YAP and TAZ Regulate Cc2d1b and Purβ in Schwann Cells

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Schwann cells (SCs) 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 expressions such as integrins and G-proteins. Yet, the characterization of the signaling downstream YAP/TAZ in SCs is incomplete. Myelin sheath production by SC 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 SC myelin gene transcription and differentiation. Furthermore, we link YAP/TAZ to two DNA binding proteins, Cc2d1b and Purβ, which have no described roles in myelinating glial cells. We demonstrate that silencing of either Cc2d1b or Purβ 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.

Keywords: Yap, Taz, Cc2d1b, Purβ, myelin, Schwann cell

MAIN POINTS
YAP and TAZ regulate positive and negative myelin regulators. Cc2d1b and Purβ are necessary for Schwann cell myelination in vitro.

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; Monk et al., 2015). 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 (Micha1ov et al., 2004; Feltri and Wrabetz, 2005; Taveggia et al., 2005; Belin et al., 2017). These signals regulate an intricate network of transcription factors that control differentiation of SCs and myelination (e.g., EGR2, YY1, ZEB2, Topilko et al., 1994; Nagarajan et al., 2001; He et al., 2010; Weng et al., 2012; Quintes et al., 2016; Wu et al., 2016).
The identification and characterization of the complete repertoire of transcription factors that modulate myelination is still incomplete (Svaren and Meijer, 2008; Fulton et al., 2011).

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 (Poitelon et al., 2016). YAP/TAZ are two transcriptional activators of the HIPPO pathway, and play important roles in controlling organ growth, cell differentiation, proliferation and survival (Dupont, 2016). Mechanical stimulation can regulate YAP/TAZ through signals involving FAK, Src, PI3K and JNK pathways (Codelia et al., 2014; Mohseni et al., 2015), or the formation of actomyosin filaments and accumulation of F-actin (Dupont et al., 2011; Aragona et al., 2013). In addition, YAP/TAZ in SCs can be activated through Crb/Amoll proteins and laminin/G-protein signaling (Colciago et al., 2015; Fernando et al., 2016; Poitelon et al., 2016; Deng et al., 2017). 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 (Kim et al., 2018). TEADs role in myelination is unknown (Hung et al., 2015; Lopez-Anido et al., 2015), but TEAD1 binding to transcriptional enhancers is induced during myelination (Lopez-Anido et al., 2016). 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 (Poitelon et al., 2016). Finally, it was suggested that YAP/TAZ and TEAD1 regulate myelin wrapping in cooperation with master myelin regulators EGR2 and SOX10 (Lopez-Anido et al., 2016; Poitelon et al., 2016).

To identify novel regulators that are essential for myelination, we integrated RNA-seq and bioinformatics analyses and looked for DNA binding proteins dysregulated in Yap cHet; Taz cKO sciatic nerves at 3 days of age. We identified two highly expressed proteins, i.e., Cc2d1b and Purβ, downregulated in Yap cHet; Taz cKO sciatic nerves. We found that silencing of Cc2d1b or Purβ in vitro significantly decreased the number of myelin segments and silencing of Purβ also significantly decreased the length of myelin segments, independently for effect on SC number, proliferation or apoptosis. These data demonstrate that CC2D1B and PURβ are necessary for myelination in vitro.

MATERIALS AND METHODS

Cell Culture

Primary rat SCs were produced as described (Poitelon and Feltrì, 2018) 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. SCs 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 (Poitelon and Feltrì, 2018). DRGs cultures were then cycled with fluoroxide (FUDDR, Sigma-Aldrich) to eliminate all non-neuronal cells. Once all non-neuronal cell remove, rat SCs 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 SCs 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 Poitelon et al. (2016). 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 Poitelon et al. (2015). shRNA targeting Cc2d1b (#1, TTTGCTGCTCATCCGCCCTTGG), (#2, ATGGACTGGAATAGCACTCC and Purβ (#1, AACTCGATGAGGCGCTCATCCC), (#2, TGTCATTGGCTTAGTGTTAGGTG) and control (non-targeting) were bought from Dharmacon SMARTvector library. SCs were infected with five virions per cells, incubated for 72 h 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 (Poitelon et al., 2012). Total RNA was prepared from sciatic nerve or SCs with TRIzol (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 the stable expression of Actb. The primers and probe used are the following: 18S (F: ctcaacaggggaaactcct, R: cgcctcaacactaaag, cg, probe #77), mouse Actb (F: aagggcacaagtcagatgat, R: gttgta gcagcagcatt, probe #56), mouse Cc2d1b (F: ccactcagggg aaacgc, R: cgtgctgcagctctcaat, probe #4); mouse Purβ (F: aat tatgcaatctgccgttt, R: tttgcagatgtaagttaattaaggttt, probe #71); rat Cc2d1b (F: gcactcaggggaaacg, R: cggctcaactttctcatgg, probe #4); rat Purβ (F: aagggcacaagtcagcact, R: agactcctgacaggtt, probe #56).
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 being used for immunohistochemistry. TUNEL assays were performed on cover slips of culture as described in Poitelon et al. (2016). 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 ImageJ software from two random fields of each culture at the 10× objective, as described in Ghidinelli et al. (2017).

Bioinformatics
RNAseq data were obtained from NCBI GEO: GSE79115 (Poitelon et al., 2016). 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 ± standard error of the mean (SEM) or SD. No statistical methods were used to determine sample sizes, but our sample sizes are similar to those generally employed in the field. Two-tailed Student’s t-test, One-way analysis of variance (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 ≤ 0.05 were considered to represent a significant difference.

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 2,071 misregulated transcripts (Figure 1, Poitelon et al., 2016). We narrowed our analysis to DNA-binding proteins and identified that ablation of Yap/Taz dysregulated 64 genes (Figure 1B). Genes encoding for DNA-binding proteins were then categorized according to their level of expression (Figure 1B, black/white heatmap). Signature genes normally expressed in neural crest cells (Tbx2) and immature SCs (Oct6/Pou3f1/Scip), as well as genes inhibiting differentiation (Id4) and myelin formation (Sox2) were highly expressed (Figure 1B, black) and upregulated in Yap cHet; Taz cKO sciatic nerves (Figure 1B, magenta; Arroyo et al., 1998; Jang et al., 2010; Ma et al., 2015; Roberts et al., 2017). Genes involved in myelination were highly expressed and downregulated (Figure 1C). Among the 10 most expressed DNA-binding proteins that were downregulated in Yap cHet; Taz cKO sciatic nerves, eight were already shown or suggested to play a role in myelination: Egr2, Nr2f1, Srebf2, Zeb2, Klf6, Hif1α, Nfe2l2 and Cers4 (Figure 1C; Topilko et al., 1994; Nagarajan et al., 2001; Yamaguchi et al., 2004; Leblanc et al., 2005; Imgrund et al., 2009; Verheijen et al., 2009; Ginkel et al., 2012; Weng et al., 2012; Zhang et al., 2013; Yuen et al., 2014; Lopez-Anido et al., 2015; Laitman et al., 2016; Quintes et al., 2016; Wu et al., 2016; Huppke et al., 2017). Excitingly, the remaining two DNA-binding proteins Cc2d1b and Purβ, have no known roles in peripheral nervous system development or myelination.

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 (Zhang et al., 2014). Purβ encodes for the Purine Rich element binding protein B. Purβ binding elements have already been characterized in numerous genes, including Mbp and Plp1 (Tretiakova et al., 1999; Dobretsova et al., 2008), 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 (Figure 2A). Because dysregulation of gene expression in sciatic nerves can be due to alterations of mRNa level in SCs, axons, perineurial or endothelial cells or fibroblasts, we confirmed that Cc2d1b and Purβ are expressed by primary SCs (Figures 2B,E). Finally, we showed that treatment of primary rat SCs 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 (Figures 2C,D). Altogether, these data indicate that CC2D1B and PURβ are expressed by SCs and regulated by YAP/TAZ/TEAD.

Cc2d1b and Purβ Regulate Myelination in vitro
To determine the function of CC2D1B and PURβ in SCs, we asked if silencing the expression of Cc2d1b and Purβ in SCs would affect the capability of SC to myelinate axons. SCs 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.
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. Log, fold-change in Yap cHet; Taz cKO vs. 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 $\leq$ 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) indicates 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 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, 2,071 out of 18,016 genes are dysregulated (Poitelon et al., 2016). 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 1,445 are dysregulated. Average logCPM were calculated as log$_2$(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 1,445 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 DNA-binding proteins repressed Yap cHet; Taz cKO sciatic nerves according to their expression levels in wildtype sciatic nerves (segment width indicates expression level). Among the 10 most expressed DNA-binding proteins (Egr2, Nr2f1, Srebf2, Pur$\beta$, Zeb2, Cc2d1b, Klf6, Hif1a, Nle2l2, Cers4), eight have a role to play in myelin formation (blue). The role of Pur$\beta$ and Cc2d1b in myelination is unknown (brown).

(Figures 3A,B). In addition, we show that silencing Cc2d1b or Pur$\beta$ does not affect the protein level of their homolog CC2D1A and PURa (Figure 3B). When SCs silenced for Cc2d1b and Pur$\beta$ were seeded on DRG neurons and cocultured in myelinating conditions by adding ascorbic acid to the cultures, myelination of axons was impaired (Figures 3C,D). Because a defect of myelination can be caused by a reduced number of SCs attached to axons, we asked whether silencing Cc2d1b or Pur$\beta$ caused changes in apoptosis or proliferation. However, silencing of Cc2d1b and Pur$\beta$ did not affect SC proliferation or apoptosis (Figure 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 (Notterpek et al., 1999). We showed that Pur$\beta$ expression was not significantly altered during SC development (Figure 3E) but appears, in vitro, to spike after 3 days in culture, before the start of myelination (Figure 3F). In contrast, Cc2d1b was highly expressed in vivo between postnatal day 5 (P5) and P20 (Figure 3E), and in vitro after 5 days in culture (Figure 3F), when SCs myelinate axons. Taken together, these data show that CC2D1B or PUR$\beta$ is required for SC myelination in vitro.
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 (Poitelon et al., 2016), 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 sciatric 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 sciatric 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 SC 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 a 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 (Basel-Vanagaite et al., 2006; Manzini et al., 2014). Cc2d1b is expressed in myelinating glial cells and peripheral nerves4,5 (Zhang et al., 2014), 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 (Hadjighassem et al., 2009, 2011). Yet, the transcriptional role of CC2D1 remains controversial, as other studies showed that both CC2D1 are confined to the cytoplasm and perinuclear endosomes (Drusenheimer et al., 2015). 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 might not be fully redundant with Cc2d1a.

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 is also involved in remyelination following injury. Thus, it will be critical to characterize the role of CC2D1B and PURβ in peripheral nerve repair.

Finally, our study extends regulatory mechanisms directing SC myelinosogenesis (Hung et al., 2015; Boerboom et al., 2017; Quintes and Brinkmann, 2017) 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 SC development as well as suggest novel therapeutic strategies to potentiate their regenerative capacity.

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4www.mousebrain.org
5www.gtexportal.org
DATA AVAILABILITY

All datasets generated for this study are included in the manuscript and/or the supplementary files.

AUTHOR CONTRIBUTIONS

BS and PoY designed research and interpreted data. BS and PoY performed experiments with HJ assistance. PaY contributed to the manuscript. PoY wrote the manuscript with BS assistance. BS and PoY analyzed the data. PaY, VJ, and FL critically reviewed the manuscript.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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