

*Research article***A Unique TGFB1-Driven Genomic Program Links Astrocytosis, Low-Grade Inflammation and Partial Demyelination in Periplaques of Spinal Cords from Progressive Multiple Sclerosis Patients**Serge Nataf<sup>1,2\*</sup>, Marc Barritault<sup>3,4</sup> and Laurent Pays<sup>1,2</sup>

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**Abstract:** We previously reported that in multiple sclerosis (MS) patients with a progressive form of the disease, spinal cord periplaques extend distance away from plaque borders and are characterized by the co-occurrence of partial demyelination, astrocytosis and low-grade inflammation. However, transcriptomic analyses comparing periplaques to adjacent normal-appearing white matter (NAWM) areas did not allow providing a comprehensive view of molecular events in astrocytes vs oligodendrocytes. Here, we re-assessed our transcriptomic data with the aim of identifying functionally-relevant co-expression networks that would reflect astrocyte vs oligodendrocyte molecular signatures in periplaques. We identified an astrocytosis-related gene module comprising GFAP, the hub gene *CX43/GJA1* and a set of transcripts forming a *TGFB/SMAD1/SMAD2* genomic signature. Partial demyelination was characterized by a co-expression network which, besides myelin genes, comprised a highly significant number of translation/elongation-related genes. Interestingly, the main oligodendrocyte-related hub we identified was *NDRG1*, a gene previously shown to be specifically silenced in the NAWM of MS patients. This result indicated that *NDRG1* down-regulation could be an important event in the process of periplaque partial demyelination. To establish a putative link between *NDRG1* down-regulation and a cytokine/chemokine signature, we then sought to identify cytokine/chemokine genes whose mRNA levels inversely correlated with those of *NDRG1*. Following this approach we found 5 candidate immune-related genes whose up-regulation associated with *NDRG1* down-regulation: *TGFB1*, *PDGFC*, *IL-17D*, *IL-33*, and *IL-12A*. From these results we propose that in the spinal cord of MS patients with progressive forms of the disease, *TGFB1* may

limit acute inflammation but concurrently induce astrogliosis and an alteration of oligodendrocytes terminal differentiation.

**Keywords:** multiple sclerosis – neuroinflammation – astrocytes – myelin – bioinformatics

## 1. Introduction

Multiple sclerosis (MS) is a neuroinflammatory disorder of the central nervous system (CNS) and the leading cause of permanent neurological disability in young adults [1]. While autoimmune mechanisms targeting the myelin sheath have been extensively demonstrated, there are still uncertainties regarding the pathophysiology of MS progressive forms [2–5]. Importantly, patients suffering from secondary or primary progressive multiple sclerosis (SPMS and PPMS respectively) frequently exhibit slowly evolving spinal cord-related symptoms and studies based on magnetic resonance imaging (MRI) or neuropathological analyses demonstrated a high occurrence of spinal cord lesions as compared to patients with a relapsing-remitting form of the disease (RRMS)[6,7]. In this context, we recently described a particular type of tissue alteration in the spinal cord of MS patients with SPMS or PPMS [8]. We found that periplaques i.e. areas that surround the borders of fully-demyelinated lesions, are characterized by a partial loss of myelin, a low-grade inflammatory process and an astrogliosis signed by a constant upregulation of the astrocyte markers *Cx43/GJA1* and *AQP4*. Spinal cord periplaques extend distance away from the plaque borders and appear to evolve, at least in part, independently from plaque activity. However, when performing paired comparisons between periplaques and areas of adjacent normal-appearing white matter (NAWM), whole genome transcriptomic analyses did not allow providing a comprehensive picture of the molecular events driving astrogliosis, inflammation and partial demyelination in periplaques. Indeed, only 34 genes were found to be constantly up-regulated in periplaques and 57 constantly down-regulated. A potential caveat of the differential expression approach we opted for, relies on the varied levels of molecular alterations that may occur in areas of NAWM [9,10]. In particular, previous studies demonstrated that NAWM in MS brains or spinal cords exhibit; i) signs of low-grade inflammation [9,10] and astrogliosis [11–13] ii) an abnormal lipid and protein composition of myelin sheaths [14–16] and iii) an epigenetic silencing of the oligodendrocyte gene *NDRG1* [17]. Thus, when performing paired comparisons between periplaques and NAWM, the genomic alterations occurring in NAWM might have masked functionally-relevant molecular events taking place in both periplaques and NAWM.

In the present paper, we re-assessed our transcriptomic data with the aim of identifying gene co-expression networks that would link astrogliosis, inflammation and myelin alterations in spinal cord periplaques. In a first step, we identified *Cx43/GJA1* as the only astrocyte-specific hub gene among genes that constantly co-upregulated in periplaques. To identify a molecular signature of astrogliosis in periplaques, we then used *Cx43/GJA1* as a "bait" and determined the top-200 genes that more closely co-upregulated with *Cx43/GJA1*. Interestingly, besides *GFAP*, this *Cx43/GJA1* genomic

module comprised the two cytokines *IL17D* and *IL33* and a set of genes forming a *TGFB1/SMAD1/SMAD2* proglial signature. In parallel, we identified *NDRG1* as the only oligodendrocyte-specific hub gene among genes that constantly co-downregulated in spinal cord periplaques. To identify a molecular signature of oligodendrocytes in periplaques and NAWM, we then used *NDRG1* as a "bait" and determined the top-200 genes that more closely co-downregulated with *NDRG1*. Interestingly, *NDRG1* co-expression module comprised not only the myelin genes *PLP1*, *MBP* and *MOBP* but a highly significant number of transcripts involved in the process of mRNA translation and elongation. Finally, to unravel molecular mechanisms potentially linking partial demyelination to inflammation, we established a list of 193 candidate genes coding for cytokines, chemokines or growth factors from which we identified the top-5 genes whose mRNA levels inversely correlated with those of *NDRG1* in periplaques. We found that *NDRG1* mRNA levels inversely correlated with *TGFB1*, *IL33*, *IL17D*, *IL12A* and *PDGFC*.

## 2. Results

### 2.1. Workflow and results summary

Our workflow and main results are summarized in Figures 1 and 2.

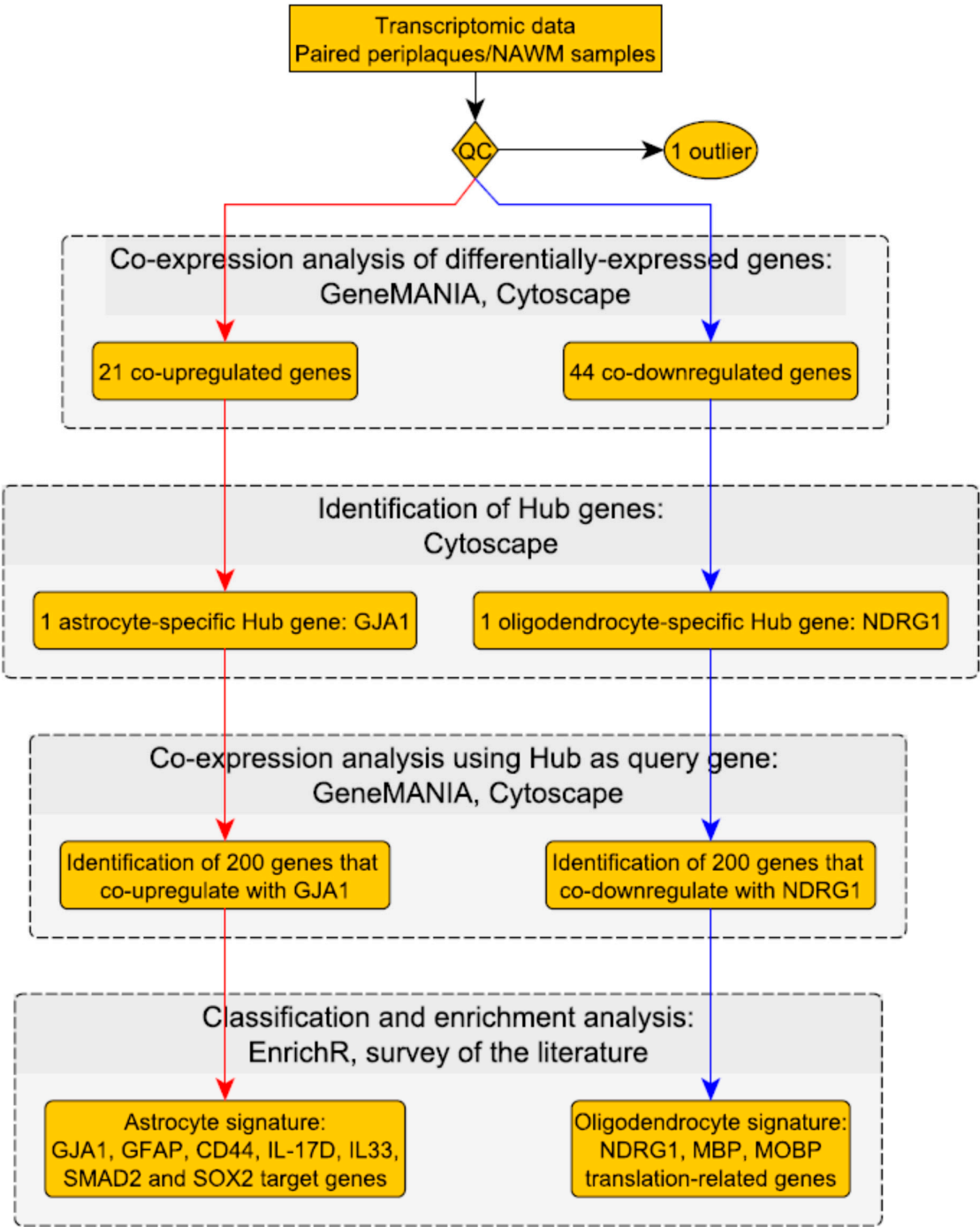


Figure 1: Workflow N°1

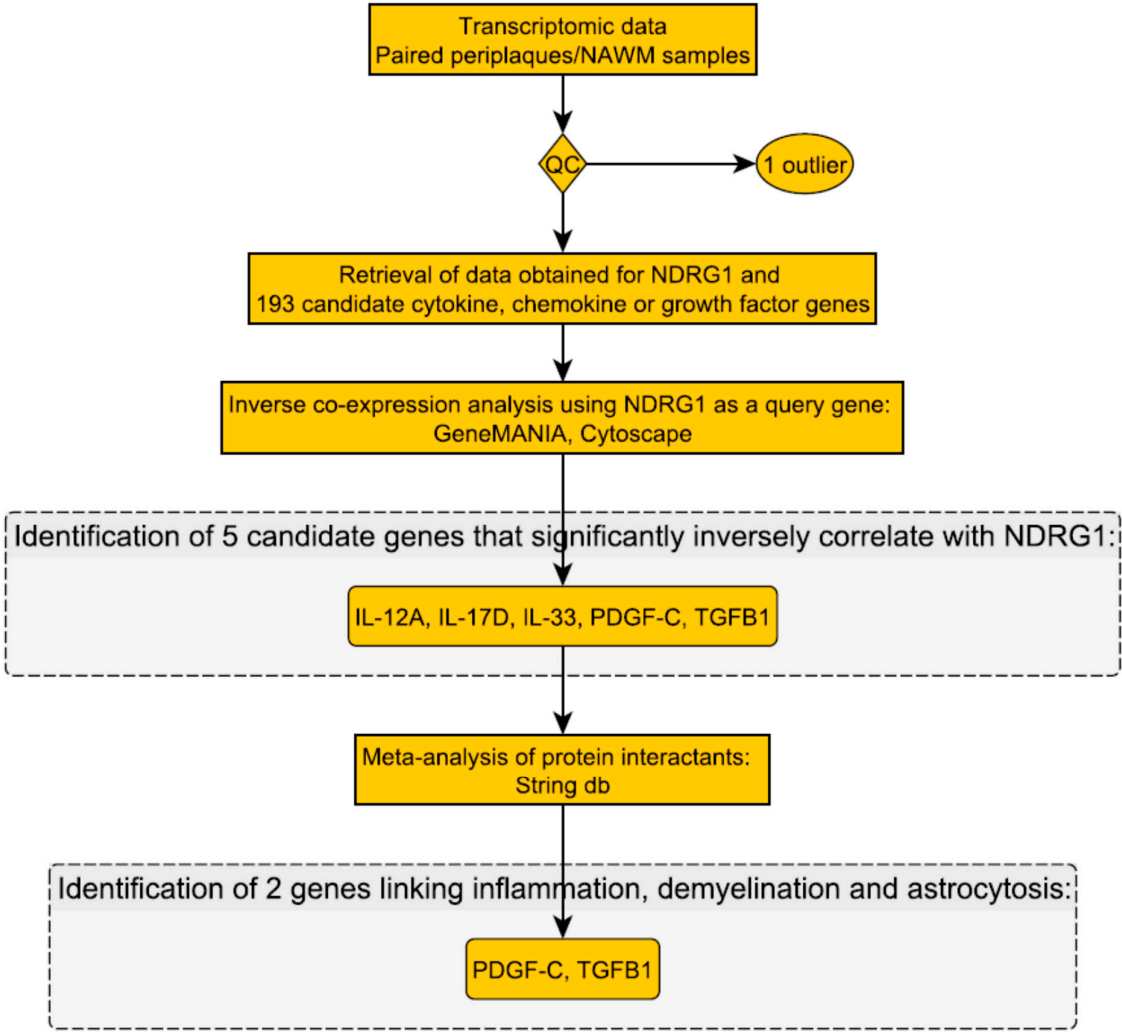
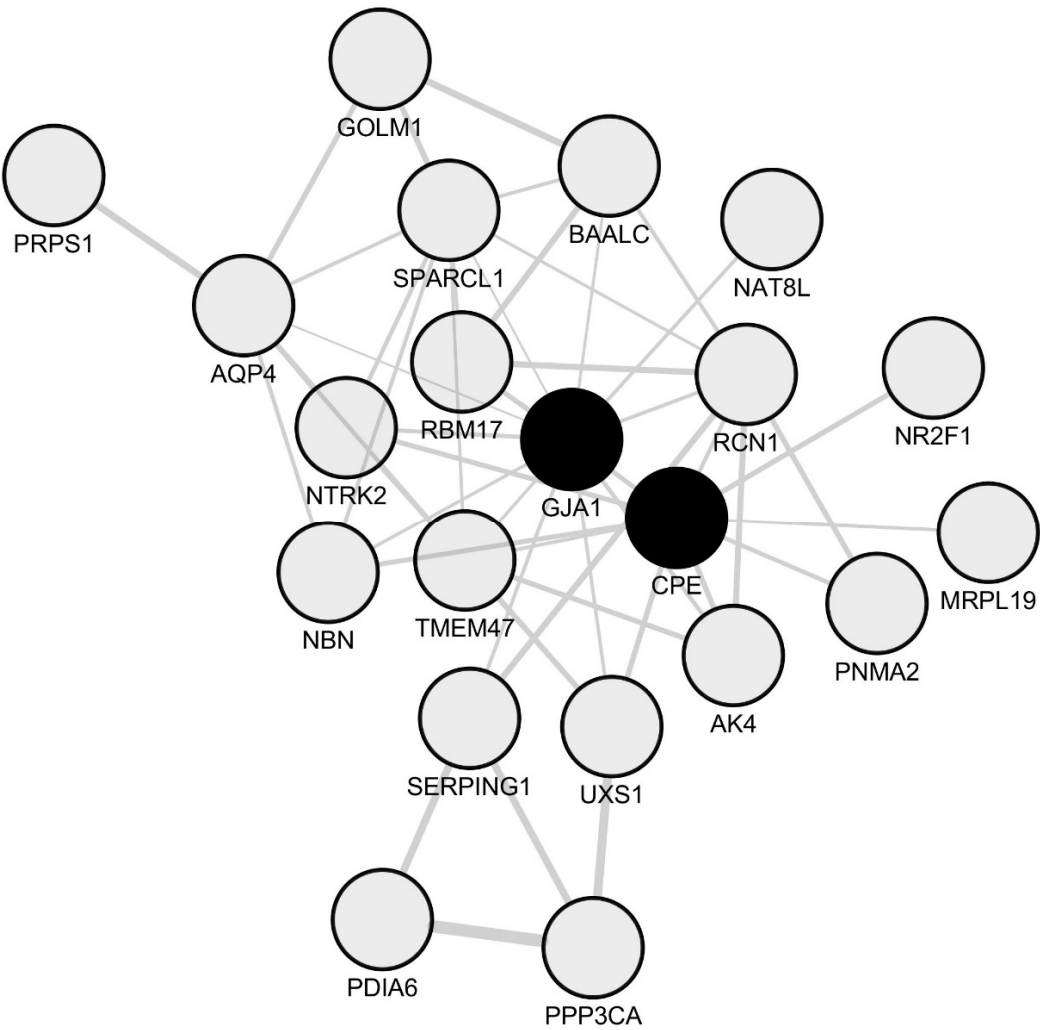


Figure 2: Workflow N°2

2.2. Identification of Cx43/GJA1 as an upregulated astrocyte-related hub gene in spinal cord periplaques

Based on the data we previously obtained by paired comparisons of periplaques vs NAWM transcriptomic profiles, we assessed whether the 34 coding genes identified as constantly upregulated in periplaques were indeed co-upregulated in periplaques. We identified a set of 21 co-upregulated mRNA species among which Cx43/GJA1 (Gap junction protein alpha 1 also named Connexin 43) was the only astrocyte-related hub gene (Figure 3). Importantly, Cx43 on astrocytes was previously reported to be up-regulated in the NAWM of MS patients as compared to controls [18].



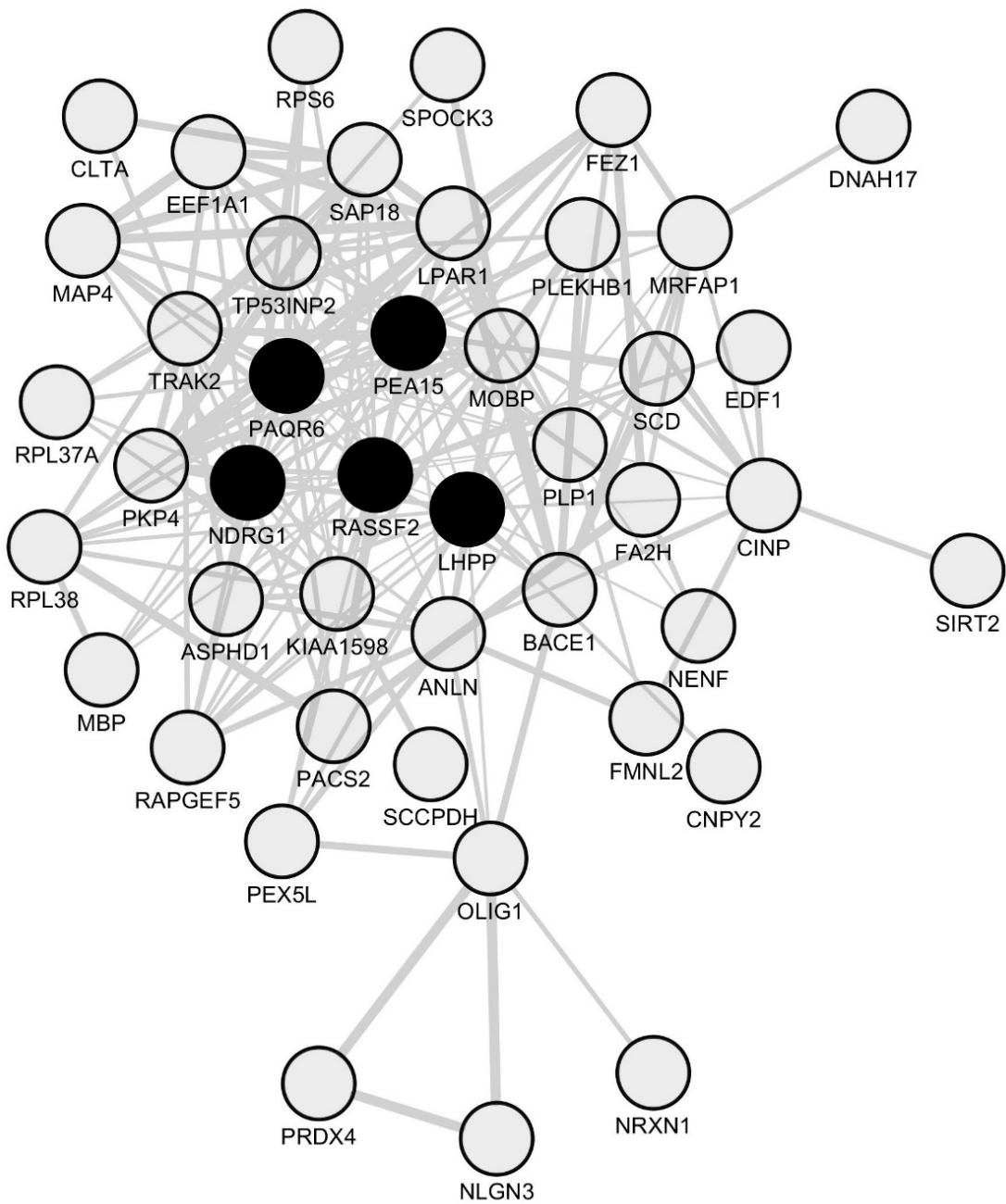
**Figure 3: Network of co-upregulated genes in spinal cord periplaques.** A co-expression network was generated with the set of genes that are constantly upregulated in

periplaques as compared to adjacent NAWM. In this co-expression module, hubs were defined as genes (nodes) being connected to at least half of the total number of genes (nodes) forming the network. Two hub genes (black-filled nodes) were identified, namely CPE (Carboxypeptidase E) and the astrocyte-specific gene marker Cx43/GJA1 (Gap junction protein alpha 1 also named Connexin 43). Genes are designated by their gene symbols.

## *2.2. Identification of NDRG1 as a downregulated oligodendrocyte-related hub gene in spinal cord periplaques*

We then assessed whether the 57 coding genes identified as constantly downregulated in paired comparisons of periplaques vs NAWM were co-downregulated in periplaques. We identified a set of 44 co-downregulated genes (Figure 4 and Table 1) among which NDRG1 was the only hub gene previously recognized as being specifically expressed by oligodendrocytes in the central nervous system [19–21].





**Figure 4: Network of co-downregulated genes in spinal cord periplaques.** A co-expression network was generated with the set of genes that are constantly downregulated in periplaques as compared to adjacent NAWM. In this co-expression module, hubs were defined as genes (nodes) being connected to at least half of the total number of genes (nodes) forming the network. Five hub genes (black-filled nodes) were identified, namely *NDRG1* (N-myc downstream regulated 1), *PEA15* (phosphoprotein enriched in astrocytes 15), *RASSF2* (Ras association domain family member 2), *PAQR6* (progesterin and adipoQ receptor family member 6) and *LHPP* (phospholysine phosphohistidine inorganic



pyrophosphate phosphatase). Genes are designated by their gene symbols. Full names of functionally-relevant genes are provided in Table 1.

**Table 1: Hub genes and oligodendrocyte-related genes that co-downregulate in spinal cord periplaques as compared to NAWM**

**Hub genes**

LHPP	Phospholysine phosphohistidine inorganic pyrophosphate phosphatase
NDRG1	N-myc downstream regulated 1
PAQR6	Progestin and adipoQ receptor family member VI
PEA15	Phosphoprotein enriched in astrocytes 15
RASSF2	Ras association (RalGDS/AF-6) domain family member 2

**Oligodendrocyte-related genes**

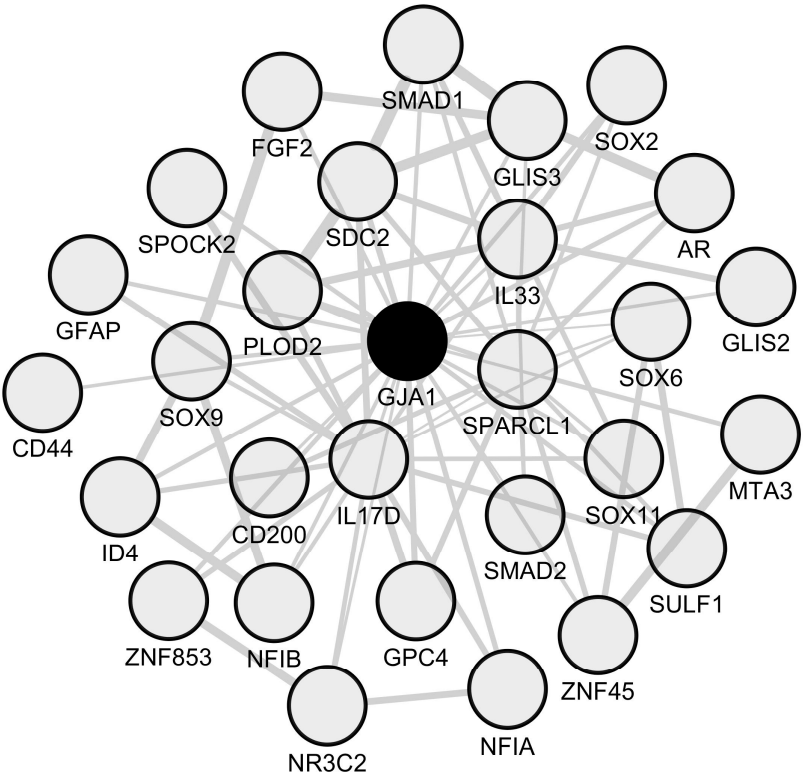
NDRG1	N-myc downstream regulated 1
MOBP	Myelin-associated oligodendrocyte basic protein
MBP	Myelin basic protein
PLP1	Proteolipid protein 1
OLIG1	Oligodendrocyte transcription factor 1
SIRT2	Sirtuin (silent mating type information regulation 2 homolog) 2 ( <i>S. cerevisiae</i> )
FA2H	Fatty acid 2-hydroxylase

**Legend:** A co-expression network was generated with the set of genes that constantly downregulated in periplaques as compared to adjacent NAWM. In this co-expression module, hubs were defined as genes (nodes) being connected to at least half of the total number of genes (nodes) forming the network.

*2.3. A unique set of genes co-upregulate with CX43/GJA1 in periplaques of MS spinal cords*

To identify a putative astrocyte molecular signature in periplaque areas of MS spinal cords, we used the astrocyte-specific hub gene *Cx43/GJA1* as a "bait" and retrieved the top-200 mRNA species that more closely co-expressed with *Cx43/GJA1* in our whole set of

data. Functionally-relevant genes are shown in Table 2 and Figure 5. The full list of *Cx43/GJA1* co-expressed genes is provided in data supplement 2.



**Figure 5: Astrocyte signature inferred from the *GJA1/Cx43* co-expression module in spinal cord periplaques.** To identify an astrocyte molecular signature in spinal cord periplaques, we used the astrocyte-specific hub gene *Cx43/GJA1* as a "bait" and retrieved the top-200 mRNA species that most closely co-upregulated with *Cx43/GJA1*. The co-expression network formed by functionally-relevant genes is shown. Genes are designated by their gene symbols. Gene full names are provided in Table 2.

**Table 2:** List of functionally-relevant genes that co-express with *GJA1/Cx43* in spinal cord periplaque areas

**Transcription factors**

AR	Androgen receptor
NR3C2	Nuclear receptor subfamily 3 group C member 2 (mineralocorticoid receptor )
SMAD1	SMAD family member 1

SMAD2	SMAD family member 2
SOX2	SRY (sex determining region Y)-box 2
SOX6	SRY (sex determining region Y)-box 6
SOX9	SRY (sex determining region Y)-box 9
SOX11	SRY (sex determining region Y)-box 11
ZNF45	Zinc finger protein 175
ZNF853	Zinc finger protein 853
GLIS2	GLIS family zinc finger 2
GLIS3	GLIS family zinc finger 3
NFIA	Nuclear factor I/A
NFIB	Nuclear factor I/B
ID4	Inhibitor of DNA binding 4, dominant negative helix-loop-helix protein
MTA3	Metastasis associated 1 family, member 3

### Cytokines

IL-33	Interleukin 33
IL-17D	Interleukin 17D

### ECM-related genes

SPARCL1	SPARC-like 1 (hevin)
SDC2	Syndecan 2
PLOD2	Procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2
GPC4	Glypican 4
SULF1	Sulfatase 1
SPOCK2	Sparc/osteonectin, cwcv and kazal-like domains proteoglycan (testican) 2

### Others

GFAP	Glial fibrillary acidic protein
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CD44	CD44 molecule (Indian blood group)
CD200	CD200 molecule
FGF2	Fibroblast growth factor 2 (basic)

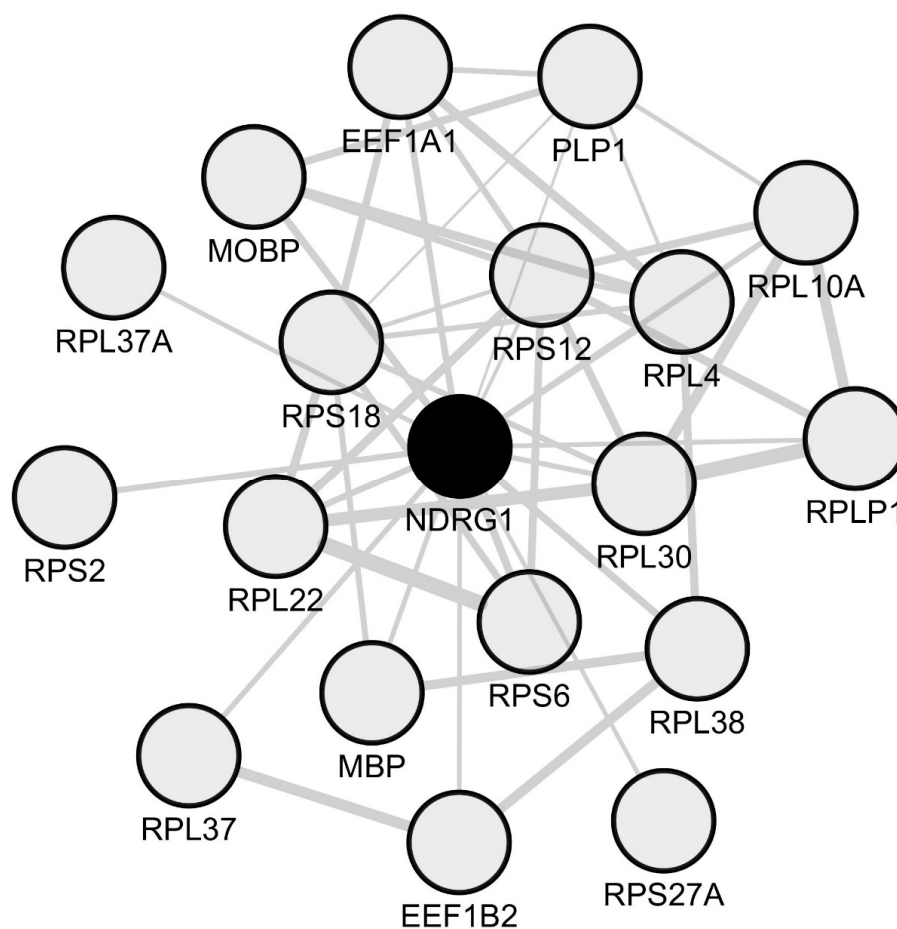
**Legend:** A co-expression analysis was performed in which the astrocyte-specific gene *CX43/GJA1* was used as a "bait" to identify a putative molecular signature of reactive astrocytes in periplaque areas of MS spinal cords. The top-200 genes that more closely co-expressed with *CX43/GJA1* were identified. Listed are genes considered as functionally-relevant in the context of astrogliosis and inflammation.

Besides *GFAP*, such a genomic network comprised 3 groups of genes that we found of particular relevance in the context of astrogliosis, inflammation and tissue remodeling: i) transcription factors (TFs), ii) cytokines and iii) genes coding for extra-cellular matrix (ECM) molecules (Figure 3 and Table 2). Interestingly, the TFs that co-expressed with *Cx43/GJA1* comprised *SMAD1* and *SMAD2*, two major signaling components of the TGF-beta and Bone Morphogenetic Proteins (BMPs) pro-gliotic pathways [22–25]. Moreover, TFs that co-expressed with *Cx43/GJA1* also comprised *SOX2* a stem cell-related gene expressed by proliferating astrocytes [26,27] and *NFIA*, a positive regulator of *GFAP* transcription [28]. On the other hand, the androgen receptor (*AR*), which was also found to co-express with *Cx43/GJA1*, was previously shown to inhibit reactive astrogliosis under varied CNS conditions [29–34]. Of note, *AR* co-expressed also with *Cx43/GJA1* when excluding the 2 male patients from our analysis (data supplement 3). To establish a potential link between *Cx43/GJA1* co-expressed TFs and other genes of the *CX43/GJA1* module, we performed an enrichment analysis using ChEA2016, a Chip-Seq and Chip-Chip database and webtool [35]. We found that the *GJA1/Cx43* module was significantly enriched (adjusted p-value < 0.01) in previously identified gene targets of *AR* or *SOX2* in human cells (data supplement 4). However, the *GJA1/Cx43* module was not enriched in known functional pathways as assessed with the Reactome 2016 database and webtool [36]. Surprisingly, we found that only 2 cytokines, namely *IL-17D* and *IL33*, co-upregulated with *Cx43/GJA1* in spinal cord periplaque areas. Other inflammation-related genes comprised notably *CD44* and *CD200* that were both previously demonstrated on reactive astrocytes [37,38]. Finally, the *GJA1/Cx43* module comprised several ECM-related genes that are expressed by reactive astrocytes: *GPC4* (Allen NJ et al. Nature 2012), *SULF1* (Higginson JR et al J Neurosci 2012) and *SPARCL1* (Lively S et al. J Neuropathol Exp Neurol 2011).

2.4. A unique set of genes co-express with *NDRG1* in periplaque areas of MS spinal cords

To unravel a molecular signature of oligodendrocytes in periplaque areas of MS spinal cords, we used the oligodendrocyte-related hub gene *NDRG1* as a "bait" and retrieved the top-200 mRNA species that more closely co-expressed with *NDRG1*. The full list of *NDRG1*

co-expressed genes is provided in data supplement 5. As expected, such a module comprised genes coding for myelin proteins, namely *MBP*, *MOBP* and *PLP1*. Similarly to the *Cx43/GJA1* module, the *NDRG1* module also comprised several TFs. However, the network of *NDRG1* co-expressed genes was not enriched in known targets of these TFs, as assessed with the ChEA2016 database and webtool. A survey of the "TargetScan microRNA" [39] database showed that the *NDRG1* co-expression module was not significantly enriched in known targets of miRNAs. However, pathway analysis with the webtool "Reactome 2016" showed a highly significant enrichment in genes involved in the functional pathway "Eukaryotic Translation Elongation" (Reactome 2016, adjusted p-value=1.37E-9)(Figure 6 and Table 3).



**Figure 6: Oligodendrocyte signature inferred from the *NDRG1* co-expression module in spinal cord periplaques.** To identify an oligodendrocyte molecular signature in spinal cord periplaques, we used the oligodendrocyte-specific hub gene *NDRG1* as a "bait" and retrieved the top-200 mRNA species that most closely co-downregulated with *NDRG1*. The co-expression network formed by functionally-relevant genes is shown. Genes are designated by their gene symbols. Gene full names are provided in Table 3.

**Table 3:** List of functionally-relevant genes that co-express with *NDRG1* in spinal cord periplaque areas

**Myelin-related genes**

MBP	Myelin basic protein
MOBP	Myelin-associated oligodendrocyte basic protein
PLP1	Proteolipid protein 1

**Genes involved in the « Eukaryotic Translation Elongation » pathway (Reactome 2016, p = 1.37E-09)**

EEF1A1	Eukaryotic translation elongation factor 1 alpha 1
EEF1B2	Eukaryotic translation elongation factor 1 beta 2
RPLP1	Ribosomal protein, large, P1
RPL4	Ribosomal protein L4
RPL10A	Ribosomal protein L10a
RPL22	Ribosomal protein L22
RPL30	Ribosomal protein L30
RPL37	Ribosomal protein L37
RPL37A	Ribosomal protein L37a
RPL38	Ribosomal protein L38
RPS2	Ribosomal protein S2
RPS6	Ribosomal protein S6
RPS12	Ribosomal protein S12
RPS18	Ribosomal protein S18
RPS27A	Ribosomal protein S27A

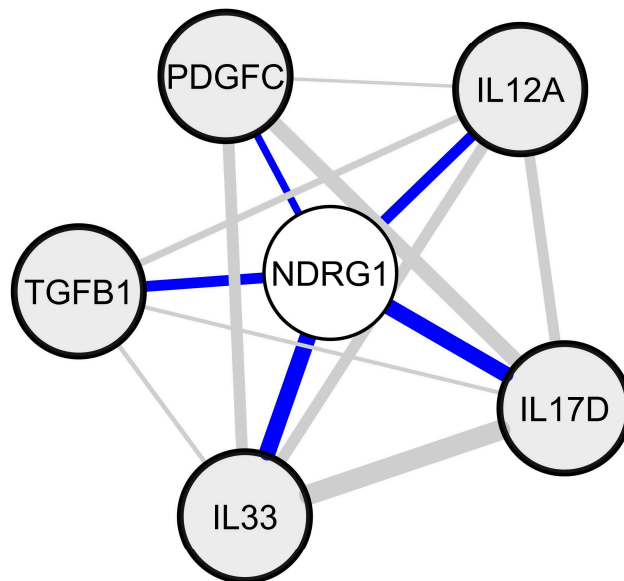
**Legend:** A co-expression analysis was performed in which the oligodendrocyte-specific gene *NDRG1* was used as a "bait" to identify a putative molecular signature of oligodendrocytes in spinal cord periplaques. The top-200 genes that more closely co-express with *NDRG1* in spinal cord periplaque areas were identified. Listed are genes considered as functionally-relevant in the context of myelin loss.

This finding appears of particular interest since, in oligodendrocytes, the translation of *MBP* and *MOBP* was previously shown to rely on specific mechanisms requiring a transport of the protein translation machinery (including ribosomal proteins) along the cell processes of oligodendrocytes [40–45]. To explore the existence of potential links between *NDRG1* and the processes of translation/elongation, we performed a meta-analysis of *NDRG1* protein interactants. While the STRING database allowed to recover only 20 direct protein interactants with no significant enrichment relating with translation/elongation, the Wiki-Pi database, gathering data from high throughput technologies, listed 64 *NDRG1* interactants (data supplement 6) that showed a highly significant enrichment in the "Translation" pathway (Reactome 2016, adjusted P-value = 4.80E-11). Similar results were obtained when assessing *NDRG1* interactants in the protein interactome database TissueNet2 [46] (data not shown). This result points to a potential link between *NDRG1* down-regulation in oligodendrocytes and a subsequent altered translation/elongation of myelin genes leading to partial demyelination.

#### *2.5. Identification of candidate soluble factors that may trigger NDRG1 silencing in periplaque areas of MS spinal cords*

While a previous study showed that *NDRG1* is specifically silenced in the NAWM of MS patients [19–21], we found that *NDRG1* was constantly downregulated in spinal cord periplaques as compared to adjacent NAWM. We thus sought to identify candidate soluble factors (in particular immune molecules) that would be potentially involved in a repression of *NDRG1* expression in periplaque areas of MS spinal cords. To this aim, we first established a list of 193 candidate genes coding for cytokines, chemokines or growth factors. We then used the GeneMANIA software to identify the top-5 candidate genes whose mRNA levels harbored the more closely inverse correlation with *NDRG1* mRNA levels in our whole set of data. This top-5 genes was established as follows: *IL17D*, *IL33*, *IL12A*, *PDGF-C* and *TGFB1* (Figure 7).

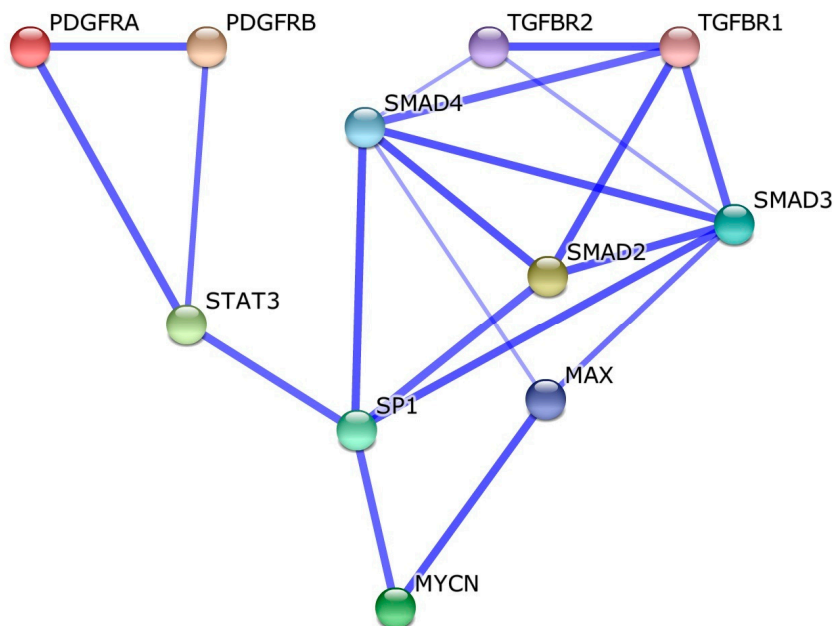




**Figure 7: Identification of candidate soluble factors that putatively repress *NDRG1* expression.** Starting from a list of 193 candidate genes coding for cytokines, chemokines or growth factors, we used the GeneMANIA software to identify mRNA species whose levels inversely correlated with those of *NDRG1* in spinal cord periplaques. The top 5 genes whose expression levels inversely correlated with *NDRG1* were: *IL17D*, *IL33*, *IL12A*, *PDGF-C* and *TGFB1*.

## 2.6. Identification of a molecular pathway linking MYCN to PDGFC and TGFB1

*NDRG1* was originally identified as a major target gene repressed by the transcription factor MYCN (also named *N-Myc*) in neuroblastoma cells [47,48]. Since then, in multiple cell types, MYCN was shown to silence *NDRG1* gene expression via a process involving a hypermethylation of the *NDRG1* promoter [47–52]. On this basis, we performed a meta-analysis of the human interactome to determine whether signaling pathways could link MYCN to the identified candidate molecules (i.e. *IL17D*, *IL33*, *IL12A*, *PDGF-C* and *TGFB1*). We found that PDGFRA (a PDGFC receptor) and TGFBR1 (a TGFB1 receptor) are second shell interactants of MAX (Myc associated factor X) and SP1 (Sp1 transcription factor) (Figure 8), two TFs that are recruited by MYCN during the transcriptional repression of specific target genes [49].



**Figure 8: Identification of molecular pathways linking PDGFC and TGFB1 to MYCN.** A meta-analysis of the human interactome was performed to determine whether signaling pathways could link MYCN (a known repressor of NDRG1 expression) to the identified candidate IL17D, IL33, IL12A, PDGF-C and TGFB1. Data show that PDGFRA (a PDGFC receptor) and TGFB1 (a TGFB1 receptor) are second shell interactants of MAX (Myc associated factor X) and SP1 (Sp1 transcription factor), two transcription factors that are recruited by MYCN during the transcriptional repression of specific target genes.

### 3. Discussion

In this paper, gene co-expression analyses were performed in order to infer astrocytes vs oligodendrocytes molecular signatures in spinal cord periplaques of MS patients. Regarding astrocytes, genes that were found to co-upregulate with *GJA1/Cx43* in periplaques formed a pro-gliotic signature which composition is compatible with a combined TGFB/SAMD1/SMAD2- and SOX2-driven program. While TGFB and SOX2 were both previously identified as astrocytosis-promoting factors [22–25], TGFB was further shown to induce an astrocyte-specific developmental program that, in turn, prevents effective remyelination in MS plaques and periplaques [53]. Superimposing to the TGFB/SMAD1/SMAD2 and SOX2 progliotic signatures, we also found that the *GJA1/Cx43* module was characterized by a highly significant enrichment in genes that are regulated by the AR. Interestingly, even when excluding males from our study, the AR was still co-expressed with *GJA1/Cx43*. As androgens are known to prevent astrocytosis [29–34], our results raise the possibility that in males and females suffering from progressive forms of MS, activation of the AR is somehow defective and fails to downregulate a large set of progliotic genes induced by the TGFB/SMAD1/SMAD2 pathway. This could be due to an

age-related decrease of circulating testosterone or of the circulating androgen precursors (Dehydroepiandrosterone and Androstendiol) that can be intracellularly metabolized into testosterone [54–57]. Another explanation, not exclusive from the former one, could be that the AR signaling pathway might be hampered or disrupted in periplaque astrocytes despite a normal level of circulating or intracrine testosterone. Supporting this view, a meta-analysis of the human AR interactome shows that AR interactants are significantly enriched in SMAD-binding proteins (data not shown). Moreover, mechanisms of mutual exclusion between AR and TGFB signaling pathways were previously demonstrated in different cell types [58–63].

With regard to inflammation, we found that in spinal cord periplaques, the astrocyte signature formed by *GJA1/Cx43* gene module comprised only two cytokines, namely *IL17D* and *IL33*. *IL17D* is a pro-inflammatory cytokine that binds to a yet unknown receptor and directs *in vivo* the intra-tissular recruitment of NK T-cells [64,65]. Of note, *IL17D*, as all the members of the *IL17* family, is also a potent inducer of TGFB secretion in many cell types [66,67]. Interestingly also, the astrocytic expression of *IL-33* was recently demonstrated in MS plaques and *IL33* was shown to inhibit the *de novo* myelination of rat axons *in vitro* [68]. However, other works reported that *IL-33* promotes remyelination [69] and exerts potent anti-inflammatory and neuroprotective functions [70]. Finally, besides TFs and immune genes, we observed that *GJA1/Cx43* module included several key extra-cellular matrix (ECM)-related genes involved in tissue remodeling. Among these, Procollagen Lysyl Hydroxylase 2 (*PLOD2*) is a TGFB-induced enzyme [71–73] that promotes fibrosis via the crosslinking of collagen molecules [74–76].

The second aim of our work was to identify a molecular signature of oligodendrocytes in the spinal cord periplaques of MS patients. Among the network of genes that were constantly down-regulated in periplaques, *NDRG1* was identified as the only oligodendrocyte-specific hub gene. Such a finding has to be interpreted in light of a recent work demonstrating that *NDRG1* is also the only oligodendrocyte-specific gene whose silencing was demonstrated in the NAWM of MS patients. These data, while further supporting the role of *NDRG1* as a master regulator of oligodendrocyte differentiation and myelin maintenance [19–21], suggest also that *NDRG1* may be a major target of a yet uncharacterized process leading to diffuse myelin alterations in periplaques. When using *NDRG1* as a bait gene to identify a large co-expression module in oligodendrocytes, we found that only 3 genes coding for myelin proteins co-downregulated with *NDRG1* in periplaques: *MBP*, *MOBP* and *PLP1*. Interestingly, as opposed to the *GJA1/Cx43* module, no specific enrichment in known targets of co-expressed TF could be evidenced in the *NDRG1* module. Moreover, we were neither able to identify enrichment in apoptosis-related pathways nor in miRNA targets, which would have provided molecular schemes explaining oligodendrocyte cell loss or altered myelin gene expression. In contrast, the *NDRG1* co-expression module was highly significantly enriched in genes involved in translation/elongation of mRNAs. Although, at this stage, one may only extrapolate on the significance of such an enrichment, it is important to underscore that the translation of both *MBP* and *MOBP* mRNAs in oligodendrocytes rely on a unique mechanism during which various components of the protein translation machinery

(including ribosomal proteins) are transported along the cell processes of oligodendrocytes [40–45]. Strikingly, when performing a meta-analysis of human *NDRG1* protein interactants, we found a highly significant enrichment in proteins involved in the "Translation" pathway (Reactome 2016, adjusted p-value =  $1.80 \times 10^{-11}$ ) (data supplement 6). On this basis, one may thus propose that *NDRG1* silencing in oligodendrocytes may hamper myelination via the coordinated downregulation of a specific subset of ribosomal genes supporting the translation/elongation of *MBP* and *MOBP* mRNAs.

Finally, we attempted to identify candidate soluble molecules that could be responsible for *NDRG1* down-regulation in spinal cord periplaques. Our analysis pointed to a network of 5 genes whose expression levels inversely correlated with those of *NDRG1* with the highest levels of confidence: i) *IL-17D* and *IL-33*, the two cytokines found to co-upregulate with *GJA1/Cx43* in periplaques, ii) *IL12-A*, a potent inducer of TH1 (T-Helper 1) polarization [77], iii) *PDGFC* (Platelet-derived growth factor C), a profibrotic gene [78,79] and a regulator of the neurovascular unit [80] and iv) *TGFB1* (Transforming growth factor-1). As a matter of fact, these correlations do not allow establishing a functional causative link between an up-regulation of these cytokines and the down-regulation of *NDRG1* expression. Again, we used bioinformatics tools to explore such putative links and performed a meta-analysis of the protein interactants potentially linking the identified candidate cytokines with N-Myc. Indeed, a well identified mechanism of *NDRG1* repression relies on the binding of N-Myc to the promoter region of *NDRG1* and the subsequent recruitment of histone deacetylases leading to promoter hypermethylation and an epigenetic silencing of *NDRG1* [47–52]. Our meta-analysis showed that only *TGFBR1* (an oligodendrocyte-expressed receptor to *TGFB1*) and *PDGRA* (an oligodendrocyte-expressed receptor to *PDGFC*) formed a close (second shell) protein network with N-Myc. Overall, since the *NDRG1* promoter was shown to be hypermethylated in the NAWM of MS patients [20], our results indicate that *TGFB1* and/or *PDGFC* released in MS periplaques could be responsible for a silencing of *NDRG1* in periplaques and, to a lesser extent, in the NAWM.

Overall, our work points to a major role of TGF-beta1 in the development of astrocytosis and diffuse myelin alterations in the spinal cord periplaques of MS patients. In particular, we propose a new pathophysiological scheme where TGF-beta1 would be central to a self-perpetuated process during which: i) IL17-D, synthesized by reactive astrocytes, triggers the production of *TGFB1* and ii) astrocyte-derived *TGFB1* fuels chronic gliosis and hamper myelination via a transcriptional repression of *NDRG1*. While further studies are needed to test this hypothesis, it is important to notice that the monoclonal antibody Fresolimumab directed against all *TGFB* isoforms is available for clinical trials [81], cross the blood brain barrier [82] and is currently under evaluation in patients suffering from systemic sclerosis [83].

## 4. Materials and Methods

### 4.1. Transcriptomic data

Co-expression networks were generated from a previously published set of transcriptomic data that our group obtained by whole genome analyses of paired NAWM and periplaque samples derived from 8 SPMS patients [8]. Data are available at the public gene array repository bank GEO DataSets (Ref: GSE52139). To ensure the highest level of confidence to co-expression analyses, we first submitted transcriptomic data to a stringent quality control using the arrayQualityMetrics Bioconductor package (v3.28.2) [84] in R (v3.3.0). We removed from further analyses the transcriptomic profile obtained from 1 out of 16 samples as it was detected as an outlier by both boxplots and MA plots criteria.

#### *4.2. Generation of a global co-expression network.*

An expression file was generated that comprised an "OBJECT" column in which gene symbols were provided for each row and 15 "SAMPLE" columns compiling normalized data values for each sample and each transcript. This expression file was then imported in the GeneMANIA Cytoscape plugin (version 3.3.4 data version 2014-08-02-core) [85,86] allowing the generation of a co-expression network that, based on the Pearson correlation test, gathered 735 240 interactions.

#### *4.3. Connectivity analysis of gene co-expression networks*

Starting from the global co-expression network generated from periplaque and NAWM samples, we investigated the correlation links that could be established between genes that were previously found to be differentially regulated in paired comparisons between periplaques and NAWM samples. Using the GeneMANIA plugin in Cytoscape software, 2 sets of genes were entered separately as independent queries: i) the 34 genes found to be constantly upregulated in periplaques [8] and ii) the 57 genes found to be constantly downregulated in periplaques [8]. For each analysis, the number of additional connected genes to be identified was set to zero in order to limit the search for co-expression links to query genes only. Then, to identify hub genes among these modules, we used the "Network analysis" tool in Cytoscape software. A hub gene was defined as a gene (node) being connected to at least half of the total number of genes (nodes) forming the network.

#### *4.4. Identification and analysis of the Cx43/GJA1 co-upregulated gene network*

Starting from the global expression network loaded on Cytoscape/GeneMANIA, *CX43/GJA1* (*GJA1*) was entered as a query gene, and the number of connected genes to be identified was set to 200. The generated list was then assessed with regard to the presence of genes considered as functionally relevant in the context of astrogliosis, inflammation and tissue remodeling. These notably included genes coding for: i) cytokines, ii) extracellular matrix (ECM) or iii) transcription factors (TF). Identification of functionally-relevant genes was performed by a survey of the literature, on the basis of GO terms annotation, and using the TF database "AnimalTFDB" [87]. Finally, the 200 genes

forming the *Cx43/GJA1* co-expression network were also analyzed with regard to their enrichment in: i) genes involved in specific pathways, ii) genes identified as known targets of human transcription factors (TF), iii) genes identified as known targets of miRNA. These enrichment analyses were performed using the EnrichR webtools [88]: "ChEA2016" [35] (allowing to survey a total of 622 ChIP-ChIP or ChIP-seq sets of data that were manually curated), "TargetScan microRNA" [39] and "Reactome 2016" [36] .

#### 4.5. Identification and analysis of the *NDRG1* co-downregulated gene network

Starting from the global expression network loaded on Cytoscape/GeneMANIA, *NDRG1* was entered as a query gene, and the number of connected genes to be identified was set to 200. The generated list was then assessed with regard to the presence of genes coding for myelin or oligodendrocyte-related molecules as determined by GO terms annotation and a survey of the literature. The complete list of genes forming the *NDRG1* co-expression network was also analyzed with regard to its enrichment in: i) genes involved in specific pathways, ii) genes identified as known targets of human transcription factors (TF), iii) genes identified as known targets of miRNA. These enrichments analysis were performed using the open source website EnrichR [88] and the same webtools as described above.

#### 4.6. Identification of a network of *NDRG1* inversely correlated genes coding for cytokines/chemokines or growth factors

As GeneMANIA recognizes only positive correlation links, its use for the identification of mRNA species that inversely correlate with a given query gene requires the prior replacement of the corresponding mRNA values by opposite values. To identify a network of cytokines, chemokines or growth factors that inversely correlated with *NDRG1*, we first established a list of 193 candidate genes (data supplement 1) that included notably 38 interleukins, 17 interferons, 36 chemokines, 35 growth factors, 22 metalloproteases, 6 angiogenic factors and 5 granzymes. GeneMANIA was then loaded with an expression file that comprised: i) the original values obtained for each of the probes corresponding to the above-described list of cytokines/chemokines or growth factors, ii) the values opposite to the original ones obtained with the *NDRG1* probe. Again, the number of additional connected genes to be identified was set to zero. *NDRG1* was entered as a query gene, and the number of connected genes to be identified was set to 10. As GeneMANIA was conceived to process large sets of data, its algorithms include a sparsification process during which only the 50 strongest correlation links for each object are retained [85]. Therefore, for expression files comprising a low number of objects, correlation links being ranked in the top 50 might still not reach significance. To circumvent this possible drawback, for each of the 10 genes that were initially identified as inversely correlated with *NDRG1* with the GeneMANIA software, the statistical significance of the inverse correlation was checked using the Pearson correlation test. Only genes for which p values were  $\leq 0.01$  were taken into account.



#### 4.7. Meta-analysis of MYCN protein interactants

When needed, the open source websites "STRING" [89] and Wiki-Pi [90] were used to perform meta-analyses of protein-protein interactions. STRING allows protein interaction networks to be generated via a survey of distinct databases that list known protein interactions from published and manually-curated biochemical studies [89]. Here, we used STRING to identify interactants that would link MYCN (also named N-Myc) to the receptors IL12RB1, IL12RB2, IL1RL1 (IL33 receptor), PDGFRA (PDGFC receptor), PDGFRB (PDGFC receptor), TGFBR1, TGFBR2 and TGFBR3. Several round of analyses were performed in which two query proteins were entered for each analysis: MYCN and one of the above-mentioned receptors. The number of first-shell protein interactants to be identified was set to 500 and only high confidence interactions (score  $\geq 0.7$  according to the classification provided by the STRING website) retrieved from published and manually-curated biochemical studies were taken into account. Selected interaction networks were then loaded in Cytoscape in order to facilitate the visualization of identified pathways. In another set of analyses, we used the Wiki-Pi database to retrieve NDRG1 protein interactants. Wiki-Pi is a resource that is not manually-curated but allows a large survey of both Biogrid [91] and HPRD [92] databases obtained by high throughput technologies. Wiki-Pi compiles 48419 unique binary biophysical interactions among 10492 human proteins.

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#### Abbreviations

MS	multiple sclerosis
RRMS	Relapsing-remitting multiple sclerosis
SPMS	secondary progressive multiple sclerosis
PPMS	primary progressive multiple sclerosis
NAWM	normal-appearing white matter



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