

***JAB1* deletion in oligodendrocytes causes senescence-induced inflammation and neurodegeneration in mice**

Cristina Rivellini¹, Emanuela Porrello¹, Giorgia Dina¹, Simona Mrakic-Sposta², Alessandra Vezzoli², Marco Bacigaluppi¹, Giorgia Serena Gullotta¹, Linda Chaabane^{1,3}, Letizia Leocani^{1,4}, Silvia Marenni¹, Emanuela Colombo¹, Cinthia Farina¹, Jia Newcombe⁵, Klaus-Armin Nave⁶, Ruggero Pardi⁷, Angelo Quattrini¹, Stefano Carlo Previtali^{1*}

¹Institute of Experimental Neurology (INSPE), Division of Neuroscience; IRCCS San Raffaele Scientific Institute, 20132 Milan, Italy.

²Institute of Clinical Physiology National Research Council, ICF-CNR, Milan, Italy.

³Experimental Imaging Center (CIS), IRCCS San Raffaele Scientific Institute, 20132 Milan, Italy.

⁴University Vita-Salute San Raffaele, Milan, Italy.

⁵NeuroResource, Department of Neuroinflammation, UCL Queen Square Institute of Neurology, London, England.

⁶Department of Neurogenetics, Max Planck Institute of Experimental Medicine, Göttingen, Germany.

⁷Division of Immunology, Transplantation, and Infectious Disease; IRCCS San Raffaele Scientific Institute, 20132 Milan, Italy.

***Correspondence:** Stefano C. Previtali, InSpe and Division of Neuroscience, Ospedale San Raffaele, Via Olgettina 60, 20132 Milano. previtali.stefano@hsr.it

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Abstract

Oligodendrocytes are the primary target of demyelinating disorders and progressive neurodegenerative changes may evolve in the CNS. DNA damage and oxidative stress are considered key pathogenic events, but the underlying molecular mechanisms remain unclear. Moreover, animal models do not fully recapitulate human diseases, complicating the path to effective treatments. Here we report that mice with cell autonomous deletion of the nuclear COP9 signalosome component *CSN5* (JAB1) in oligodendrocytes develop DNA damage and defective DNA repair in myelinating glial cells. Interestingly, oligodendrocytes lacking JAB1 expression underwent a senescence-like phenotype that fostered chronic inflammation and oxidative stress. These mutants developed progressive CNS demyelination, microglia inflammation and neurodegeneration, with severe motor deficits and premature death. Notably, blocking microglia inflammation did not prevent neurodegeneration, whereas the deletion of p21^{CIP1} but not p16^{INK4a} pathway ameliorated the disease. We suggest that senescence is key to sustaining neurodegeneration in demyelinating disorders and may be considered a potential therapeutic target.

Introduction

Myelin is a highly specialized protein and lipid-rich membrane that wraps around axons in the central (CNS) and peripheral nervous system (PNS). It is made by oligodendrocytes in the CNS and Schwann cells in the PNS. The main function of myelin is to enable energy-efficient saltatory conduction (1), though recent studies have highlighted its critical importance in neuronal survival and in providing trophic support for axon integrity (2). Accordingly, white matter disorders in which mature or myelinating oligodendrocytes are damaged can evolve into devastating neurodegenerative disorders, such as multiple sclerosis (MS) and leukodystrophies (3, 4). Thus, a better understanding of the processes regulating oligodendrocyte myelination, maintenance, and axon-glia interaction is crucial to unravel mechanisms underlying neurodegeneration and to develop potential treatments.

Oligodendrocytes are the primary target of white matter disorders, but it is still unclear how this damage is related to axonal loss. Although demyelination is considered the primary cause for axonal degeneration, axonal damage may even precede or parallel demyelination (5, 6). Indeed, demyelination *per se* not necessarily results in axonal damage (7). Oligodendrocytes are particularly sensitive to DNA damage and oxidative stress, events that have been associated with progressive axonal loss and are considered to be key elements in MS pathology and many neurodegenerative disorders (8–11). Despite their critical role, it is still unclear how DNA damage and oxidative stress are molecularly linked and can foster progressive axonal loss.

JAB1 (Jun activation domain-binding protein 1, or CSN5) is a multifunctional nuclear protein involved in controlling several aspects of the cell function through gene transcription and protein degradation (12). Of note, along with the control of cell-cycle progression (12, 13), JAB1 regulates DNA damage and repair (14, 15). JAB1 is the catalytic subunit of the COP9 (constitutive photomorphogenesis 9) signalosome (CSN), a modifier of the cullin-RING ubiquitin ligase (CRL) complexes. The main CSN function is the enzymatic removal of NEDD8 from the cullin component of the ligase, a requirement for proper functioning of CRL complexes. CRL-controlled substrates (e.g MYC,

p27^{kip1}, β -catenin, cyclin E) are key regulators of cell cycle progression and DNA repair (16).

We previously reported that in the PNS, JAB1 autonomously controls Schwann cell-axon interaction and its loss results in demyelination and axonal degeneration of peripheral nerves (17). Since oligodendrocytes are sensitive to DNA damage, oxidative stress and perturbation of cell cycle progression, we now investigated whether JAB1 in the oligodendrocyte lineage might control CNS myelination and maintenance. Here we show that oligodendrocyte conditional deletion of *Jab1* - with two different transgenes - causes progressive CNS demyelination, inflammation and axonal degeneration. Rescue of the inflammatory response did not prevent neurodegeneration. The molecular mechanism was shown to reside in the activation of the senescence pathway by DNA damage in mature oligodendrocytes.

Results

JAB1 expressed in oligodendrocytes is required for oligodendrocyte growth and survival

To define the role of JAB1 in myelin formation, we generated mutant mice specifically lacking its expression in oligodendrocytes. Thus, we crossed *Jab1*^{fl/fl} mice with *Cnp1-Cre* transgene mice (Figure 1, A and B) (18). The latter is active in the late phase of oligodendrocyte precursor (OPCs) development and in differentiated oligodendrocytes, and ensures efficient recombination (19, 20). O1⁺ FACS-sorted oligodendrocytes from the mutant mouse brains showed significant reduction of *Jab1* mRNA (Figure 1C, and Supplemental Figure 1A for gating strategy of FACS-sorting). In addition, JAB1 protein was significantly reduced in western blot analysis in mutant optic nerves (Figure 1D). Moreover, breeding of *Cnp-Cre:Jab1*^{fl/fl} with *YFP-Rosa26* mice revealed high rate of recombination: 58% in NG2⁺ OPCs and 80% in CC1⁺ oligodendrocytes (Figure 1E).

Cnp-Cre:Jab1^{fl/fl} mice appeared phenotypically normal until post-natal day (P) 20. After P40, mutant mice showed progressive motor impairment (tremor, unsteady gait), along with reduced size and weight as compared to controls

(Figure 1G). Remarkably, all *Jab1* mutant mice died within P150 and already at P70 we observed 50% mortality in the colony (Figure 1F).

We also measured motor performances (strength and coordination) by rotarod analysis. We observed normal function at P20, whereas significant impairment in *Cnp-Cre:Jab1^{fl/fl}* mice from P40 (Figure 1H).

***Jab1* mutant mice are characterized by progressive CNS demyelination and axonal loss**

In agreement with their phenotypic appearance, P20 *Cnp-Cre:Jab1^{fl/fl}* mice were slightly hypomyelinated in optic nerves, with morphologically normal myelin sheath and no alterations in the number of fibers (Figure 2, A and C and Supplemental Figure 2A). Accordingly, the amounts of myelin basic protein (MBP) and myelin associated glycoprotein (MAG) were comparable to controls in P20 optic nerves, corpus callosum and spinal cord (Supplemental Figure 2, B and C, and data not shown).

From P40 onwards, we observed progressive demyelination in optic nerve, corpus callosum and spinal cord of *Cnp-Cre:Jab1^{fl/fl}* mice (Figure 2A), as corroborated by increased *g*-ratio values (Figure 2C) and consistent reduction in myelin protein expression (Supplemental Figure 2B and C). At P60, almost all fibers in the CNS were devoid of myelin (Figure 2A). Notably, starting at P40, *Cnp-Cre:Jab1^{fl/fl}* mice also showed progressive neurodegeneration (Figure 2A), with signs of active axonal degeneration (Figure 2B) and significant loss of myelinated axons (Figure 2C).

To corroborate findings of axonal degeneration, we evaluated ganglion cells as major output cells of the retina to form the optic nerve. We stained the retina with anti BRN3A antibody to identify ganglion cells, and we quantified cell numbers at different time points. We did not observe significant differences at P20 and P40, whilst the number of ganglion cells was significantly reduced in *Cnp-Cre:Jab1^{fl/fl}* mice at P60 (Supplemental Figure 3, A and B). Moreover, we examined levels of non-phosphorylated neurofilaments as a marker of axonal degeneration in the brain, as previously reported (21,22). As depicted in Supplemental Figure 3 (C and D), we observed a significant increase in the amount of non-phosphorylated neurofilaments in brain homogenates of *Cnp-Cre:Jab1^{fl/fl}* as compared to Wt mice.

Finally, as *Cnp-Cre* transgene also recombines in Schwann cells, we evaluated peripheral nerves of mutant mice. As previously reported with *P0-Cre* transgene, which recombines specifically in Schwann cells (17), *Cnp-Cre:Jab1^{fl/fl}* mice also developed a dysmyelinating neuropathy with axonal sorting defects (Supplemental Figure 4A). As peripheral neuropathy may influence rotarod analysis, we compared P60 *Cnp-Cre:Jab1^{fl/fl}* with *P0-Cre:Jab1^{fl/fl}* mice, revealing that motor deficit was significantly worse in *Cnp-Cre:Jab1^{fl/fl}* mice as a consequence of CNS neurodegeneration (Supplemental Figure 4B).

Functional analyses in *Jab1* mutant mice

To further evaluate demyelination and neurodegeneration, we performed MRI using magnetization transfer (MT) contrast method to estimate myelination and diffusion tensor imaging (DTI) to measure the fractional anisotropy (FA) to evaluate axonal integrity. At P30, *Cnp-Cre:Jab1^{fl/fl}* mice were almost the same as controls (Figure 3, A-C). However, at P45 the corpus callosum of mutant mice showed significant hyperintense signals in MT sequences, which were even more pronounced at P65 (Figure 3, A and C). Similarly, on DTI the normal control corpus callosum was well defined, but we observed a significant reduction in FA (both mean and axial diffusivity) in mutant mice at P45 and P65 (Figure 3, B and C). These changes correlated with progressive demyelination and axonal loss in the corpus callosum of *Cnp-Cre:Jab1^{fl/fl}* mice.

Finally, we performed visual evoked potentials (VEPs) to functionally test optic nerve activity. While at P30, *Cnp-Cre:Jab1^{fl/fl}* mice showed detectable VEPs with significant increased latency and reduced amplitude (Figure 3, D and E), and at P45 and at P60 both latency and amplitude could not be detected in mutant mice (Figure 3, D and E), suggesting severe functional deficit.

CNS degeneration in *Cnp-Cre:Jab1^{fl/fl}* mice is associated with inflammation

Between P40 and P60 in addition to demyelination and neurodegeneration, we observed in *Cnp-Cre:Jab1^{fl/fl}* mice a diffuse microglial/macrophage inflammatory infiltrate, as revealed by anti-IBA1 staining (Figure 4A). To further characterize the inflammatory infiltrate, we FACS-sorted inflammatory cells

from brains. We confirmed that mutant mice had a significant increase both in microglia (CD45^{lo}CD11b⁺Ly6G⁻Ly6C⁻) and macrophages (CD45^{hi}CD11b⁺Ly6G⁻Ly6C⁻), which represented the majority of the inflammatory cells (Figure 4B and Supplemental Figure 1B). Interestingly, in *Cnp-Cre:Jab1^{fl/fl}* mice, microglia displayed high CD11c positivity, were smaller in size (Farnaud scatter, FSC-A MFI) and had higher granularity (SSC-A MFI), all hallmarks of activation (Figure 4B and Supplemental Figure 1C). Although significantly increased in number, lymphocytes and monocytes represented a smaller proportion of the inflammatory cells, whereas neutrophils did not differ from controls (Figure 4B and Supplemental Figure 1B). In addition, we also observed moderate astrogliosis, as glial fibrillary acidic protein (GFAP) staining was increased in optic nerves of *Jab1* mutant mice (Figure 4C).

Inhibition of monocyte recruitment or depletion of microglia is not sufficient to rescue neurodegeneration in *Cnp-Cre:Jab1^{fl/fl}* mice

To determine if the observed inflammatory infiltrate could contribute to demyelination and neurodegeneration and to discriminate the role of monocytes/macrophages versus microglia, we first crossed C-C chemokine receptor type 2 knock-out (*Ccr2^{-/-}*) with *Jab1* mutant mice. CCR2 is in fact required for the egress of monocytes from the bone marrow and has a prevalent role on monocyte/macrophage recruitment. Furthermore *Ccr2^{-/-}* mice are resistant to experimental autoimmune encephalitis (EAE) (23, 24). Indeed, P50 *Cnp-Cre:Jab1^{fl/fl}/Ccr2^{-/-}* mutants did not show differences to *Cnp-Cre:Jab1^{fl/fl}* mice in the numbers of inflammatory cells, myelinated fibers and degenerating fibers (Figure 5A).

Since inhibition of monocyte/macrophage recruitments in *Cnp-Cre:Jab1^{fl/fl}* brain did not seem responsible for the observed neurodegeneration, we next explored the role of microglia. To test this hypothesis, we depleted microglia in *Cnp-Cre:Jab1^{fl/fl}* mice by using PLX3397, a selective inhibitor of colony-stimulating factor 1 receptor (CSF1R) able to eradicate CNS microglia (25, 26). Although, PLX3397 treatment resulted in a significant reduction in IBA1⁺ microglia in both *Cnp-Cre:Jab1^{fl/fl}* and control brains, we did not observe any amelioration in the number of myelinated or degenerating axons in mutant mice (Figure 5B).

Based on these results, we posit that neurodegeneration in *Cnp-Cre:Jab1^{fl/fl}* mice is not due to an overt role of microglia and monocytes, since its inhibition does not ameliorate disease course.

***Jab1* mutant oligodendrocytes develop and differentiate normally**

We previously reported that in the PNS JAB1 controls p27^{KIP1} levels in Schwann cells and their cell cycle progression, differentiation and survival (17). Thus, we next determined whether the degeneration in *Cnp-Cre:Jab1^{fl/fl}* mice could be the result of reduced oligodendrocyte number, survival and/or differentiation.

We first counted the total number of cells in optic nerves from P20 to P90. We did not observe any difference until P60, when this was increased in *Jab1* mutant mice (Supplemental Figure 5A), most likely because of the inflammatory infiltrates. Next, we specifically assessed the number of OPCs (NG2 positive) and mature oligodendrocytes (CC1 positive) in optic nerves. While the number of mature oligodendrocytes did not change at any time point analyzed, OPCs were similar to control until P60 and then increased (Supplemental Figure 5B), suggesting that JAB1 deletion did not affect cell cycle progression and/or the proliferation/survival ratio.

To determine whether in the absence of JAB1, OPC/oligodendrocyte proliferation was affected, we performed BrdU incorporation assay and Ki67 staining in optic nerves. Whereas at P20, BrdU incorporation was comparable to controls, we observed an overall significant increase in all cells and in NG2⁺ OPCs in P60 optic nerves of mutant mice (Supplemental Figure 5C), as confirmed also by Ki67 staining (Supplemental Figure 5D). Next, we investigated whether JAB1 ablation might lead to altered survival in the oligodendrocyte lineage. Caspase-3 staining in P40 and P60 optic nerves did not show any significant difference between mutants and controls while at P90 we observed a significant number of apoptotic oligodendrocytes in mutant nerves (Supplemental Figure 5E).

Finally, we investigated whether JAB1 might control OPCs terminal differentiation. We injected BrdU for 3 consecutive days in P20 mice that were then analyzed at P30 (Supplemental Figure 5F). Quantification of the number of double positive BrdU/CC1 oligodendrocytes at P30 was similar in *Jab1*

mutant mice and controls (Supplemental Figure 5F), suggesting that OPC differentiate normally into mature oligodendrocytes.

Collectively, these results suggest that unlike Schwann cells, JAB1 is not required for oligodendrocyte survival and differentiation. Accordingly, p27^{KIP1} levels did not change in optic nerves of *Jab1* mutants (Supplemental Figure 6).

***Jab1* mutant oligodendrocytes activate senescence-like program**

Since JAB1 has a critical role in DNA integrity and DNA damage repair (14), we next determined whether these processes are hampered in oligodendrocytes lacking JAB1 expression. Thus, we performed immunohistochemical analyses with anti-phospho histone H2AX (pH2AX). While pH2AX in optic nerves of control mice was virtually absent at all analyzed time points, we observed pH2AX in a significant number of mutant oligodendrocytes, particularly at P40 and P60 (Figure 6A).

To further corroborate these results, we next investigated nonhomologous end-joining (NHEJ) mechanism of repair, which is operating in non-dividing mature oligodendrocytes (27). As under physiological conditions there was no DNA damage, and to examine differences between controls and *Jab1* mutant oligodendrocytes, we artificially induced DNA damage by X-ray irradiation. Optic nerves from control and *Cnp-Cre:Jab1^{fl/fl}* mice were cultured *in vitro* and X-ray irradiated (3 Gy) to induce massive DNA damage. The activation of DNA-PK, the primary nuclease complex involved in NHEJ, was significantly reduced in *Cnp-Cre:Jab1^{fl/fl}* oligodendrocytes (Figure 6B), though a similar number of mature oligodendrocytes (CC1⁺) showed DNA damage in both *Jab1* mutant and control optic nerves.

As a result of DNA damage, JAB1 regulates cell fate through DNA repair. In the absence of JAB1, the cell fate could vary depending on the extent of DNA damage and may result in apoptosis or in senescence (28). Since we did not observe apoptosis in *Jab1* null oligodendrocytes until late stage (P90; Supplemental Figure 5E), we next investigated whether in mature oligodendrocytes JAB1 might control senescence. We thus evaluated specific markers such as senescence associated β -galactosidase (SA- β -gal), p16^{INK4a} and p21^{CIP1}. As expected, we observed SA- β -gal staining in oligodendrocytes

already at P40 and significantly increased at P60 in optic nerve and corpus callosum of *Jab1* mutant mice, but not in controls (Figure 6C). Accordingly, we also detected significant increases of p16^{INK4a} and of p21^{CIP1} in P40-P60 optic nerves of *Jab1* mutant mice (Figure 6, D and E). Interestingly, p21^{CIP1} and p53 (p16^{INK4a} was not detectable; Supplemental Figure 4C) and did not change in the peripheral nerve of *Cnp-Cre:Jab1^{fl/fl}* mice. Also we could not detect SA- β -Gal staining in Schwann cells (Supplemental Figure 4D), confirming that the neuropathy due to the lack of JAB1 is caused by a different mechanism, and that lack of JAB1 is not necessarily a common mechanism to induce senescence in the CNS and PNS.

It has been reported that senescent cells release soluble inflammatory factors (senescence associated secretory phenotype, or SASP) and produce reactive oxygen species (ROS), which may amplify cell/tissue damage (28). Thus, we measured levels of SASP molecules in optic nerves of *Cnp-Cre:Jab1^{fl/fl}* mice and controls by quantitative PCR analyses. We observed significant increase in interleukin-1 β (IL1 β), tumor growth factor- β 1 (TGF β 1), chemokine (C-X-C motif) ligand 1 (CXCL1), and granulocyte-macrophage colony-stimulating factor (GM-CSF) mRNA expression specifically in mutant mice (Figure 6F). Accordingly, high mobility group box 1 (HMGB1), a further component of the secretory pathway of senescent cells and possibly involved in perpetrating senescence (29), was significantly increased in optic nerve homogenates from mutant mice (Figure 6G) and, as expected, mobilized in the cytoplasm of senescent oligodendrocytes, differently from the nuclear localization normally present in healthy cells (Figure 6G). Moreover, ROS levels were increased starting at P40 in *Jab1* mutant optic nerves and corpus callosum (Figure 6H), and at P90 in the spinal cord (data not shown), when measured by electron paramagnetic resonance (EPR) spectroscopy.

To confirm the above results and to demonstrate that SASP originated from mutant oligodendrocytes, we FACS-sorted O1+ oligodendrocytes from P40 brain of *Cnp-Cre:Jab1^{fl/fl}* and control mice and performed RNAseq and quantitative PCR analyses. We observed a significant number of genes that were down- (930) and up-regulated (1576; Supplemental Figure 7A and Supplemental Table 1). Interestingly, we revealed a significant up-regulation of

genes associated to senescence and SASP/inflammation in mutant oligodendrocytes (Supplemental Figure 7B, and Figure 8, G), as also confirmed by quantitative PCR analysis (Supplemental Figure 7C and Supplemental Figure 12B, C and D).

As a complementary result, to confirm that the elevation of SASP/inflammation and ROS in mutant mice was due to senescent oligodendrocytes and not by activated microglia, we measured these molecules in PLX-treated *Cnp-Cre:Jab1^{fl/fl}* mice, thus ablated of microglia. SASP-related genes and ROS remained significantly elevated in mutant mice treated with PLX as compared to Wt controls (treated and not treated; Supplemental Figure 8, A and B).

Collectively, loss of JAB1 induces a progressive DNA damage in mutant oligodendrocytes that undergo a senescence-like program. Further, senescent oligodendrocytes trigger inflammation and promote diffuse oxidative stress leading to neurodegeneration.

***Jab1* deletion in adult mice induces neurodegeneration, neuroinflammation and senescence**

To further confirm the role of JAB1 in the regulation of the senescence program we also deleted *Jab1* in mature oligodendrocytes using the inducible *Plp-CreErt2* transgene (30). *Plp-CreErt2:Jab1^{fl/fl}* mice were tamoxifen treated from P40 to P45. Breeding with *EYFP-Rosa26* mice, PCR and western blot analyses for JAB1 confirmed efficient recombination in optic nerves (Supplemental Figure 9, A-B and C). At 60 days post injection (dpi), tamoxifen-treated mice were phenotypically undistinguishable from controls in terms of behaviour, weight, number of IBA1⁺ cells, CNS morphology (optic nerve, corpus callosum and spinal cord) and myelination (Figure 7A, B and C and Supplemental Figure 9D). However, at 180 dpi, tamoxifen-treated mice displayed reduced size and weight (Figure 7A). Morphological analyses showed diffuse CNS demyelination, loss of myelinated fibers and axonal degeneration (Figure 7, B and C and Supplemental Figure 9D). Further, these mice also showed increase in IBA1⁺ microglia (Figure 7D). Similarly to *Cnp-Cre:Jab1^{fl/fl}* mice, tamoxifen-treated *Plp-CreErt2:Jab1^{fl/fl}* mice had increased number of oligodendrocytes (Supplemental Figure 9E) that expressed SA- β -gal (Figure 7E), and increased

expression of p21^{CIP1}, p16^{INK4a} and SASP (Figure 7F and Supplemental Figure 9F).

Genetic depletion of p21^{CIP1} but not p16^{INK4a} ameliorates neurodegeneration in *Jab1* mutants

To test the hypothesis that senescence, through p16^{INK4a} and/or p21^{CIP1} pathway, is responsible for neurodegeneration in *Jab1* mutant mice, we ablated p16^{INK4a} or p21^{CIP1} in *Cnp-Cre:Jab1^{fl/fl}* mice.

Adult *Cnp-Cre:Jab1^{fl/fl};p16^{INK4a}^{-/-}* did not show amelioration of demyelination and neurodegeneration of the CNS, as confirmed by quantification of myelinated fibers and g-ratio in the optic nerve (Supplemental Figure 10, A and B). Similarly, the inflammatory infiltrate did not change (Supplemental Figure 10C). Not surprisingly, double mutant oligodendrocytes (deleted for *Jab1* and p16^{INK4a}) showed senescence markers, including SA-β-gal activity and high p21^{CIP1} expression (Supplemental Figure 10, D and E), and SASP levels were unchanged in the CNS tissue as compared to *Cnp-Cre:Jab1^{fl/fl}* mice (Supplemental Figure 10F).

Conversely, deletion of p21^{CIP1} in *Cnp-Cre:Jab1^{fl/fl}* induced amelioration of the early phase of the phenotype and delay in neurodegeneration. Rotarod analysis showed better performances in *Cnp-Cre:Jab1^{fl/fl};p21^{CIP1}^{-/-}* as compared to *Cnp-Cre:Jab1^{fl/fl}* mice, significantly at P40 (Figure 8A) – a time point of active neurodegeneration in *Jab1* mutant mice (see also Figure 1H). Mouse body weight was similar between Wt and p21^{CIP1}^{-/-} mice, and between *Cnp-Cre:Jab1^{fl/fl}* and *Cnp-Cre:Jab1^{fl/fl};p21^{CIP1}^{-/-}* mice (Supplemental Figure 11A). When we analyzed optic nerves at P60, we confirmed amelioration of the histopathological findings, including significant increase of myelin thickness as sustained by g-ratio quantification (Figure 8B and Supplemental Figure 11, B and C). We then quantified the number of ganglion cells in the retina, this was significantly higher in *Cnp-Cre:Jab1^{fl/fl};p21^{CIP1}^{-/-}* as compared to *Cnp-Cre:Jab1^{fl/fl}* mice and not different to controls (Figure 8C). Accordingly, the number of myelinated fibers in the spinal cord was also significantly increased in double mutants, with significantly reduced numbers of degenerating axons (Figure 8D). Similarly, the levels of non-phosphorylated neurofilaments in the

brains of double mutant mice were rescued (Figure 8E and Supplemental Figure 11D). Conversely, the amount of microglia did not differ in double mutants as compared to *Cnp-Cre:Jab1^{fl/fl}* mice (Supplemental Figure 11E).

When we investigated senescent oligodendrocytes, we observed a significant reduction of SA- β -gal positivity in P60 optic nerve of *Cnp-Cre:Jab1^{fl/fl};p21^{CIP1}^{-/-}* as compared to *Cnp-Cre:Jab1^{fl/fl}* mice (Figure 8F). Consistently, we also observed a significant reduction of cytoplasmic HMGB1 expression in double mutant oligodendrocytes and reduced levels of SASP molecules (Supplemental Figure 11, F and H). The level of p16^{INK4a} (*Cdkn2a*) was unchanged in double mutants as compared to *Cnp-Cre:Jab1^{fl/fl}* mice (Supplemental Figure 11G), suggesting that it might partially compensate in the absence of p21^{CIP1}.

Finally, we performed RNA-seq analysis in FACS-sorted (O1⁺) oligodendrocytes to evaluate cell rescue of senescence and SASP/inflammatory markers. In parallel with findings in CNS tissue, we observed a rescue of senescence-associated and SASP/inflammatory molecules in *Cnp-Cre:Jab1^{fl/fl};p21^{CIP1}^{-/-}* as compared to *Cnp-Cre:Jab1^{fl/fl}* oligodendrocytes, which had a pattern more similar to controls (Figure 8G, Supplemental Table 2 and Supplemental Table 3). Also markers for oligodendrocytes maturation in double mutant oligodendrocytes were more similar to controls as compared to *Cnp-Cre:Jab1^{fl/fl}* ones (Supplemental Figure 12A). As expected, qPCR for p21^{CIP1}^{-/-} (*Cdkn1a*) was undetectable in *Cre:Jab1^{fl/fl};p21^{CIP1}^{-/-}* oligodendrocytes (Supplemental Figure 12B). qPCR for selected markers of senescence and SASP (included in the heatmaps) confirmed RNA-seq results (Supplemental Figure 12, C and D).

Overall, these above results suggest that interfering with the senescence pathway may ameliorate/delay neurodegeneration in *Jab1* mutant mice.

JAB1 expression in human brain

As pathological findings in our mutant mice resemble those described in MS pathology, to strengthen the clinical relevance of our results we investigated JAB1 expression in brain samples from MS patients and controls. Immunofluorescence analysis revealed JAB1 expression in all the

oligodendrocyte nuclei of normal control brain samples (Figure 9). Conversely, normal-appearing white matter surrounding chronic inactive MS lesions showed a significant reduction of oligodendrocytes which had JAB1 positive nuclei (Figure 9, A and B).

Discussion

Oligodendrocyte DNA damage and oxidative stress are among the main pathological mechanisms driving neurodegeneration in white matter disorders, such as progressive MS and leukodystrophies (8–11). However, the underlying molecular mechanisms have not been fully clarified, also because mouse models do not completely recapitulate human disorders and/or pathogenesis (31, 32). We now report that genetic deletion of JAB1 in oligodendrocyte is sufficient to cause CNS demyelination, inflammation and progressive neurodegeneration by inducing cell-autonomous DNA damage. Mutant oligodendrocytes undergo a senescence-like process that results in diffuse oxidative stress and pro-inflammatory cytokines release into the CNS. These events promote inflammation and neurodegeneration leading to mouse death in few months. Moreover, our data strongly indicate that suppression of microglia activation does not ameliorate neurodegeneration. We provide a proof-of-principle that interfering with the senescence pathway ameliorates/delays the disease.

The role of JAB1 in oligodendrocytes has been corroborated using two different transgenes, confirming the cell-intrinsic role of oligodendrocytes in the pathogenesis of CNS demyelination, inflammation and neurodegeneration. We posit a predominant role for post-mitotic oligodendrocytes in causing these effects. Indeed, although recombination with the *Cnp-Cre* transgene has been reported also in NG2⁺ OPCs (30), and JAB1 has been previously associated in controlling cell cycle progression and proliferation in Schwann cells (17), we did not observe any defect in OPC proliferation and differentiation. The different role of JAB1 in peripheral and central myelinating glia is additionally demonstrated by the fact that p27^{KIP1} levels did not change in oligodendrocytes, in contrast to Schwann cells (17), and conversely that Schwann cells did not elevate the senescence markers p16^{INK4a} and p21^{CIP1}. Moreover, unlike

Schwann cells that depend on JAB1 expression for survival (17), oligodendrocytes do not undergo apoptosis (at least until late stages and in low percentage). Indeed, oligodendrocytes survive despite extensive DNA damage, raising the possibility of their entry into a senescence-like program.

Cellular senescence is a programmed, transient phenomenon that normally contributes to tissue remodeling and homeostasis during development. In advanced age, senescent cells may accumulate following declining/disruption of the immune function, decreased stability of p53 (p21^{CIP1}) and accumulation of DNA damage, which however does not reach a threshold level to induce cell death (33). Cellular senescence has been well investigated in mitotic cells and is mediated by the activation of either one or both p53/p21^{CIP1} and p16^{INK4A}/pRB (retinoblastoma protein) tumor suppressor pathways (34). Both these pathways are complex, involve side branches, and are interlinked with extensive crosstalk. Activated p53 regulates the expression of many anti-proliferative genes, primarily through p21^{CIP1}. p21^{CIP1} arrests the cell cycle (by inhibition of various cyclin-dependent kinases, CDKs), facilitates DNA damage response, induces the secretion of SASP and promotes ROS elevation (35). p16^{INK4A} overexpression is also induced during senescence. p16^{INK4A} directly binds to CDK4/6, prevents the phosphorylation of pRB and promotes the expression of EF2-target genes, acting as cell cycle brake. p16^{INK4A} has also non-canonical (pRB-independent) roles by regulating mitochondria biogenesis, oxidative stress and inflammatory molecules (36). These chronic senescent cells promote or amplify cell/tissue damage through the release of inflammatory factors (SASP) and ROS (37). Based on the results of our study, we posit that *Jab1*^{-/-} senescent oligodendrocytes couple CNS demyelination to chronic inflammation and elevated ROS production, which eventually result in axonal damage and loss. Accordingly, demyelination per se does not necessarily induce axonal degeneration as reported in *shiverer* (*Mbp*^{-/-}) mutants, which are devoid of CNS myelin but present preserved axons (7), indicating that further mechanisms may trigger neurodegeneration.

It is intriguing to note that senescent progenitors have been identified in brains of progressive MS patients as well as in induced pluripotent stem-derived neural precursors cells obtained from the same patients (38), providing evidences that cellular senescence is an active process during

neurodegeneration. Of note, lack of a reliable model reproducing key clinical and pathological features of primary progressive MS is an absolute requirement to validate possible therapeutic treatments for these patients (31), who are characterized by activated microglia, robust chronic demyelination and autoimmune-independent neurodegeneration (31). These are all features that we observed in *Jab1* mutant mice. In this context, oligodendrocyte-conditional *Jab1*^{-/-} mice would promote cell-intrinsic DNA damage and defective DNA-damage repair, which may represent a possible common mechanism of oligodendrocyte-driven neurodegeneration in different white matter disorders, such as progressive MS and leukodystrophies. Accordingly, we observed reduced JAB1 expression in oligodendrocytes of brain samples from MS patients. Moreover, oligodendrocyte DNA damage, senescence, oxidative stress, and defective in DNA damage repair have all been described in progressive MS (8, 38–40) and leukodystrophy (41–44).

We consistently observed neuroinflammation in oligodendrocyte-conditional *Jab1*^{-/-} mice as a consequence of primary oligodendrocyte dysfunction. Inflammation was primarily related to microglia activation, though lymphocytes, monocytes and macrophages later contributed to the process. Predominant microglia activation is a common finding in both primary progressive MS and leukodystrophies (3, 39, 45, 46), and observed in other models featuring oligodendrocyte dysfunction (10, 18). Given the phenotypic characteristics of our mutant, we could test whether microglia actively contributed to neurodegeneration. Interestingly, microglia depletion did not ameliorate demyelination nor neurodegeneration, suggesting that this is not a key event in disease progression. This result is in line with the failure in the use of immunomodulatory/immunosuppressive drugs to treat progressive MS patients (47). Our data indicate that cells other than inflammatory, likely senescent oligodendrocytes, with or without the contribution of reactive astrocytes, promote neurodegeneration. Nevertheless, we cannot exclude that enforcing neuroprotective microglia subtypes could constitute an alternative approach to rescue neurodegeneration in chronic white matter disorders (48).

Collectively, we propose that oligodendrocyte senescence cell autonomously represents a key element in the pathogenesis of neurodegeneration in chronic white matter disorders. Thus, targeting senescent

cells might constitute a primary objective to develop innovative therapeutic strategies. Accordingly, our results show that interfering with the senescence pathway may ameliorate/delay the disease, as described also in other contexts (49, 50). Of note, this was observed with deletion of p21^{CIP1} but not p16^{INK4a}. One explanation can reside in compensation. Not surprisingly, in both double mutants, the expression of the other senescence marker (p21^{CIP1} in *Cnp-Cre:Jab1^{fl/fl};p16^{INK4a}-/-* and p16^{INK4a} in *Cnp-Cre:Jab1^{fl/fl};p21^{CIP1}-/-* mice) was maintained at increased levels, and this may suggest that both these genes should be deleted to completely rescue the phenotype. Moreover, differences in the experimental rescue may be due to a specific function or pathway originated by the two cyclin inhibitors. For example, it is known that p21^{CIP1} is (mainly) required for the induction of senescence while p16^{INK4A} is required for its maintenance (34). Epigenetically induced senescence mostly acts through p16^{INK4A} as opposed to DNA damage-induced senescence which mainly relies on p21^{CIP1} (51). Moreover, specific functions of p21^{CIP1} and p16^{INK4A} in post-mitotic cells, such as oligodendrocytes is still poorly understood. We cannot exclude that senescence in CNS cells (such as oligodendrocytes) might be sustained also by other molecules that may partially overtake the function of p21^{CIP1}, and/or may not include p16^{INK4A}.

Unfortunately, senolytic drugs still present limits in terms of potency and applicability (52, 53). However, recent findings gave promising results by eliminating senescent cells with antibodies or CAR-T cells (54, 55), suggesting possible future development of alternative treatments for these devastating disorders of the CNS.

Methods

Animals

The generation of floxed *Jab1*, *Cnp-Cre*, *Plp-CreErt2*, and *EYFP-Rosa26* mice has been previously described (18, 30, 56, 57). *Ccr2* and p21^{CIP1} (*Cdkn1a*) KO mice were purchased from Jackson Laboratory, and p16^{INK4a} (*Cdkn2a*) KO mice from the National-Cancer-Institute-Mouse-Models of Human-Cancer-Consortium (Supplemental Table 4). For genotyping, we isolated genomic DNA from tail biopsies using DirectPCR solution (ViaGen), according to the

manufacturer's directions. All primer sequences are available in Supplemental Table 5.

PLX3397 administration

The compound Pexidartinib PLX3397 (S7818) was purchased by Selleckchem and formulated in irradiated D10001i Rodent Diet standard chow by Research Diets at a dose of 290 mg per kg of chow. At P20 Wt and Cnp-Cre:Jab1^{fl/fl} mice were fed with standard diet or PLX3397 (290 mg/kg) chow for 21 days to reduce microglia cells by >99% in the CNS, as previously described (25).

Behavioral test

Rotarod: mice at different ages were placed on a 3 cm diameter horizontal rotating rod with acceleration from 4 rpm to a maximum of 40 rpm after 300 seconds (4 rpm every 30 seconds). The maximum time on the rod was 600 seconds. The latency to fall was measured on the moment that mouse fell down or stopped walking (clinging the rod) for two consecutive turns. Mice were trained the first day with a constant speed of 20 rpm for 5 minutes. On day 2 and 3, mice were directly subjected to 3 trials in the morning and 3 trials in the afternoon (each trial was separated at least by 15 minutes).

MRI

For each scan session, mice were anesthetized using gas anesthesia (1-2% isoflurane in O₂) with the respiration rate and external body temperature monitored during imaging using an MR-compatible small animal monitoring and gating system. External body temperature was maintained at 36-37 °C with a heating circulator bath. With a dedicated phased-array surface coil placed on the heads of the mice, MRI acquisitions were performed on a 7-Tesla scanner (70/30 cm, Bruker BioSpin MRI GmbH) using different MR sequence. Following standard anatomical images (T2 weighted) in the 3 orthogonal planes, 16 coronal sections (0.9 mm thickness) were positioned to cover the entire mouse brains. Magnetization transfer (MT) data were acquired using a fast low angle shot (FLASH) sequence (TR/TE = 550/2.9 ms; 6 averages; matrix = 192×128) with a saturation pulse (frequency offset = 3 000 Hz; RF power = 5 μT) and with

a high spatial resolution ($75.5 \times 70.3 \mu\text{m}^2$). The percentage of MT was quantified from the difference of signal in images with and without saturation pulse. Diffusion tensor imaging (DTI) was acquired with an echo planar imaging (EPI) sequence (TR/TE = 4500/23 ms; matrix = 128×128) with diffusion gradients applied in 30 directions ($b = 1200 \text{ s/mm}^2$ and duration/separation = 5/10 ms) or without ($n = 5$, $b = 0$). From DTI, the main diffusion directions and the magnitude of diffusivity were calculated in all three directions using standard algorithms (Bruker-Paravision 5.1) that provide maps of the fractional anisotropy (FA), mean diffusivity (MD), axial diffusivity ($\lambda_{\parallel}$) and radial diffusivity ($\lambda_{\perp}$). The total imaging session was approximately 1 hour. For each animal scan, MRI parameters were measured by manually drawing ROIs in the medial corpus callosum (CC) over different coronal sections. FA map was used for ROI limits definition as the CC was clearly visible. All measured values were normalized to the mean value of healthy controls (100%).

VEPs recording

Non-invasive epidermal VEPs were recorded using a 6 mm Ø Ag/AgCl cup electrode (SEI EMG s.r.l.) placed on the shaved scalp over V1 (1 mm anterior to interaural line and 2.5 mm contralateral to stimulation) fixed with electroconductive adhesive paste (Elefix EEG paste). A needle was inserted on the nose as reference. Mice were anesthetized (80 mg/kg ketamine, 10 mg/kg xylazine) at adequate level verified as absence of tail-pinching reflex. Body temperature was maintained at $36.5 \pm 0.5^\circ\text{C}$ by a homeothermic blanket system with a rectal thermometer probe. Pupils were dilated with 1% tropicamide (Visumidriatic, Visufarma s.p.a.) and eyes protected with ophthalmic gel (Gel 4000). VEPs were acquired after 5 minutes of dark adaptation in a darkened Faraday cage. For each VEP recording session, 3 trains of 20 flash stimuli (with intensity 260 mJ, 10 μs duration and 1 Hz frequency) were delivered with a flash photostimulator (Micromed) placed at 15 cm from the stimulated eye, while the contralateral was covered. Data were acquired by Micromed SystemPlus Evolution software at a sampling frequency of 4096 Hz (bandpass-filtered 0.16-1024), coded with 16 bits and bandpass-filtered (5–100 Hz, notch filter 50 Hz). When no VEP response was identified (signal lower than 5 μV), an additional recording was performed, keeping the flash outside the Faraday

cage to ensure noise reduction below that level. Latency of the first negative peak (N1) and amplitude with respect of the following positive peak (P2) were measured with the same software.

Histology and morphometric analysis

Semithin and ultrathin morphological experiments were performed as described (58). Briefly, brain, spinal cord and optic nerves were dissected from mice transcardially perfused with 4% (vol/vol) paraformaldehyde and fixed with 2% (vol/vol) glutaraldehyde and 4% (vol/vol) paraformaldehyde in 0.12 M phosphate buffer overnight, postfixed with 1% (vol/vol) osmium tetroxide and embedded in epon (Fluka). Semithin sections (0.5 μm thick) were stained with toluidine blue and examined by light microscopy (Olympus BX51, Leica Microsystems). Ultrathin sections (100–120 nm thick) were stained with uranyl acetate and lead citrate and examined by electron microscopy (FEI Talos L120C G2 Transmission Electron Microscope). Morphometric analysis on ultrastructural sections of optic nerve was done on 25 digitalized images per animal, randomly photographed at 5300X, whereas for spinal cord we analyzed images of semithin sections at 100x, 5 images per mouse. G-ratio value were determined by manually measuring axon and fiber diameters, while fibers count was defined by absolute number of fibers per area (mm^2) with ImageJ software (v1.6 <http://rsbweb.nih.gov/ij/download>).

Immunohistochemistry

Mice were anaesthetized and perfused with 4% (vol/vol) paraformaldehyde. Brain, spinal cord and optic nerves were isolated and post-fixed overnight in 4% (vol/vol) paraformaldehyde and incubated prior to cryoprotection in 30% sucrose/PBS at 4°C overnight. Tissue was embedded in OCT and sectioned. 8 μm -thick cryosections were permeabilized in cold acetone or methanol depending on antibodies used. Sections were incubated in blocking solution containing 10% of NGS (Normal Goat Serum, Vector Laboratories) in BSA 1% (Bovine Serum Albumine, Sigma-Aldrich) with 1% of TritonTM-X100 (Sigma-Aldrich), if required. Primary antibodies (Supplemental Table 6) were diluted in blocking solution containing 1% of Triton-X100, if required, while secondary

antibody (Supplemental Table 7) in phosphate buffer. Slices were then mounted with Vectashield with Dapi (H-1200, Vector Laboratories). For immunoperoxidase staining, sections were processed with UltraVision Quanto Detection System HRP-DAB (Thermo Scientific) following manufacturer's instructions. Briefly samples were incubated for 10 min with UltraVision Hydrogen Peroxidase Block, washed 2 times with TBS-T (0.05% Tween®-20, Sigma-Aldrich), incubated 5 minutes with UltraVision Protein Block, and successively with primary antibody for 1 hour at room temperature. After washing, sections were incubated 10 minutes with Primary Antibody Amplifier Quanto, and then with HRP Polymer Quanto for further 10 minutes. Staining was revealed by DAB Quanto Chromogen (5 minutes) to detect HRP. Slides were examined with confocal (Leica SP5 or SP2; Leica Microsystems), fluorescent or light microscope (Olympus BX51, Leica Microsystem). Quantification analysis was performed by counting absolute number of positive cells per area (mm²) in 5 adjacent images per tissue section, in 2-3 serial sections.

Retina isolation and Immunofluorescence of Retinal Ganglion Cells

Mice were anaesthetized and perfused with 4% (vol/vol) paraformaldehyde. The eyes were dissected out and post-fixed with 4% (vol/vol) paraformaldehyde for 60 min. Retinas were isolated under dissecting microscope. Briefly, after making a circumferential cut of the cornea following the limbus, the cornea and lens were removed and retina was visualized as a white surface covering the inside of the posterior eye cup. Eye cups were equilibrated in 30% Sucrose/PBS overnight, embedded in OCT and frozen in liquid nitrogen. Retina 10 μ m-thick cryosections were processed for immunofluorescence as described above. To count RGCs, four non-overlapping images from three different retinal sections were taken at the fluorescence microscope (Olympus BX5, Leica Microsystem) using 20X objective. RGCs were manually counted as a ratio of RGCs cells per mm and the average value was calculated for each sample.

BrdU assays

Optic nerves were evaluated *in vivo* by BrdU incorporation (5-Bromo-2'-deoxyuridine, Roche), as previously described (17). To evaluate OPC differentiation into mature CC1 positive oligodendrocytes, we injected BrdU (i.p., 100 mg/g body weight) in P20 mice for 3 consecutive days and analyzed mice at P30. Briefly, cryosections were fixed in cold methanol, treated with 2N HCl for 15 min at 37°C, and neutralized with 0.1M sodium borate pH 8.5 for 10 min. Slides were then incubated overnight with pAb anti NG2 or mAb anti CC1 and mAb anti-BrdU to identify proliferating cells at P20 and differentiated oligodendrocytes at P30. After staining with secondary antibodies, nuclei were counterstained with Dapi (Vectashield, Vector Laboratories). Quantification was performed by counting the absolute number of positive cells per area (mm²) in 5 adjacent images per nerve section, in 2-3 serial sections (at least 4000 cells per animal).

Senescence β -gal staining

Mice were anaesthetized and perfused with 4% (vol/vol) paraformaldehyde. Optic nerves were isolated and post-fixed overnight in 4% (vol/vol) paraformaldehyde and incubated prior to cryoprotection in 30% sucrose/PBS at 4°C overnight. Tissue was embedded in OCT and sectioned. 8 μ m-thick cryosections were processed according to the manufacturer's instructions of CellEvent™ Senescence Green detection Kit (Invitrogen) or Senescence Cell Histochemical Staining Kit (Sigma-Aldrich). Briefly, optic nerve cryosections were post-fixed 15 minutes in 4% (vol/vol) paraformaldehyde then washed and incubated at 37°C in a humid chamber for 2 hours with probe diluted into the pre-warmed staining mixture. Finally, slides were processed for immunohistochemistry as previously described.

ROS detection by Electron Paramagnetic Resonance

ROS were measured by Electron Paramagnetic Resonance (EPR) spectroscopy, as previously described (59, 60). Briefly, tissue samples (optic nerve, spinal cord and brain) were dissected and immediately incubated at 37°C in Krebs-Hepes buffer (KHB) containing 25 μ M deferroxamine methane-sulfonate salt (DF) chelating agent and 5 μ M sodium diethyldithio-carbamate

trihydrate (DETC) at pH 7.4 with 1mM 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine (CMH, Noxygen Science Transfer & Diagnostics) as spin-probe. After 30 minutes the isolated tissues were placed in the center of 1 mL plastic syringe, in according to Dikalov et al (60), snap frozen and stored at -80°C. Then frozen block was removed by gentle push from the warmed-up syringe and analyzed in the quartz Dewar with liquid N₂. Spectra were recorded at 77 K by using an X-band (9 GHz) EPR spectroscopy (E-scan-Bruker BioSpin GmbH). The EPR signal, generated by the reaction of the spin probe (CMH) with whole tissue ROS, was then recorded: acquisition parameters: modulation amplitude: 5 G; centered field: 2.0023 g, sweep time 10 s, field sweep 60 G, microwave power: 1 mW, number of scans: 10; receiver gain: 1*10³. Spectra were recorded and analyzed by using Win EPR software (2.11 version) standardly supplied by Bruker. EPR measurements allowed us to attain a relative quantitative determination of ROS production rate in samples. All data were, in turn, converted into absolute concentration levels ($\mu\text{mol}\cdot\text{g}^{-1}$) by adopting CP[•] (3-Carboxy-2,2,5,5-tetramethyl-1-pyrrolidinyloxy) stable radical as external reference.

Western blotting

Proteins were isolated from snap-frozen tissue, homogenized in lysis buffer, containing 2% (vol/vol) SDS, 25mM Tris-HCl pH 7.5, 95mM NaCl, 10mM EDTA pH 8, phosphatase inhibitor (PhoSTOP, Roche), and protease inhibitors (Complete-Mini EDTA free, Roche). Homogenates were sonicated and centrifuged for 10 min at 12.000 rpm at 4°C. Protein concentrations were determined by BCA Assay (Pierce). Equal amounts of homogenates were fractionated by SDS-PAGE and blotted onto PVDF (Millipore) or nitrocellulose (Biorad). Membranes were blocked in 5% no-fat dry milk in PBS-T (0.1% Tween®-20, Sigma-Aldrich, in PBS) incubated with specific primary and secondary antibodies (Supplemental Table 6 and Supplemental Table 7), washed in PBS-T and developed with the Clarity Western ECL substrate (BioRad) on films or analyzed by ChemiDoc Imaging System (BioRad) or using the Odyssey Infrared Imaging System (LI-COR Biosciences) according to the manufacturer's instructions. Densitometric analysis were performed with the ImageJ software (v1.6 <http://rsbweb.nih.gov/ij/download>) for membranes

developed by chemiluminescence and the Odyssey v3 CLX infrared Imaging System when fluorescent secondary is used. Each antigen was normalized as respect to the housekeeping protein.

FACS analysis and cell sorting

Mice were anesthetized, perfused with saline and the brain removed. Brains from 8 Wt and 8 *Cnp1-Cre:Jab1^{fl/fl}* mice at P40 and P60 were dissociated with 0.4 mg/ml of collagenase type IV (Sigma-Aldrich) and through a 18G syringe to obtain a homogeneous cell suspension. Cell suspensions were further enriched by an isotonic Percoll gradient, myelin was removed, cells washed in PBS and finally centrifuged and resuspended in MACS Buffer as described (61). Cells were stained with specific antibodies (Supplemental Table 6). Zombie Nir (BioLegend) was added to exclude non-viable cells. Samples were sorted on FACS Aria Fusion (BD Biosciences) and analyzed with Flow-jo (Three star). Schematic representation of gating strategy for FACS-sorting is depicted in Supplemental Figure 1. Mature oligodendrocytes were identified as single live cells CD45⁻O1⁺, activated microglia were determined as single live cells CD45⁺/loCD11b⁺Ly6G⁻Ly6C⁻CD11c⁺, macrophages were identified as single live CD45^{hi}CD11b⁺Ly6G⁻Ly6C⁻ cells, monocytes as single live CD45⁺CD11b⁺Ly6G⁻Ly6C⁺, lymphocytes as single live CD45⁺CD11b⁻, and neutrophils as single live CD45⁺CD11b⁺Ly6G⁺ cells. On FACS sorting experiments re-analysis by cytometry did not show overt CD45⁺ cell contamination (0% of CD45⁺ cells) in all the groups tested (Wt, *Cnp-Cre:Jab1^{fl/fl}* and *Cnp-Cre:Jab1^{fl/fl};p21^{CIP1-/-}*). Purity of sorted O1⁺ cells ranged between 80-90% on reanalysis. Due to the FACS sorting technique, to the material tested (central nervous system tissue after dissociation) and to the cells studied (CNS oligodendrocytes) a small contamination by other cells cannot be entirely excluded.

Tissue irradiation

Mice were anesthetized, perfused with saline and optic nerves dissected and placed in 500µl of DMEM supplemented with 5% FBS (FBS Good, PANTM Biotech) on ice in 4 wells plate. Optic nerves were cut to obtain 5 mm segments,

then segments were irradiated (3Gy) by BIOBEAM GM 8000 gamma irradiator. Optic nerves were maintained in cells incubator at 37°C, 5% CO₂ for 8 hours and gently shaken every two hours. After 8 hours, optic nerves were embedded in OCT and snap-frozen in liquid nitrogen. Longitudinal 8-µm-thick cryosections were processed for immunohistochemistry as described above.

Quantitative RT-PCR

Total RNA was isolated from optic nerves or sorted oligodendrocyte using TriPure Isolation Reagent (Roche) according to the manufacturer's instructions. Briefly, cells or nerves were homogenized in the presence of TriPure Isolation Reagent, total RNA was extracted with chloroform and precipitated with isopropanol. A portion (350 ng) of optic nerves total RNA was reverse transcribed using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystem). RNA from sorted oligodendrocyte was amplified and reverse transcribed by SMART-seq V4 Ultra Low Input RNA Kit (Takara Bio), 1 µl of the amplified cDNA were validated using the Agilent 2100 Bioanalyzer and Agilent's High Sensitivity DNA Kit (Agilent, Cat. No. 5067-4626). Quantitative RT-PCR analyses were performed on Applied Biosystems 7900HT Real-Time PCR System using the 2x Taqman PCR Mastermix (Applied Biosystems) according to manufacturer's recommendations. The primers used were TaqMan® Gene Expression Assays by Applied Biosystems: *Ccl5* ID:Mm01302427-m1, *Cdkn1a* ID:Mm04205640-g1, *Cdkn2a* ID: Mm00494449-m, *Cops5* Mm00489065-m1, *Csf2* ID:Mm01290062-m1, *Cxcl1* ID:Mm04207460-m1, *Gapdh* ID:Mm99999915-g1, *Il1b* ID:Mm00434228-m1, *Serpine1* ID:Mm00435858-m1, *Tgfb1* ID:Mm03024053-m1, *Trim10* ID:Mm00600002-m1, *Trp53* ID: Mm01731287-m1. Levels of gene expression were determined with the comparative cycle threshold ($\Delta\Delta C_t$) method. The mRNA level of each gene of interest was normalized to the level of *Gapdh* mRNA. Each time point is the average of 4-5 experiments (each experiment was performed with two optic nerves, one brain hemisphere, or sorted oligodendrocyte derived from two mouse brains).

RNA sequencing

Total RNA was isolated from sorted oligodendrocytes using ReliaPrep™ RNA Cell Miniprep System (Promega) according to the manufacturer's instructions. Four sample per genotype of Wt, *Cnp-Cre:Jab1^{fl/fl}* and *Cnp-Cre:Jab1^{fl/fl};p21^{CIP1}^{-/-}* (one sample derived from 2 different P40 mouse brains) were analyzed. Libraries were prepared using the SMART-Seq® v4 Ultra® Low Input RNA Kit for Sequencing (Takara Bio USA), according to the manufacturer's instructions. Sequencing was performed on a Illumina NovaSeq 6000 machine (Illumina, San Diego, CA) obtaining an average of 100 millions of paired-end reads per sample. The raw reads produced from sequencing were trimmed using Trimmomatic, version 0.32, to remove adapters and to exclude low-quality reads from the analysis. The remaining reads were then aligned to the murine genome GRCm38 using STAR, version 2.5.3a. Reads were eventually assigned to the corresponding genomic features using featureCounts, according to the Gencode basic annotations (Gencode version M22). Quality of sequencing and alignment was assessed using the FastQC, RseQC and MultiQC tools. Expressed genes were defined as those genes showing at least 1 Count Per Million reads (CPM) on at least 4 samples, corresponding to the sample size of each group of samples (62). Low-expressed genes that did not match this criteria were excluded from the dataset. Gene expression read counts were exported and analyzed in the R environment (v. 3.5.1) to identify differentially expressed genes (DEGs), using the DESeq2 Bioconductor library (v. 1.22.1, <https://doi.org/10.1186/s13059-014-0550-8>). The p-values were adjusted using a threshold for false discovery rate (FDR) <0.05 (63). Functional enrichment analysis was conducted using the enrichR R package (v. 1.0, <https://doi.org/10.1093/nar/gkw377>), starting from the lists of differentially expressed genes as defined by FDR<0.05. The enrichment with a subset of selected gene-sets was further evaluated using the Pre-ranked Gene Set Enrichment Analysis (Pre-ranked GSEA), as reported (64). The gene-sets included in the GSEA analyses were retrieved from the Gene Ontology Biological Process collection as reported in the MSigDB database, and from the senescence pathway as described (65).

Human brain samples

Post-mortem brain white matter samples of 6 multiple sclerosis (MS) chronic inactive plaques (1 female and 5 male, mean age 64 ± 10) and 6 normal control tissue donors (2 female and 4 male, mean age 60 ± 20) were conserved as snap-frozen tissue blocks at the NeuroResource tissue bank, UCL Queen Square Institute of Neurology, London, England. For lesion classification, tissue sections were analyzed by immunohistochemistry/immunofluorescence for myelin basic protein (MBP), CD68 (microglia/macrophages), GFAP (astrocytes) and CD3 (T cells), as previously reported (66)(67). The chronic inactive lesions were fully demyelinated, lacked T cell infiltration in the parenchyma, and presented sharp lesion borders, strong astrogliosis and low myeloid cell numbers. These human samples were donated with informed consent using documentation approved by the NHS (NRES Committee London–Central, UK), and stored under a Research Sector Licence from the UK Human Tissue Authority (HTA). These studies were approved by the Ethical Committee at San Raffaele Scientific Institute and performed in accordance with the ethical standards as laid down in the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards.

Human brain sample immunostaining

Twelve μm -thick serial sections were prepared from human snap-frozen brain tissue blocks. Sections were fixed in 4% (vol/vol) paraformaldehyde and non-specific binding was blocked in PBS/1% BSA/5% FCS. Slides were then incubated with primary antibodies and appropriate species-specific Alexa Fluor 488/594-conjugated secondary antibodies (Thermo Fisher scientific). The slides were counterstained with 4',6-diamidino-2-phenylindole (Dapi, Sigma) and mounted with fluorescent mounting medium (Agilent). For primary antibodies see Supplemental Table 6. As negative controls, mouse IgG1 (Sigma-Aldrich) or rabbit immunoglobulins (Ig) (Thermo Fisher scientific) were used at the same concentration of the primary antibodies. Fluorescence images were captured at Leica TCS SP5 confocal laser-scanning microscope equipped with 40 $\times$ and 63 $\times$ oil objectives for tissue stainings. Single stacks were acquired for image quantification. LASAF software was used for image acquisition and ImageJ (v1.6.0 <http://rsbweb.nih.gov/ij/download>) software was used for image analysis. To quantify nuclear JAB1, DAPI images were converted to 8-bit, and

regions of interest (ROIs) were generated to select (Dapi positive) nuclei. Then ROIs were applied to the corresponding OLIG2 or JAB1 images, and the number of positive nuclei was counted after setting of appropriate fluorescence threshold.

Statistical analysis

Data were analyzed by log-rank (Mantel-Cox) test for Kaplan-Maier Curve, Two-way ANOVA with Bonferroni post-correction for longitudinal study while unpaired T-test, Two-tailed nonparametric Mann–Whitney U-test to compare two groups at the same time point. Normalized data were analyzed using one-sample Two-Tailed Student's T-Test. From data sets from more than two groups, dedicated p-value were corrected for multiple testing using One-way ANOVA with Bonferroni's multiple comparison test. All these tests were done using GraphPad Prism version 5. Differences were considered significant when $p < 0.05$. Data are presented as mean $\pm$ s.e.m. and level of significance are depicted by asterisks in the figures * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Study approval

All animal experiments were approved and performed in compliance with the guidelines of San Raffaele Institutional Animal Care and Use Committee.

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The Authors declare no competing interests.

Author contribution: SCP designed and supervised the study. CR, GD, EC performed histological and morphological analyses. CR performed immunohistochemistry, beta-gal and Western blot analyses. CR, MB, GSG performed the FACS analysis. CR and EP performed RT and qPCR experiments. LL and SM performed VEP analysis. LC performed MRI studies. SMS and AV performed ROS quantification. CF, EC, JN performed studies in human samples. SCP, CR, AQ, MB, KAN, US, RP, CF interpreted the results. SCP wrote the paper with input from all the Authors. SCP, CR, AQ, CF made the figures.

The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (Edgar et al.,2002) and are accessible through GEO Series accession number GSE184573 (<http://ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE184573>)

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Figure legends

Figure 1. Generation of oligodendrocyte-conditional *Jab1* null mice

(A) *Cnp-Cre:Jab1^{fl/fl}* mouse showing reduced size as compared to littermate control. (B) Genotyping PCR using optic nerve-derived DNA providing genomic recombination only in *Cnp-Cre:Jab1^{fl/fl}* mice. (C) qPCR for *Cops5* (*Jab1*) expression in sorted oligodendrocyte (O1+) showing reduced expression in *Cnp-Cre:Jab1^{fl/fl}* as compared to Wt mice (* $p < 0.05$, $n = 4$; Two-tailed nonparametric Mann–Whitney U-test). (D) Western blot analysis and quantification of optic nerve homogenate showing JAB1 reduction in *Cnp-Cre:Jab1^{fl/fl}* mice (**** $p < 0.0001$; $n = 6$; one sample two-tailed Student's t-test; Wt is reported as =1). (E) Confocal images of the corpus callosum (within dotted lines) of *CnpCre Jab1^{fl/fl} Rosa26* mice stained for GFP, CC1, NG2 and Dapi. Recombined OPC positive for GFP and NG2 (arrows) and oligodendrocytes positive for GFP and CC1 (arrowheads) are labelled in the inset magnification. (F) Kaplan-Meier curve showing reduced survival in mutant mice ($n = 100$ controls and 72 mutants; **** $p < 0.0001$, Chi-square Mantel-Cox test). (G) Mutant mice showing significant body weight reduction overtime (* $p < 0.05$, *** $p < 0.001$; $n = 10$; Two-way ANOVA with Bonferroni post-correction). (H) Rotarod analysis showing significant motor deficits in mutant mice since P40

(**** $p < 0.0001$; Two-tailed nonparametric Mann–Whitney U-test). Scale bars, (d) 20 μm ; inset 10 μm .

Figure 2. Morphological abnormalities in *Jab1* mutant mice

(A) Electron micrographs of transverse sections of the optic nerve, corpus callosum and spinal cord from *Wt* and *Cnp-Cre:Jab1^{fl/fl}* mice at the age P20, P40 and P60 showing progressive demyelination and axonal loss. (B) Representative electron micrographs of spinal cord cross-sections from P60 *Cnp-Cre:Jab1^{fl/fl}* mice showing axonal spheroid (asterisk), swelling and accumulation of organelles and ovoids (arrow) and myelin fragmentation (arrowheads). (C) Quantification of g-ratio, number of myelinated fibers and degenerating fibers in the optic nerves from *Wt* and *Cnp-Cre:Jab1^{fl/fl}* mice at different time points; * $p < 0.05$, ** $p < 0.01$ ($n = 4$ to 5; at least 2000 fibers counted per mouse; Two-tailed nonparametric Mann–Whitney U-test).

Scale bars, (A) and (B) 2 μm .

Figure 3. Longitudinal MRI and VEPs evaluation

(A) Brain MT MRI images of *Wt* and *Cnp-Cre:Jab1^{fl/fl}* mice at different age (P30, P45 and P65). While in *Wt* mice, the corpus callosum is clearly delineated on MT images (in dark, arrows), it is hardly distinguishable in *Jab1* mutant mice in which the contrast is similar at the first exam (P30) and became hyperintense at later stages (P45 and P60, arrows). (B) With DTI (color map of FA), the corpus callosum is well discriminated in all the animals but the FA (color map) is clearly decreased at P45 and P60 in *Jab1* mutant as compared to *Wt* mice (arrows). (C) Quantitative analysis of MT and FA measured in the medial of the corpus callosum. Both MT and FA were significantly reduced with age in *Jab1* mutant mice as compared to healthy controls; * $p < 0.05$ and *** $p < 0.001$ ($n = 6$; Two-way ANOVA with Bonferroni post-correction). (D) N1 latency and amplitude measured in *Wt* and *Cnp-Cre:Jab1^{fl/fl}* mice at age P30, P45 and P65; **** $p < 0.0001$ ($n = \text{Wt } 7, \text{ mutant } 6$; Two-way ANOVA with Bonferroni post-correction). (E) Representative VEP waveforms recorded from *Wt* and *Cnp-Cre:Jab1^{fl/fl}* at different ages. Horizontal bar 50 ms and vertical bar 20 μV .

Figure 4. Inflammatory infiltration in *Jab1* mutant mice

(A) Immunolabelling of *Wt* and *Cnp-Cre:Jab1^{fl/fl}* optic nerves for IBA1 and MBP at ages P60 and P90 and relative quantification from P20 to P90 showing progressive increase in the number of IBA1 positive cells in mutant mice; * $p < 0.05$, ** $p < 0.01$ ($n = 3$ to 5 ; Two-tailed nonparametric Mann–Whitney U-test). (B) FACS analysis of P60 *Wt* and *Cnp-Cre:Jab1^{fl/fl}* brain for CD45, CD11b (representative plots), CD11c, Ly6G and Ly6C showing quantification of microglia, activated microglia, lymphocytes, monocytes, macrophages and neutrophils; ns means not significant, ** $p < 0.01$, *** $p < 0.001$ ($n = 13$ *Wt*, $n = 9$ *Cnp-Cre:Jab1^{fl/fl}*; Two-tailed nonparametric Mann–Whitney U-test). (C) Immunolabelling for GFAP and CC1 of the optic nerve from *Wt* and *Cnp-Cre:Jab1^{fl/fl}* mice at the age P60 and relative quantification showing increased number of GFAP positive reactive astrocytes in mutant mice. * $p < 0.05$ ($n = 4$ Two-tailed nonparametric Mann–Whitney U-test). Scale bars, (A) and (C) 40 μm .

Figure 5. Depletion of microglia activation does not ameliorate neurodegeneration in *Jab1* mutant mice

(A) Electron micrographs of transverse sections of P50 optic nerve from *Wt*, *CCR2^{-/-}*, *Cnp-Cre:Jab1^{fl/fl}* and *Cnp-Cre:Jab1^{fl/fl}:CCR2^{-/-}* mice and relative quantification of IBA1⁺ cells, number of myelinated fibers and of degenerating fibers, showing no significant differences between *Jab1^{-/-}* and double *Jab1^{-/-}:CCR2^{-/-}* mutant mice; ** $p < 0.01$, *** $p < 0.001$ ($n = 4$ to 5 ; One-way ANOVA with Bonferroni's multiple comparison test). (B) Confocal images (for IBA1 and MBP) and electron micrographs of P41 optic nerve from *Wt* and *Cnp-Cre:Jab1^{fl/fl}* mice treated (PLX+) or not (PLX-) for 21 days with PLX3397 (see scheme) and relative quantification of IBA1 positive cell, number of myelinated and degenerating fibers. Although the number of IBA1⁺ cells was significantly reduced in PLX treated mice, no differences for the number of myelinated and degenerating fibers is observed (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; $n = 4$ to 9 ; One-way ANOVA with Bonferroni's multiple comparison test). Scale bars, (A) 1 μm (B) 40 μm IHC and 2 μm EM.

Figure 6. DNA damage and senescence in mutant mice

(A) Confocal immunolabelling of Wt and mutant optic nerves stained for CC1 and pH2AX; quantification shows significant increase of pH2AX positive oligodendrocytes in mutant mice from P40 (* $p < 0.05$, ** $p < 0.01$; $n = 3-5$; Two-tailed nonparametric Mann–Whitney U-test). (B) Confocal images of ex vivo optic nerves X-ray irradiated to induce DNA damage stained for DNA-PKcs and CC1; the majority of Wt oligodendrocytes have nuclear expression of DNA-PK whereas this percentage is reduced in *Jab1*^{-/-} optic nerves (** $p < 0.01$; $n = 5$; Two-tailed nonparametric Mann–Whitney U-test). (C) β -gal staining and quantification of the corpus callosum and optic nerve from P60 Wt and *Cnp-Cre:Jab1*^{fl/fl} mice double stained with CC1 showing senescent oligodendrocytes in mutant mice (* $p < 0.05$, $n = 5-7$; Two-tailed nonparametric Mann–Whitney U-test). (D) qPCR for *Cdkn2a* (p16^{INK4}) in the optic nerve from Wt and *Cnp-Cre:Jab1*^{fl/fl} mice showing significant increase from P40 (** $p < 0.01$; $n = 5$ to 6; Two-tailed nonparametric Mann–Whitney U-test). (E) qPCR for *Cdkn1a* (p21^{CIP1}) in the optic nerve from Wt and *Cnp-Cre:Jab1*^{fl/fl} mice showing significant increase from P40 (* $p < 0.05$, ** $p < 0.01$; $n = 5-6$; Two-tailed nonparametric Mann–Whitney U-test). (F) qPCR for SASP in the optic nerve from P60 Wt and *Cnp-Cre:Jab1*^{fl/fl} mice showing significant elevation in mutant mice (* $p < 0.05$, ** $p < 0.01$; $n = 4-6$; Two-tailed nonparametric Mann–Whitney U-test). (G) Western blot analysis, quantification, and confocal images for HMGB1 in P60 optic nerves of Wt and *Cnp-Cre:Jab1*^{fl/fl} mice; HMGB1 is increased in mutant mice and is also translocated in the cytoplasm of mutant oligodendrocytes (arrow * $p < 0.05$, $n = 4$; Two-tailed nonparametric Mann–Whitney U-test). (H) ROS quantification in optic nerve and corpus callosum from Wt and *Cnp-Cre:Jab1*^{fl/fl} mice showing significant ROS elevation from P40 (* $p < 0.05$, ** $p < 0.01$; $n = 3-5$; Two-tailed nonparametric Mann–Whitney U-test). Scale bars, (A) 40 μ m, (B) 2 μ m, (D) 50 μ m light microscopy, 2 μ m fluorescence; (G) 20 μ m.

Figure 7. CNS demyelination, inflammation and axonal degeneration is reproduced in Plp-CreERT2:Jab1^{fl/fl} mice

(A) Tamoxifen-treated *Plp-CreERT2:Jab1*^{fl/fl} mouse showing reduced size as compared to littermate control and quantification of body weight at P60 and

P180 showing significant reduction at later time point (** $p < 0.01$; $n = 18$ at P60 and 6 at P180; Two-tailed nonparametric Mann–Whitney U-test). **(B)** schematic representation of tamoxifen administration and electron micrographs of P60 and P180 optic nerve and corpus callosum from tamoxifen-treated *Plp-CreERT2:Jab1^{fl/fl}* and control (vehicle-treated *Plp-CreERT2:Jab1^{fl/fl}*) mice showing no differences at P60 but diffuse demyelination and axonal degeneration at P180. **(C)** Quantification showing significant increase in the number of degenerating fibers and decrease of myelinated fibers in P180 tamoxifen-treated *Plp-CreERT2:Jab1^{fl/fl}* mice (** $p < 0.01$; $n = 3$ at P60 and 6 at P180; Two-tailed nonparametric Mann–Whitney U-test). **(D)** Immunolabelling for IBA1 and MBP of P180 optic nerves from Tamoxifen-treated *Plp-CreERT2:Jab1^{fl/fl}* and control mice, and relative quantification showing significant increased number of IBA1+ cells in tamoxifen-treated mice (* $p < 0.05$; $n = 4$ in controls and 5 in tamoxifen-treated mice; Two-tailed nonparametric Mann–Whitney U-test). **(E)** β -gal staining of the optic nerve from P180 tamoxifen-treated *Plp-CreERT2:Jab1^{fl/fl}* and control mice double stained with CC1 showing β -gal positive oligodendrocytes in tamoxifen-treated mice. **(F)** qPCR for *Cdkn1a* (p21^{CIP1}) and *Cdkn2a* (p16^{INK4}) showing increased expression in the optic nerve from P180 tamoxifen-treated *Plp-CreERT2:Jab1^{fl/fl}* as compared to control mice (*** $p < 0.001$; $n = 7$; Two-tailed nonparametric Mann–Whitney U-test). Scale bars, **(B)** 2 μ m **(D)** 40 μ m, **(F)** 10 μ m.

Figure 8. Deletion of *p21^{CIP1}* ameliorates the phenotype in *Jab1* mutant mice.

(A) Rotarod analysis showing amelioration of motor deficits in *Cnp-Cre:Jab1^{fl/fl};p21^{CIP1}^{-/-}* as compared to *Cnp-Cre:Jab1^{fl/fl}* mice (** $p < 0.01$ at P40 $n = 5$; Two-tailed nonparametric Mann–Whitney U-test). **(B)** Quantification of g-ratio in P60 optic nerves showing a significant reduction in *Cnp-Cre:Jab1^{fl/fl};p21^{CIP1}^{-/-}* as compared to *Cnp-Cre:Jab1^{fl/fl}* mice (* $p < 0.05$, ** $p < 0.01$, $n = 5$ to 7; at least 2000 fibers counted per mouse; One-way ANOVA with Bonferroni's multiple comparison test). **(C)** Confocal images and quantification of BRN3A-positive ganglion cells in the retina at P60 showing a significant

rescue in *Cnp-Cre:Jab1^{fl/fl};p21^{CIP1-/-}* as compared to *Cnp-Cre:Jab1^{fl/fl}* mice (** $p < 0.01$, *** $p < 0.001$, $n = 6$ to 8 ; One-way ANOVA with Bonferroni's multiple comparison test). **(D)** Quantification of myelinated fibers and degenerating fibers in the P60 spinal cord showing significant rescue in *Cnp-Cre:Jab1^{fl/fl};p21^{CIP1-/-}* as compared to *Cnp-Cre:Jab1^{fl/fl}* mice (* $p < 0.05$, *** $p < 0.001$, $n = 7$ to 9 ; One-way ANOVA with Bonferroni's multiple comparison test). **(E)** Quantification of Western blot analysis for phosphorylated (SMI-31) and non-phosphorylated (SMI-32) neurofilaments of high molecular weight (NF-H) in P60 brain homogenate showing a significant rescue in *Cnp-Cre:Jab1^{fl/fl};p21^{CIP1-/-}* as compared to *Cnp-Cre:Jab1^{fl/fl}* mice (* $p < 0.05$, $n = 6$; One-way ANOVA with Bonferroni's multiple comparison test). **(F)** Confocal immunolabelling for CC1 and β -gal staining and quantification in P60 optic nerve showing reduced number of senescent oligodendrocytes in *Cnp-Cre:Jab1^{fl/fl};p21^{CIP1-/-}* as compared to *Cnp-Cre:Jab1^{fl/fl}* mice; * $p < 0.05$ ($n = 4$; Two-tailed nonparametric Mann–Whitney U-test). **(G)** Heatmaps representing the expression values of key genes for cellular senescence and for SASP/inflammation differentially expressed in FACS-sorted O1⁺ oligodendrocytes. Scale bars, **(C)** 40 μ m, **(F)** 40 μ m.

Figure 9. Expression of Jab1 in brain of MS patients

(A) Representative immunofluorescence images for nuclear JAB1 (red) expression in OLIG2 positive cells (green) in control white matter or in normal appearing white matter surrounding chronic inactive MS lesion. Regions of interest (ROIs) were generated on DAPI images (left panels) to select nuclei and then applied to the corresponding OLIG2 and JAB1 images. Arrows highlight JAB1 positive oligodendrocytes. **(B)** Percentage of JAB1 expressing OLIG2 positive cells in control white matter (black dots) or MS normal appearing white matter (white dots). Each dot represents a single sample and bars represent mean. * $p < 0.05$ ($n = 6$; Two-tailed nonparametric Mann–Whitney U-test). Scale bar, **(A)** 10 μ m.

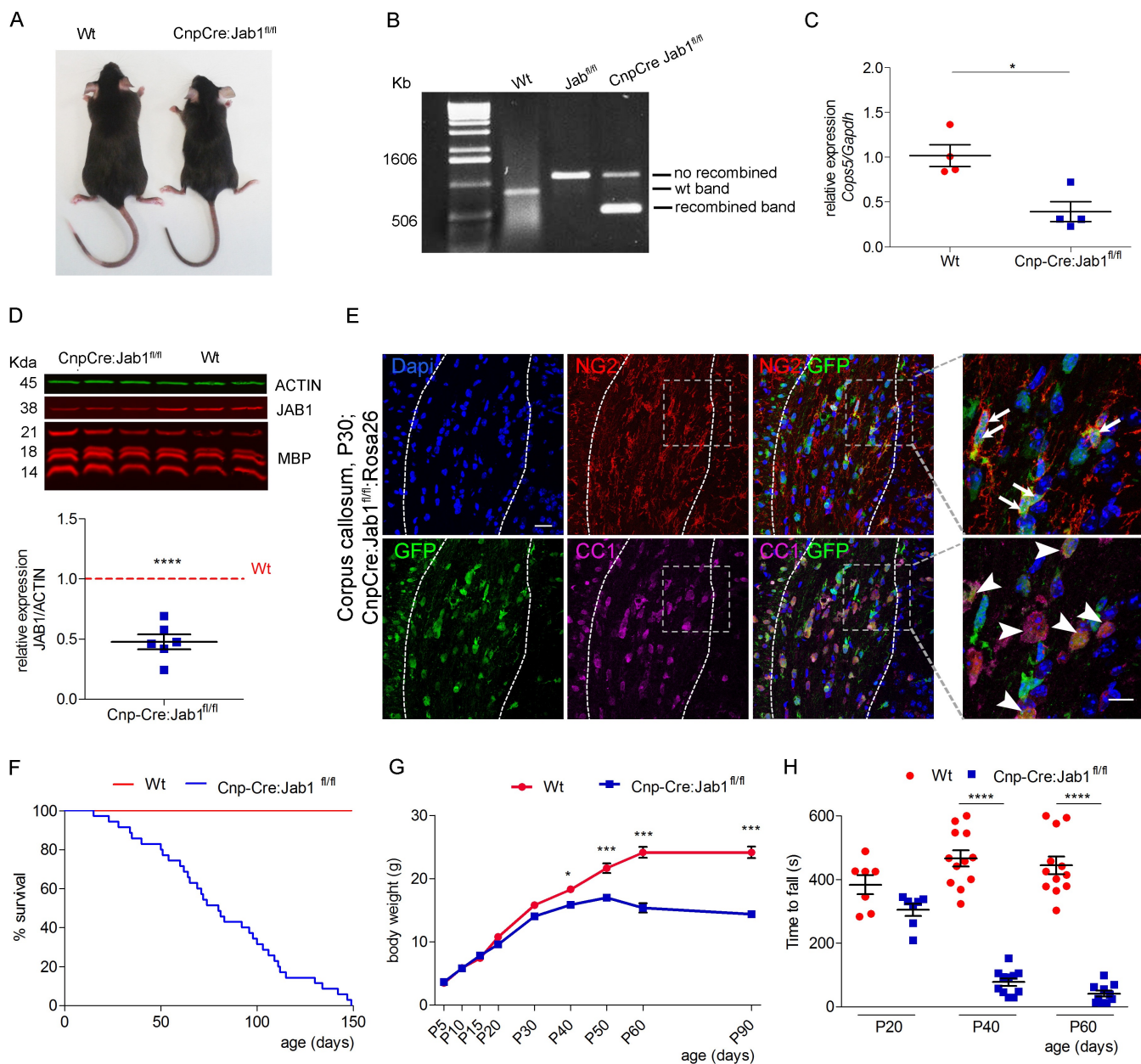


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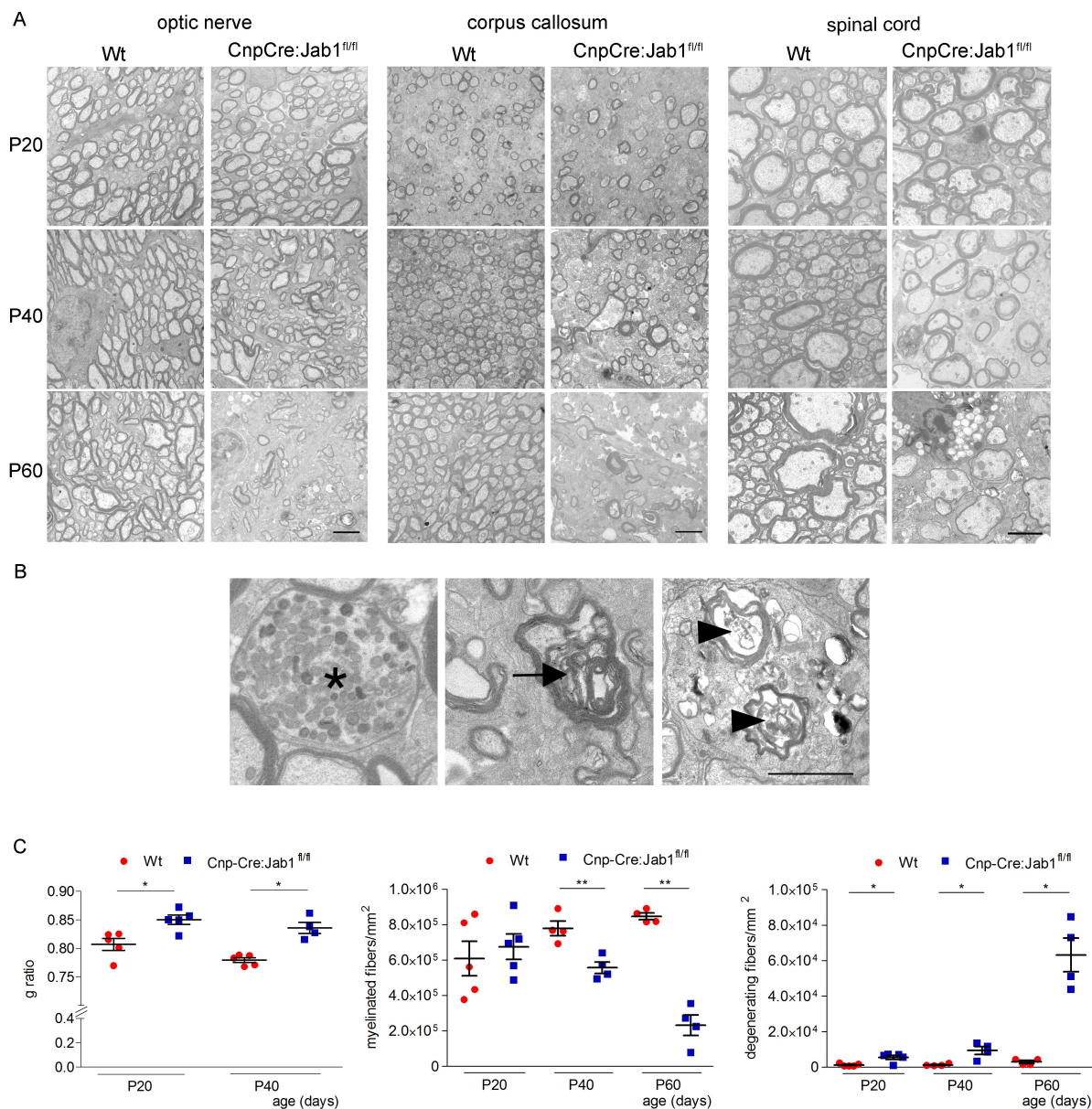


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Scale bars, (**A**) and (**B**) 2 μ m.

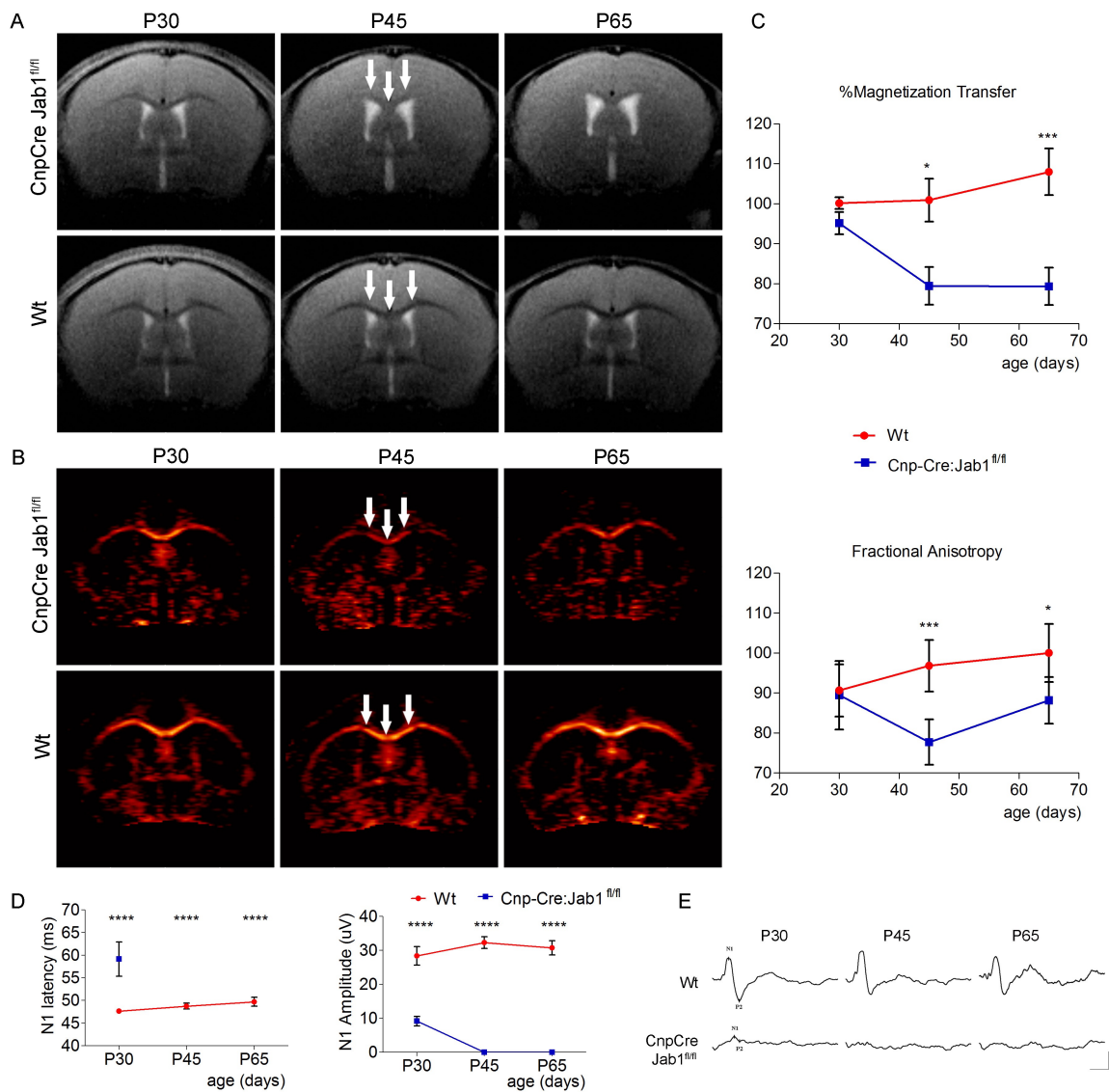


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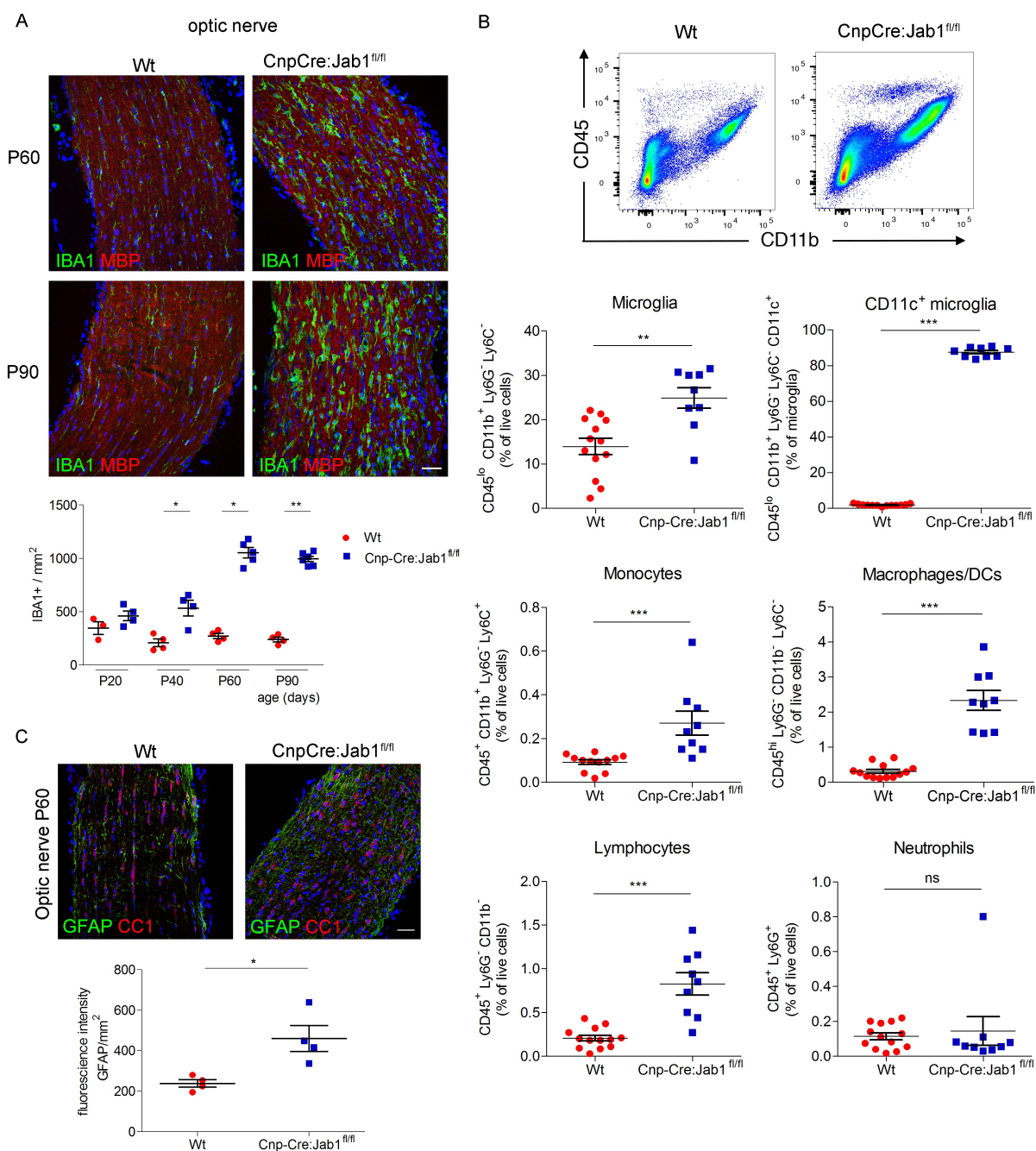


Figure 4. Inflammatory infiltration in *Jab1* mutant mice

(A) Immunolabelling of *Wt* and *Cnp-Cre:Jab1^{fl/fl}* optic nerves for IBA1 and MBP at ages P60 and P90 and relative quantification from P20 to P90 showing progressive increase in the number of IBA1 positive cells in mutant mice; * $p < 0.05$, ** $p < 0.01$ ($n = 3$ to 5 ; Two-tailed nonparametric Mann–Whitney U-test). (B) FACS analysis of P60 *Wt* and *Cnp-Cre:Jab1^{fl/fl}* brain for CD45, CD11b (representative plots), CD11c, Ly6G and Ly6C showing quantification of microglia, activated microglia, lymphocytes, monocytes, macrophages and neutrophils; ns means not significant, ** $p < 0.01$, *** $p < 0.001$ ($n = 13$ *Wt*, $n = 9$ *Cnp-Cre:Jab1^{fl/fl}*; Two-tailed nonparametric Mann–Whitney U-test). (C) Immunolabelling for GFAP and CC1 of the optic nerve from *Wt* and *Cnp-Cre:Jab1^{fl/fl}* mice at the age P60 and relative quantification showing increased number of GFAP positive reactive astrocytes in mutant mice. * $p < 0.05$ ($n = 4$ Two-tailed nonparametric Mann–Whitney U-test). Scale bar, (A) and (C) 40 μ m.

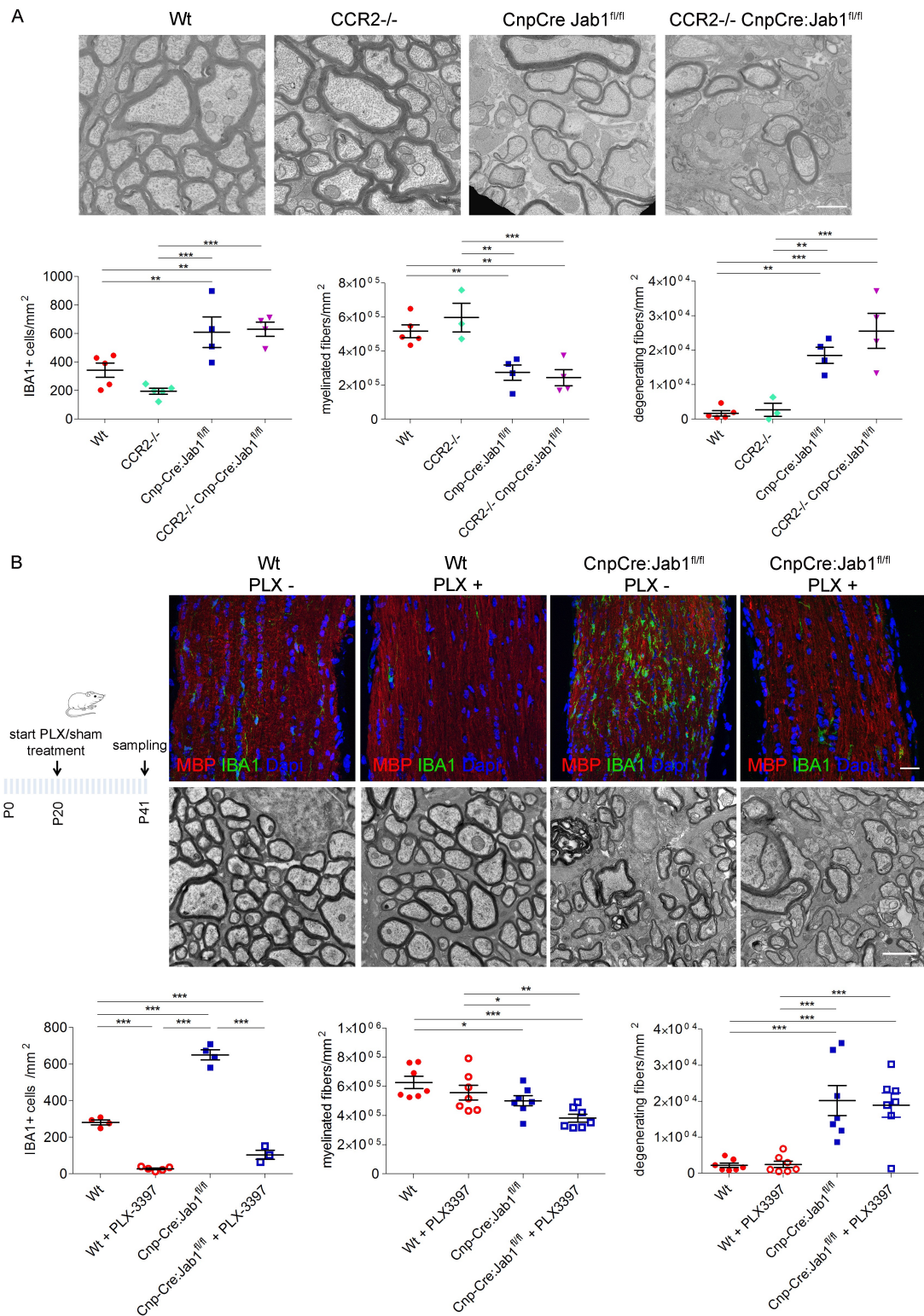


Figure 5. Depletion of microglia activation does not ameliorate neurodegeneration in Jab1 mutant mice

(A) Electron micrographs of transverse sections of P50 optic nerve from Wt, CCR2^{-/-}, Cnp-Cre:Jab1^{fl/fl} and Cnp-Cre:Jab1^{fl/fl}:CCR2^{-/-} mice and relative quantification of IBA1⁺ cells, number of myelinated fibers and of degenerating fibers, showing no significant differences between Jab1^{-/-} and double Jab1^{-/-}:CCR2^{-/-} mutant mice; **p<0.01, ***p<0.001 (n = 4 to 5; One-way ANOVA with Bonferroni's multiple comparison test). **(B)** Confocal images (for IBA1 and MBP) and electron micrographs of P41 optic nerve from Wt and Cnp-Cre:Jab1^{fl/fl} mice treated (PLX+) or not (PLX-) for 21 days with PLX3397 (see scheme) and relative quantification of IBA1 positive cell, number of myelinated and degenerating fibers. Although the number of IBA1⁺ cells was significantly reduced in PLX treated mice, no differences for the number of myelinated and degenerating fibers is observed (*p<0.05, **p<0.01, ***p<0.001; n =4 to 9; One-way ANOVA with Bonferroni's multiple comparison test). Scale bars, **(A)** 1 μ m **(B)** 40 μ m IHC and 2 μ m EM.

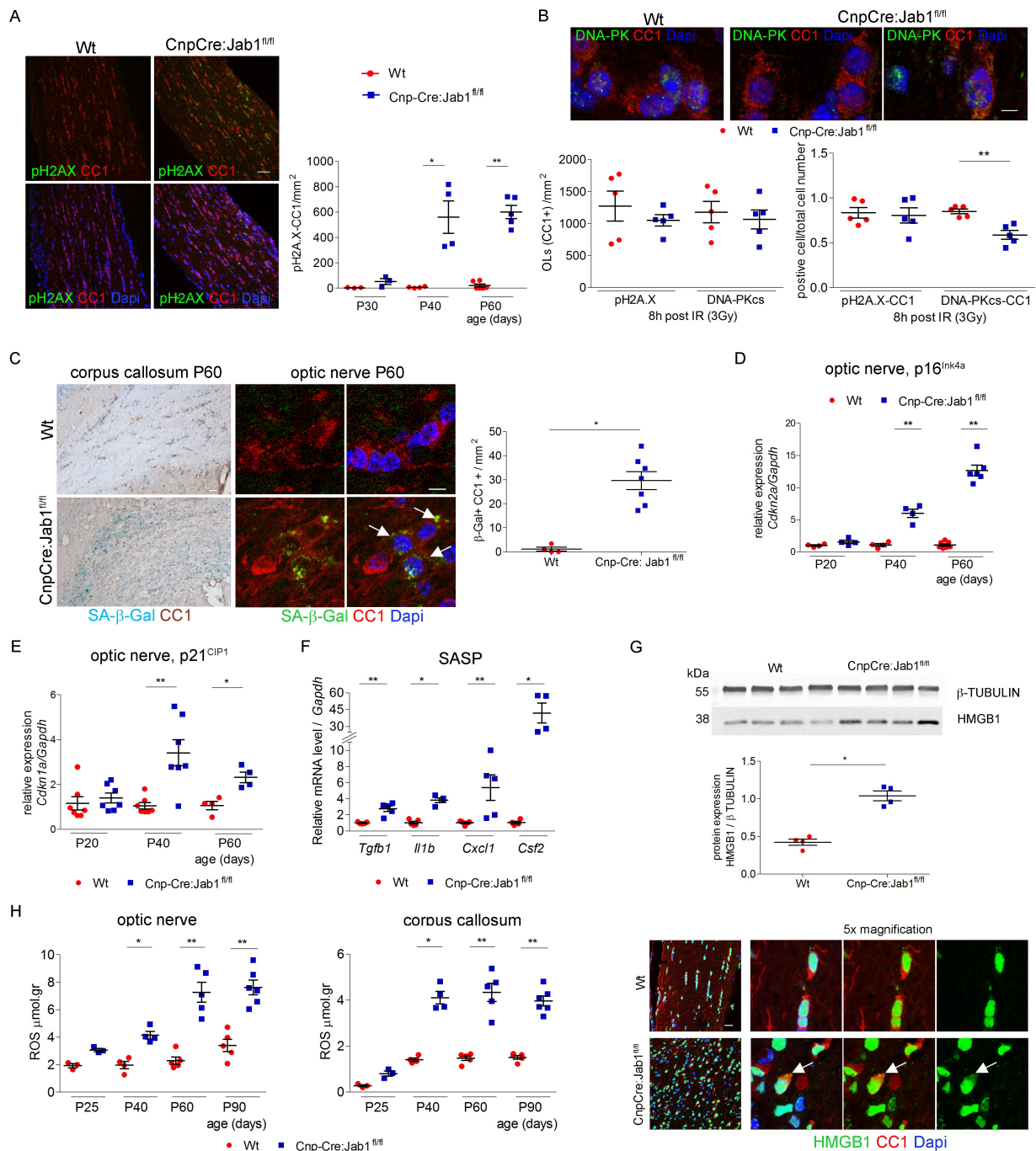


Figure 6. DNA damage and senescence in mutant mice

(A) Confocal immunolabelling of Wt and mutant optic nerves stained for CC1 and pH2AX; quantification shows significant increase of pH2AX positive oligodendrocytes in mutant mice from P40 (* $p<0.05$, ** $p<0.01$; $n=3-5$; Two-tailed nonparametric Mann–Whitney U-test). (B) Confocal images of ex vivo optic nerves X-ray irradiated to induce DNA damage stained for DNA-PKcs and CC1; the majority of Wt oligodendrocytes have nuclear expression of DNA-PK whereas this percentage is reduced in *Jab1*^{-/-} optic nerves (** $p<0.01$; $n=5$; Two-tailed nonparametric Mann–Whitney U-test). (C) β -gal staining and quantification of the corpus callosum and optic nerve from P60 Wt and *Cnp-Cre:Jab1*^{fl/fl} mice double stained with CC1 showing senescent oligodendrocytes in mutant mice (* $p<0.05$, $n=5-7$; Two-tailed nonparametric Mann–Whitney U-test). (D) qPCR for *Cdkn2a* (p16^{INK4a}) in the optic nerve from Wt and *Cnp-Cre:Jab1*^{fl/fl} mice showing significant increase from P40 (** $p<0.01$; $n=5$ to 6; Two-tailed nonparametric Mann–Whitney U-test). (E) qPCR for *Cdkn1a* (p21^{CIP1}) in the optic nerve from Wt and *Cnp-Cre:Jab1*^{fl/fl} mice showing significant increase from P40 (* $p<0.05$, ** $p<0.01$; $n=5-6$; Two-tailed nonparametric Mann–Whitney U-test). (F) qPCR for SASP in the optic nerve from P60 Wt and *Cnp-Cre:Jab1*^{fl/fl} mice showing significant elevation in mutant mice (* $p<0.05$, ** $p<0.01$; $n=4-6$; Two-tailed nonparametric Mann–Whitney U-test). (G) Western blot analysis, quantification, and confocal images for HMGB1 in P60 optic nerves of Wt and *Cnp-Cre:Jab1*^{fl/fl} mice; HMGB1 is increased in mutant mice and is also translocated in the cytoplasm of mutant oligodendrocytes (arrow * $p<0.05$, $n=4$; Two-tailed nonparametric Mann–Whitney U-test). (H) ROS quantification in optic nerve and corpus callosum from Wt and *Cnp-Cre:Jab1*^{fl/fl} mice showing significant ROS elevation from P40 (* $p<0.05$, ** $p<0.01$; $n=3-5$; Two-tailed nonparametric Mann–Whitney U-test). Scale bars, (A) 40 μ m, (B) 2 μ m, (D) 50 μ m light microscopy, 2 μ m fluorescence; (G) 20 μ m.

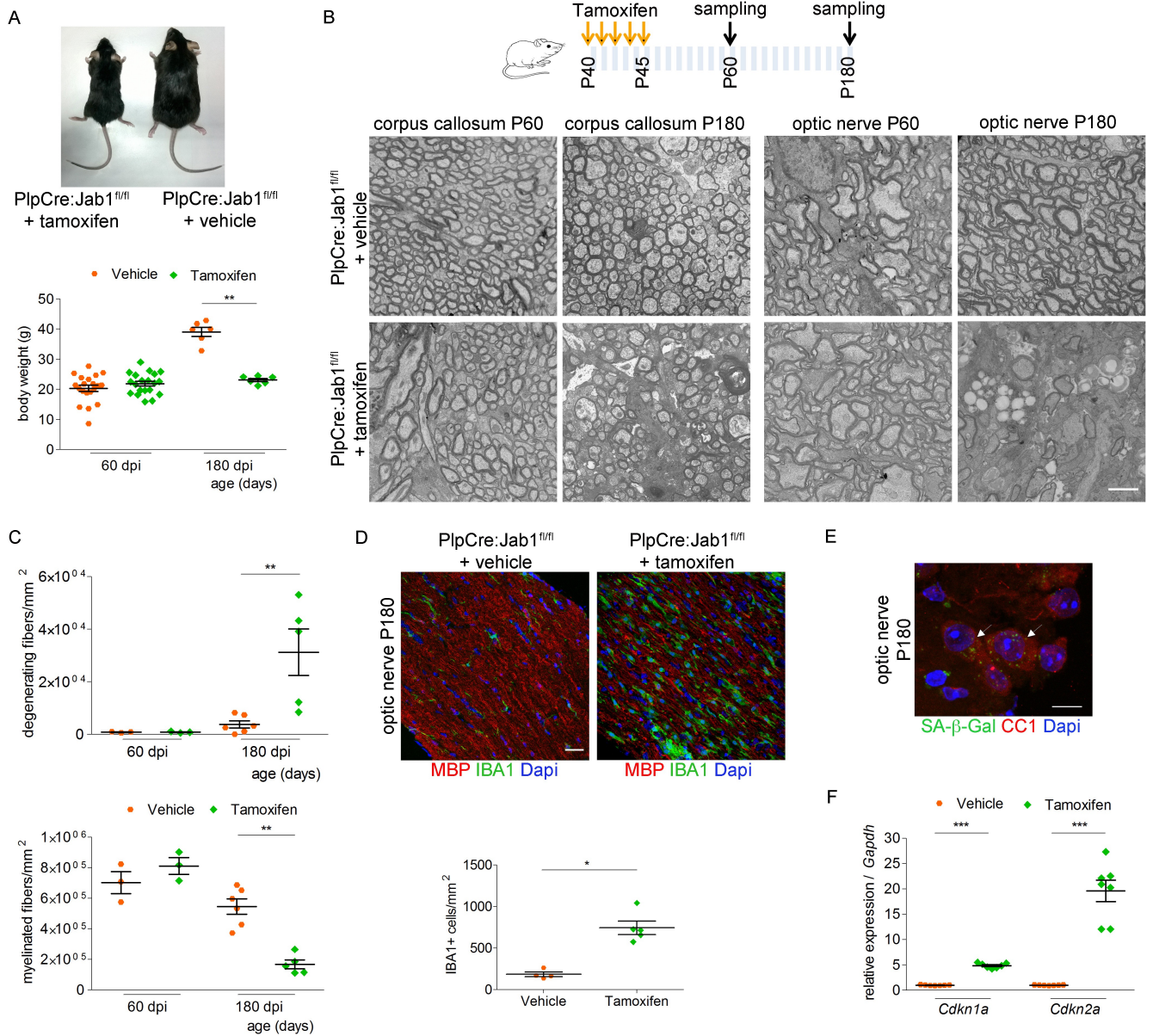


Figure 7. CNS demyelination, inflammation and axonal degeneration is reproduced in Plp-CreERT2:Jab1^{fl/fl} mice

(A) Tamoxifen-treated *Plp-CreERT2:Jab1^{fl/fl}* mouse showing reduced size as compared to littermate control and quantification of body weight at P60 and P180 showing significant reduction at later time point (** $p < 0.01$; $n = 18$ at P60 and 6 at P180; Two-tailed nonparametric Mann–Whitney U-test). (B) schematic representation of tamoxifen administration and electron micrographs of P60 and P180 optic nerve and corpus callosum from tamoxifen-treated *Plp-CreERT2:Jab1^{fl/fl}* and control (vehicle-treated *Plp-CreERT2:Jab1^{fl/fl}*) mice showing no differences at P60 but diffuse demyelination and axonal degeneration at P180. (C) Quantification showing significant increase in the number of degenerating fibers and decrease of myelinated fibers in P180 tamoxifen-treated *Plp-CreERT2:Jab1^{fl/fl}* mice (** $p < 0.01$; $n = 3$ at P60 and 6 at P180; Two-tailed nonparametric Mann–Whitney U-test). (D) Immunolabelling for IBA1 and MBP of P180 optic nerves from Tamoxifen-treated *Plp-CreERT2:Jab1^{fl/fl}* and control mice, and relative quantification showing significant increased number of IBA1+ cells in tamoxifen-treated mice (* $p < 0.05$; $n = 4$ in controls and 5 in tamoxifen-treated mice; Two-tailed nonparametric Mann–Whitney U-test). (E) β -gal staining of the optic nerve from P180 tamoxifen-treated *Plp-CreERT2:Jab1^{fl/fl}* and control mice double stained with CC1 showing β -gal. positive oligodendrocytes in tamoxifen-treated mice. (F) qPCR for *Cdkn1a* (p21^{CIP1}) and *Cdkn2a* (p16^{INK4}) showing increased expression in the optic nerve from P180 tamoxifen-treated *Plp-CreERT2:Jab1^{fl/fl}* as compared to control mice (*** $p < 0.001$; $n = 7$; Two-tailed nonparametric Mann–Whitney U-test). Scale bars, (B) 2 μ m (D) 40 μ m, (F) 10 μ m.

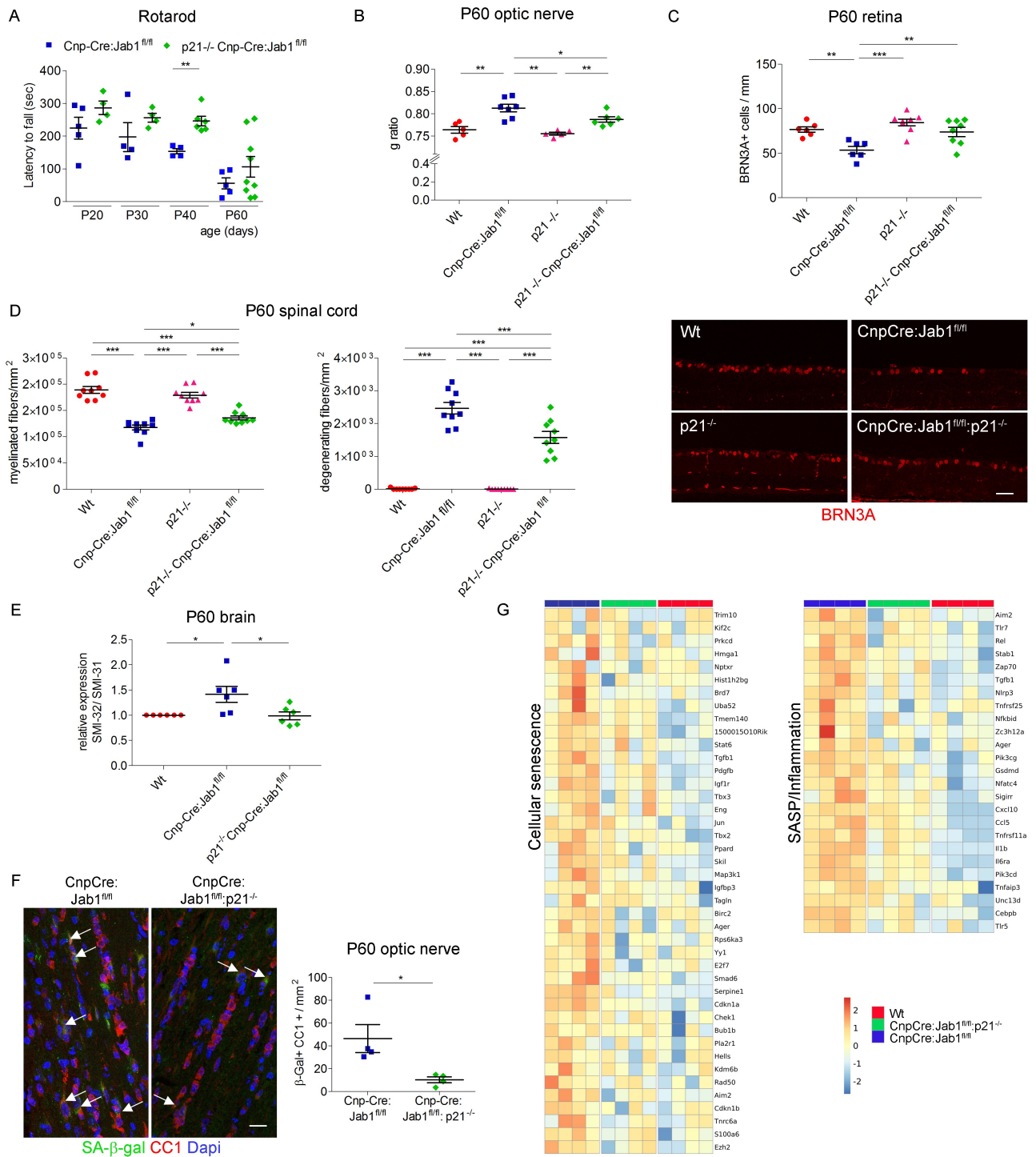


Figure 8. Deletion of *p21^{CIP1}* ameliorates the phenotype in *Jab1* mutant mice.

(A) Rotarod analysis showing amelioration of motor deficits in *Cnp-Cre:Jab1^{fl/fl};p21^{CIP1}/-* as compared to *Cnp-Cre:Jab1^{fl/fl}* mice (** $p < 0.01$ at P40 $n = 5$; Two-tailed nonparametric Mann–Whitney U-test). (B) Quantification of g-ratio in P60 optic nerves showing a significant reduction in *Cnp-Cre:Jab1^{fl/fl};p21^{CIP1}/-* as compared to *Cnp-Cre:Jab1^{fl/fl}* mice (* $p < 0.05$, ** $p < 0.01$, $n = 5$ to 7; at least 2000 fibers counted per mouse; One-way ANOVA with Bonferroni's multiple comparison test). (C) Confocal images and quantification of BRN3A-positive ganglion cells in the retina at P60 showing a significant rescue in *Cnp-Cre:Jab1^{fl/fl};p21^{CIP1}/-* as compared to *Cnp-Cre:Jab1^{fl/fl}* mice (** $p < 0.01$, *** $p < 0.001$, $n = 6$ to 8; One-way ANOVA with Bonferroni's multiple comparison test). (D) Quantification of myelinated fibers and degenerating fibers in the P60 spinal cord showing significant rescue in *Cnp-Cre:Jab1^{fl/fl};p21^{CIP1}/-* as compared to *Cnp-Cre:Jab1^{fl/fl}* mice (* $p < 0.05$, *** $p < 0.001$, $n = 7$ to 9; One-way ANOVA with Bonferroni's multiple comparison test). (E) Quantification of Western blot analysis for phosphorylated (SMI-31) and non-phosphorylated (SMI-32) neurofilaments of high molecular weight (NF-H) in P60 brain homogenate showing a significant rescue in *Cnp-Cre:Jab1^{fl/fl};p21^{CIP1}/-* as compared to *Cnp-Cre:Jab1^{fl/fl}* mice (* $p < 0.05$, $n = 6$; Oneway ANOVA with Bonferroni's multiple comparison test). (F) Confocal immunolabelling for CC1 and β -gal staining and quantification in P60 optic nerve showing reduced number of senescent oligodendrocytes in *Cnp-Cre:Jab1^{fl/fl};p21^{CIP1}/-* as compared to *Cnp-Cre:Jab1^{fl/fl}* mice; * $p < 0.05$ ($n = 4$; Two-tailed nonparametric Mann–Whitney U-test). (G) Heatmaps representing the expression values of key genes for SASP/inflammation and for cellular senescence differentially expressed in FACS-sorted O1⁺ oligodendrocytes. Scale bar, (C) 40 μ m, (F) 40 μ m.

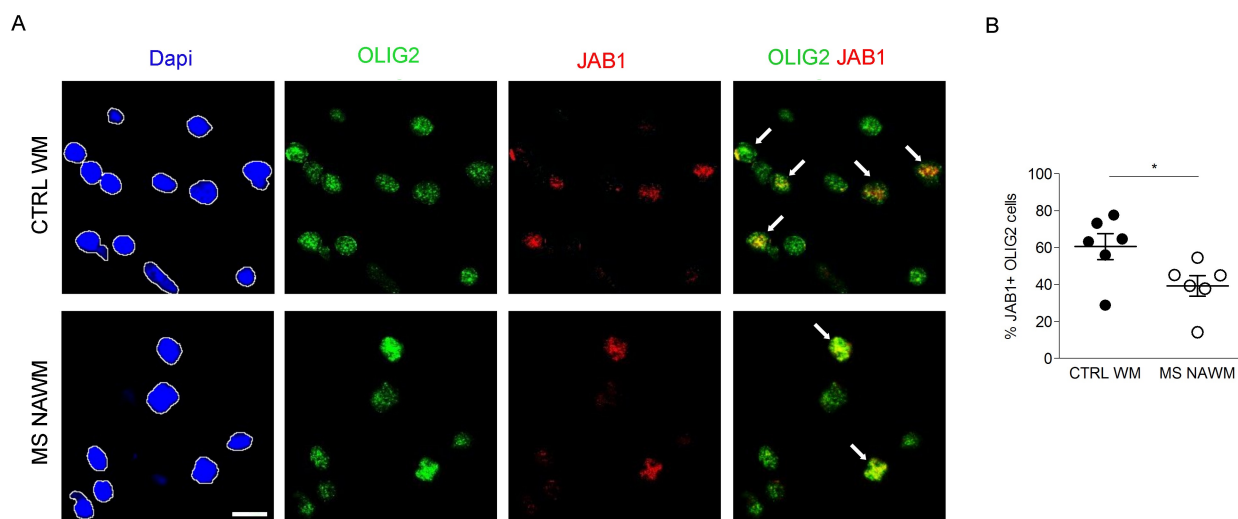


Figure 9. Expression of Jab1 in brain of MS patients

(A) Representative immunofluorescence images for nuclear JAB1 (red) expression in OLIG2 positive cells (green) in control white matter or in normal appearing white matter surrounding chronic inactive MS lesion. Regions of interest (ROIs) were generated on DAPI images (left panels) to select nuclei and then applied to the corresponding OLIG2 and JAB1 images. Arrows highlight JAB1 positive oligodendrocytes. **(B)** Percentage of JAB1 expressing OLIG2 positive cells in control white matter (black dots) or MS normal appearing white matter (white dots). Each dot represents a single sample and bars represent mean. * $p < 0.05$ ($n = 6$; Two-tailed nonparametric Mann–Whitney U-test). Scale bar: **(A)** 10 μm .