified that ablation of Yap/Taz dysregulated 64 genes (Fig.1b). Genes encoding for DNA-binding proteins were then categorized according to their level of expression (Fig.1b, black/white heatmap). Signature genes normally expressed in neural crest cells (Tbx2) and immature Schwann cells (Oct6/Pou3f1/Scip), as well as genes inhibiting differentiation (Id4) and myelin formation (Sox2) were highly expressed (Fig.1b, black) and upregulated in Yap cHet; Taz cKO sciatic nerves (Fig.1b, magenta). Genes involved in myelination were highly expressed and downregulated (Fig.1c). Among the 10 most expressed DNA-binding proteins that were downregulated in Yap cHet; Taz cKO sciatic nerves, 8 were already shown or suggested to play a role in myelination: Egr2, Nr2f1, Srebf2, Zeb2, Klf6, Hif1a, Nfe2l2 and Cers4 (Fig.1c). Excitingly, the remaining two DNA-binding proteins Cc2d1b and Purβ, have no known roles in peripheral nervous system development or myelination.
Figure 1 Genes encoding DNA-binding proteins significantly repressed in Yap cHet; Taz cKO sciatic nerves at 3 days of age. (a) Scatter plot for the comparison between genes differentially expressed in the wildtype and Yap cHet; Taz cKO sciatic nerves at 3 days of age. Log₂ fold-change in Yap cHet; Taz cKO; versus control mice was plotted against the average count size (log-counts-per-million) for every gene. Blue dots indicate statistically different genes (False Discovery Rate ≤ 0.05). The x-axis (logCPM, log counts per million) is a measure of gene expression, with higher numbers indicating genes highly expressed in sciatic nerves (e.g.: Mpz). The y-axis (logFC, log base 2 fold change) indicated if ablation of Yap/Taz upregulate or downregulate gene expression. Genes with positive values on the y-axis are positively regulated in Yap cHet; Taz cKO sciatic nerves when compared to control, while those with negative values on the y-axis are negatively regulated in in Yap cHet; Taz cKO sciatic nerves. Red lines indicate a 2-fold difference. On the left, expression levels of all genes expressed in sciatic nerves are represented (whole-genome). Among these, 2071 out of 18016 genes are dysregulated. On the right, genes encoding for a DNA-binding protein were selected for the presence of a DNA binding domain (http://www.transcriptionfactor.org). expression levels of all DNA-binding proteins expressed in sciatic nerves are represented. Among these, 64 out of 1445 are dysregulated. Average logCPM were calculated as log₂(average CPM+0.5). (b) Heatmap for the significantly dysregulated DNA-binding proteins in Yap cHet; Taz cKO sciatic nerves at 3 days of age. The differential expression of genes encoding for a DNA binding domain protein was tested in wildtype and Yap cHet; Taz cKO sciatic nerves. Of the 1445 genes tested, 64 showed statistical significance. Colors in this heatmap correspond to expression levels in Yap cHet; Taz cKO vs. control sciatic nerves, on a scale of yellow for lowest values to magenta for highest values with cyan for moderate values. Genes are categorized based on their expression levels in wildtype sciatic nerves (black denotes high expression levels, whereas white depicts low expression levels). Heatmap data are calculated using Z-score, where z=(x-mean in the samples/standard deviation in the samples). (c) The chart categorized transcription factors repressed Yap cHet; Taz cKO sciatic nerves according to their expression levels in wildtype sciatic nerves (segment width indicates expression level). Among the ten most expressed transcription factors (Egr2, Nr2f1, Srebf2, Purβ, Zeb2, Cc2d1b, Klf6, Hif1a, Nfe2l2, Cers4), 8 have a role to play in myelin formation (blue). The role of Purβ and Cc2d1b in myelination is unknown (brown).
Identification of novel myelin regulators in Schwann cells. Cc2d1b, also named Freud-2, encodes for Coiled-coil and c2 domain containing 1B protein and is highly expressed in peripheral nerves and myelinating oligodendrocytes mousebrain.org; gtexportal.org. Purβ encodes for the Purine Rich element binding protein B. Purβ binding elements have already been characterized in numerous genes, including Mbp and Plp1, yet it is unclear if Purβ is necessary for their expression.

We first confirmed our RNAseq data by qRT-PCR and showed that Cc2d1b and Purβ are downregulated in Yap cHet; Taz cKO sciatic nerves (Fig.2a). Because dysregulation of gene expression in sciatic nerves can be due to alterations of mRNA level in Schwann cells, axons, perineurial or endothelial cells or fibroblasts, we confirmed that Cc2d1b and Purβ are expressed by primary Schwann cells (Fig.2b, e). Finally, we showed that treatment of primary rat Schwann cells with verteporfin, a drug that inhibits YAP/TAZ regulation of transcription by disrupting its interactions with TEAD transcription factors, reduces expression of Cc2d1b and Purβ (Fig.2c-d). Altogether, these data indicate that CC2D1B and PURβ are expressed by Schwann cells and regulated by YAP/TAZ/TEAD.

![Figure 2](image-url)

Figure 2 - Expression of Cc2d1b and Purβ in sciatic nerves and Schwann cells. (a) Quantitative RT-PCR analysis confirmed a transcriptional repression of Cc2d1b (-29%) and Purβ (-36%) in Yap cHet; Taz cKO sciatic nerves at P3. n = 3 animals per genotype. Two-way ANOVA with Bonferroni post hoc test. F (1, 8) = 49.64, P = 0.0001, Cc2d1b P = 0.0044, Purβ P = 0.0011. Error bars indicate s.e.m. (b) Western blot analysis using the rabbit anti-CC2D1B and PURβ antibodies on wildtype rat Schwann cells (lane 1), S16 Schwann cells (lane 2) and wildtype mouse sciatic nerves (S.N.) at 30 days of age (lane 3). Calnexin was used as loading control. (c) mRNA and (d) protein levels of Cc2d1b and Purβ after verteporfin treatment (relative to DMSO-treated controls) in Schwann cells. Cc2d1b (-93%) and Purβ (-85%) mRNA are strongly reduced upon a 24 h treatment with 10 µM of verteporfin. CC2D1B (-44%) and PURβ (-38%) protein levels are also reduced after a 24 h treatment with 10 µM of verteporfin. n = 3 independent experiments with 3 independent samples per group for (a) and (c). Error bars indicate s.d. A logarithmic scale was used for the y axis of (a) and (c), and the origin was set to 1. Two-way ANOVA with Bonferroni post hoc test. F (2, 12) = 817.9, P < 0.0001, Cc2d1b P < 0.0001, Purβ P < 0.0001. (e) Immunolocalization of PURβ in cross section of sciatic nerve at P30 show localization of PURβ in Schwann cell cytoplasm and nuclei, although at P30 PURβ staining appears to be more intense in Schwann cell cytosol than in the nuclei. Scale bar = 10 µm. Insert display a magnified Schwann cells stained for PURβ. Insert scale bar = 2 µm. immunoabs displays a validation for PURβ antibody for which PURβ antibody was pre-absorbed with 30-fold excess of the PURβ protein before immunohistochemistry.
**Cc2d1b and Purβ regulate myelination in vitro.** To determine the function of CC2D1B and PURβ in Schwann cells, we asked if silencing the expression of *Cc2d1b* and *Purβ* in Schwann cells would affect the capability of Schwann cell to myelinate axons. Schwann cells were infected with viruses expressing different shRNAs for either *Cc2d1b* or *Purβ*. All shRNAs reduced expression of *Cc2d1b* or *Purβ*, as shown by quantitative RT-PCR and western blot (Fig.3a-b). In addition, we show that silencing *Cc2d1b* or *Purβ* does not affect the protein level of their homologue CC2D1A and PURα (Fig.3b). When Schwann cells silenced for *Cc2d1b* and *Purβ* were seeded on DRG neurons and cocultured in myelinating conditions by adding ascorbic acid to the cultures, myelination of axons was impaired (Fig.3c-d). Because a defect of myelination can be caused by a reduced number of Schwann cells attached to axons, we asked whether silencing *Cc2d1b* or *Purβ* caused changes in apoptosis or proliferation. However, silencing of *Cc2d1b* and *Purβ* did not affect Schwann cell proliferation or apoptosis (Fig.4). Finally, during in vivo and in vitro myelination, *Mbp* and *Mpz* gene expression peak 3 days after birth and 40 days after addition of ascorbic acid, respectively [45]. We showed that *Purβ* expression was not significantly altered during Schwann cell development (Fig.3e) but appears, in vitro, to spike after 3 days in culture, before the start of myelination (Fig.3f). In contrast, *Cc2d1b* was highly expressed in vivo between postnatal day 5 (P5) and P20 (Fig.3e), and in vitro after 5 days in culture (Fig.3f), when Schwann cells myelinate axons. Taken together, these data show that CC2D1B or PURβ is required for Schwann cell myelination in vitro.

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**Figure 3 - *Cc2d1b* and *Purβ* regulate myelination in vitro.** (a) Quantitative RT-PCR analysis showed the silencing efficiency of shRNAs targeting *Cc2d1b* and *Purβ* in primary rat Schwann cells. n = 3 experiments. One-way ANOVA with Bonferroni post hoc test. *Cc2d1b* F (3, 16) = 10.83, P = 0.0004, shCc2d1b #1 (-43%) P < 0.0001, shCc2d1b #2 (-36%) P < 0.0001. *Purβ* F (3, 16) = 35.09, P < 0.0001, shPurβ #1 (-64%) P < 0.0001, shPurβ #2 (-75%) P < 0.0001. Error bars indicate s.d. **** P < 0.0001. A logarithmic scale was used for the y-axis and the origin was set to 1. (b) Western analysis showed that silencing *Cc2d1b* and *Purβ* reduce CC2D1B (-41%) and PURβ (-80%) protein levels, but do not after their respective homologues (CC2D1A and PURα). (c-d) Immunolocalization of myelin protein revealed a defect in myelin production in Schwann cells silenced for either *Cc2d1b* or *Purβ*. Schwann cells (200,000 cells) infected with shRNA were seeded on dorsal
root ganglia neurons and allowed to myelinate for 10 days. Cultures were stained for Myelin Basic Protein (MBP, green), neurofilament H (NFH, red) and DAPI (blue). The number and length of myelin segments were quantified. A lower number of segments were observed in both shCc2d1b and shPurβ and shortened myelin segments were observed in shPurβ. n = 4 coverslips for 3 independent experiments. One-way ANOVA with Bonferroni post hoc test. # myelin segments F (3, 44) = 24.31, P < 0.0001, shCc2d1b P < 0.0001, shPurβ P < 0.0001. myelin segments length F (3,44) = 28.03, P < 0.0001, shPurβ P < 0.0001. Error bars indicate s.d.

Figure 4 - Cc2d1b and Purβ do not increase Schwann cell proliferation or apoptosis. Dapi (blue) staining on Schwann cell silenced for either Cc2d1b and Purβ show no differences in cell number. TUNEL analysis (red) and phospho-Histone 3 (p-H3 - green) staining show no increase in proliferation or apoptosis. Schwann cells were infected with 5 virions per cells, incubated for 72 hours and stained. n = 4 independent experiment. One-way ANOVA with Bonferroni post hoc test. Error bars indicate s.d.

DISCUSSION
In this study, we identify novel regulators essential for myelination. Yap cHet ; Taz cKO sciatic nerves present an arrest SC early development and an abolition of subsequent SC myelination. We first hypothesized that genes regulated by YAP/TAZ include novel regulators of myelin formation. Thus, we analyzed gene expression by RNA-Seq analysis in Yap cHet ; Taz cKO sciatic nerves. In contrast to classical analyses based on gene dysregulation, which would highlight genes highly regulated by YAP/TAZ 14, we used our dataset to look at DNA binding proteins highly expressed in Yap cHet ; Taz cKO sciatic nerves, with the secondary assumption that their level of expression would be correlated to their importance in myelin formation. In this report, we validate our hypotheses and found that global gene expression stratification allows for the identification of genes essential for myelination. We were able to identify that most of the genes known to be either activators or inhibitors of myelination are highly expressed in sciatic nerves and are dysregulated in Yap cHet ; Taz cKO. Following our reasoning, we identify two novel DNA binding proteins CC2D1B and PURβ, with previously
unsuspected role in myelinating glial cells. CC2D1B and PURβ are highly expressed in sciatic nerves and in SCs and downregulated in Yap cHet; Taz cKO. We demonstrate that CC2D1B and PURβ are required for normal myelination. Ablation of either CC2D1B or PURβ impairs myelination in vitro, independently from effects on Schwann cell proliferation or apoptosis. Altogether our data demonstrate that CC2D1B and PURβ are both involved in myelin formation in vitro.

CC2D1B and PURβ are both DNA-binding proteins, but their role as regulator in myelinating glial cells or other cells remains undefined. CC2D1B protein structure is close to its homolog CC2D1A. Cc2d1a is highly expressed in neurons and has been implicated in intellectual disability and autism spectrum disorder 46, 47. Cc2d1b is expressed in myelinating glial cells and peripheral nerves mousebrain.org; gtexportal.org, 42, which indicates that its role and function might not be fully redundant with Cc2d1a. Few studies have suggested a redundant role between both proteins for the regulation of serotonin receptors 48, 49. Yet, the transcriptional role of CC2D1 remains controversial, as other studies showed that both CC2D1 are confined to the cytoplasm and perinuclear endosomes 50. Thus, it remains unclear whether CC2D1B can directly control transcription in vivo and whether it translocates from the cytoplasm to the nucleus. Interestingly, other functions have been proposed for CC2D1 proteins independently from their DNA-binding domain. CC2D1 proteins belong to the evolutionary conserved Lgd protein family which was shown to be involved in the regulation of signaling receptor degradation via the endosomal pathway 51. Loss of Lgd function results in an ectopic and ligand-independent activation of the Notch pathway 51-53. Notch signaling promotes the early stage of Schwann cell development but inhibits myelination 54. Thus, it is possible that CC2D1B, modulates myelination through the recruitment of specific signaling complexes. Finally, Cc2d1b KO mice have been reported and present delayed memory acquisition and retention 55. There is growing evidence, both from animal studies and human neuroimaging that myelin plays a role in learning 56, 57 and it might be worthwhile to also consider the role of CC2D1B in central nervous system myelination.

PURβ belongs to the purine-rich element binding (PUR) protein family, which includes of PURα, PURβ and PURγ. There is substantial evidence for PUR role in DNA binding 58, 59. Among them, PURα was studied the most, for its implication in fragile X syndrome and PURA syndrome, a disorder characterized by intellectual disability and delayed development of speech and motor skills, such as walking 60-62. Interestingly, Pura knockout mice present with reduced myelin production and pathologic development of glial cells 63. However, no knockouts of the other PUR family genes have been reported. PURβ is known to play a role in cell differentiation and modulates transcriptional regulation of gene expression of the α- and β-myosin heavy chain and actin α-2 54, 65. Notably, TEAD-1, the main transcriptional partner of YAP/TAZ, was reported to be upstream of Purβ 66. TEAD motif-harboring enhancers (GGAAT) can be found in numerous genes dysregulated in Yap cHet; Taz cKO, including CC2D1B and PURβ. Yet, no binding motifs for EGR2 or SOX10 were found in the PURβ enhancer region 67. Thus, it is possible that PURβ is a promyelinating regulator directly downstream of YAP/TAZ/TEAD1. Finally, all PUR isoforms have been associated with neoplasia 60. Although the Cc2d1b and Purβ expression appear to be downstream YAP/TAZ/TEADs, the signals contributing to the regulation of their expression are essentially unknown. Elucidation of the upstream pathways and signals
that induce CC2D1B and PURβ will be important both for understanding the molecular control of the myelination program, but also potentially for identifying strategies to promote remyelination in demyelinating disease. Indeed, numerous transcription factor involved in developmental myelination are also involved in remyelination following injury. Thus, it will be critical to characterize the role of CC2D1B and PURB in peripheral nerve repair.

Finally, our study extends regulatory mechanisms directing Schwann cell myelinogenesis and supports the transition from a gene-centric to a network-systems view of the myelin formation. Further characterization of the transcription factor network controlling myelin gene expression should help refine our understanding of Schwann cell development as well as suggest novel therapeutic strategies to potentiate their regenerative capacity.

REFERENCES


MATERIAL AND METHODS

Cell culture. Primary rat Schwann cells were produced as described 70 and grown with DMEM supplemented with 4 g/l glucose, 2 mM L-glutamine, 5 % bovine growth serum, 2 μM forskolin, 50 ng/ml nerve growth factor, penicillin and streptomycin. Schwann cells were not used beyond the fourth passage. Rat dorsal root ganglia (DRG) neurons from Sprague Dawley rats embryos were isolated at embryonic day 14.5 embryos. DRG were dissociated by treatment with 0.25 % trypsin and mechanical trituration and 1.5 DRGs were seeded on collagen-coated glass coverslips as described 70. DRGs cultures were then cycled with fluoroxidine (FUDR, Sigma-Aldrich) to eliminate all non-neuronal cells. Once all non-neuronal cell remove, rat Schwann cells were added (200 000 cells per coverslip) to establish myelinating cocultures of DRG neurons, and myelination was initiated by supplementing the medium with 50 μg/ml ascorbic acid (Sigma-Aldrich). For verteporfin (Sigma SML0534) treatment, verteporfin was solubilized in DMSO at 20 mM, then Schwann cells were treated with either 0.5 % of DMSO; 2 or 10 μM of verteporfin for 24 h. mRNA was extracted and cDNA was analyzed by RT-qPCR, as described in 14. This study was carried out in accordance with the principles of the Basel Declaration and recommendations of ARRIVE guidelines issued by the NC3Rs and approved by the Albany Medical College Institutional Animal Care and Use Committee (no. 17-08002).

shRNA lentivirus production and infection. shRNA virions were produced as 71. shRNA targeting Cc2d1b (#1, TTGCGCTCATCCCGACTG), (#2, ATGAGCTCGAATAGCATCC) and Purβ (#1, AACTCGATGAGGCCCTGCG), (#2, TGGCATTGCGGTAGGATGG) and control (non-targeting) were bought from Dharmacon SMARTvector library. Schwann cells were infected with 5 virions per cells, incubated for 72 hours and collected for qRT-PCR analysis. Coculture experiments were done with shCc2d1b #1 and shPurβ #2.

RNA preparation and quantitative RT-PCR. Sciatic nerves were dissected, stripped of epineurium, frozen in liquid nitrogen, pulverized and processed as described 72. Total RNA was prepared from sciatic nerve or Schwann cells with TRIzol (Roche Diagnostic). 1 μg of RNA was reverse transcribed using Superscript III (Invitrogen). For each reaction, 5 μM of oligo(dT)20 and 5 ng/μl random hexamers were used. Quantitative PCR were performed using the 20 ng of cDNA combined with 1X FastStart Universal Probe Master (Roche Diagnostic). Data were analyzed using the threshold cycle (Ct) and 2(−ΔΔCt) method. Actb was used as endogenous gene of reference and 18S was used as to validate stable expression of Actb. The primers and probe used are the following: 18S (F: ctcacaacgggaacacctac, R: cgctccaccaactaagaacg, probe #77), mouse Actb (F: aagggcacaacgtgaaagat, R: ttgtaacagcagagccatc, probe #56), mouse Cc2d1b (F: cactcaaggggaacacg, R: ctgctgcagcttcatctgttcat, probe #4); mouse Purβ (F: aattatcctactccgctgtt, R: ttgagctagatcactgtctaggtt, probe #71); rat Cc2d1b (F: gactcactgggaaacacg, R: ctgccaactttcataagggtg, probe #4); rat Purβ (F: aagggactgccagcaacct, R: agactcttgccaggttg, probe #56).
**Immunofluorescence and Immunoblotting.** Immunohistochemistry, immunocytochemistry and immunoblotting were performed as described \(^{73}\). 10 µg of protein were used for western blot. The antibodies used are the following: anti-CC2D1A (Abcam, ab68302), anti-CC2D1B (Proteintech, 20774-1-AP), anti-PURα (Proteintech, 17733-1-AP), anti-PURβ (Proteintech, 18128-1-AP), anti-calnexin (Sigma, C4731), anti-phospho-histone H3 (Millipore, 06-576), anti-neurofilament H (Biolegend, 822701), anti-MBP (Biolegend, 808401). CC2D1B antibody was validated using Cc2d1b knockout mouse (Zamarbide et al., 2018). PURβ antibody was validated using purified PURβ fusion protein (Proteintech, Ag12705). Briefly, 1.5 µg of PURβ antibody was incubated with 50 µg of Purβ protein for 1 h at 37°C prior to be used for immunohistochemistry. TUNEL assays were performed on coverslips of culture as described in \(^{14}\). Myelination *in vitro* was evaluated from three different experiments, performed with two coverslips in each case, which is a standard sample size for these experiments. Images were acquired with identical acquisition parameters on an epi fluorescent Axio Imager A2 (Zeiss). Myelin segments number and length were quantified using Image J software (http://imagej.nih.gov/ij) from two random fields of each culture at the 10 × objective, as described in \(^{74}\).

**Bioinformatics.** RNAseq data were obtained from NCBI GEO: GSE79115 \(^{14}\). Genes encoding for DNA-binding protein genes were predicted thanks to transcriptionfactor.org database. The expression data for Cc2d1b and Purβ were obtained from mousebrain.org/ and gtexportal.org on 09/2018.

**Statistical analyses.** Experiments were not randomized, but data collection and analysis were performed blind to the conditions of the experiments. Data excluded are reported in the legend of the figures. Data are presented as mean ± s.e.m or s.d. No statistical methods were used to predetermine sample sizes, but our sample sizes are similar those generally employed in the field. Two-tailed Student t-test, One-way ANOVA and Two-way ANOVA were used for statistical analysis of the differences between multiple groups according to the number of sample groups. Values of \(P \leq 0.05\) were considered to represent a significant difference.