Supplemental material

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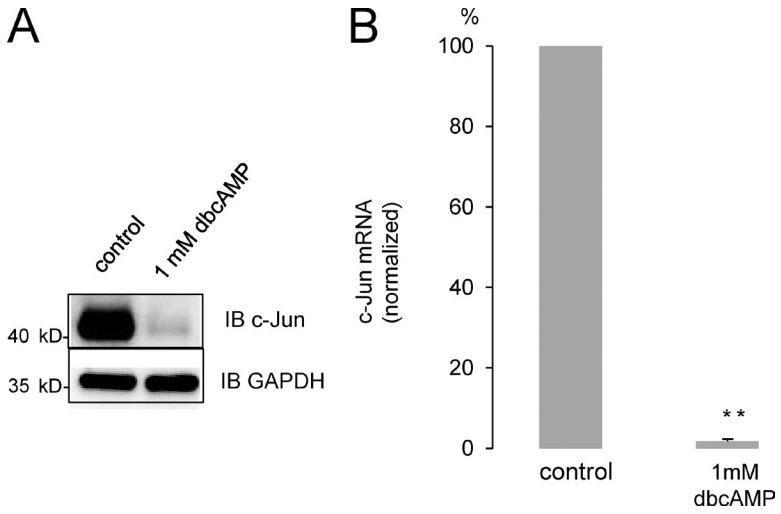

Figure S1. **c-Jun protein down-regulation by dbcAMP correlates with a decrease in mRNA. (A)** Cultured rat Schwann cells were incubated with 1 mM of dbcAMP in SATO medium for 24 h, or SATO medium alone, and lysed. An equivalent amount of protein extract was submitted to SDS-PAGE and blotted with anti–c-Jun antibody (IB c-Jun). The same membrane was blotted with anti-GAPDH as an additional loading control. **(B)** cAMP blocks the transcription of *c-Jun* mRNA: in a parallel experiment, total RNA was extracted and mRNA for *c-Jun* determined by RT-qPCR and normalized to *18S*. The result was normalized to SATO alone–incubated cultures. Data are given as mean ± SE and analyzed with the *t* test (two-sided). **, P < 0.01.

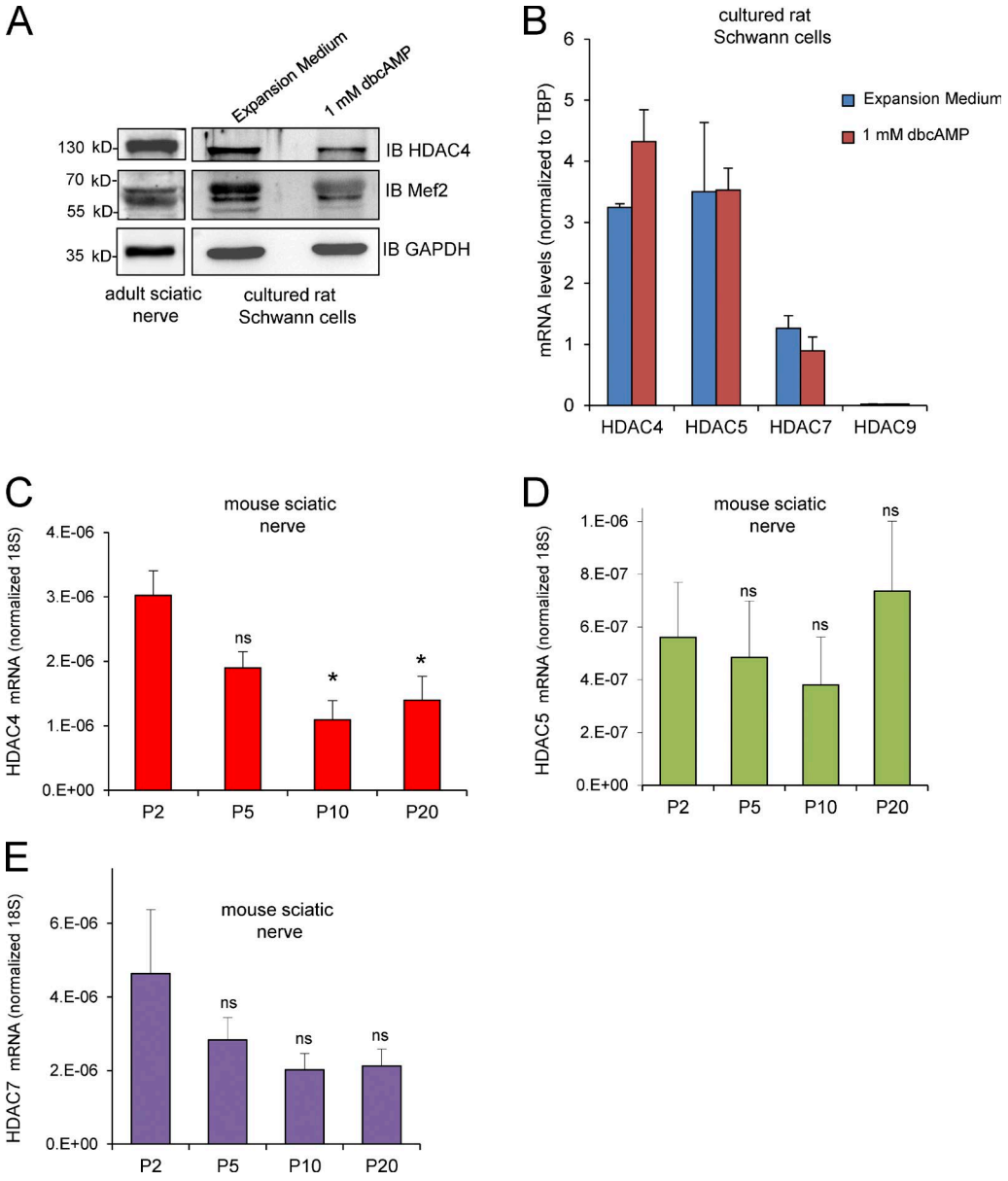

Figure S2. **Expression of *Mef2* and class IIa HDACs in mouse peripheral nerves and cultured rat Schwann cells. (A)** C57BL/6 adult mouse sciatic nerves were homogenized in RIPA buffer. Cultured rat Schwann cells were incubated in expansion medium or differentiation medium (1 mM dbcAMP in SATO) for 72 h and lysed in RIPA. Protein extracts were analyzed by Western blot with anti-HDAC4 and anti-Mef2 antibodies. As is shown, both proteins are expressed in sciatic nerves, and proliferating or differentiated Schwann cells. **(B)** Total RNA was extracted from Schwann cells in expansion or differentiation medium and retrotranscribed to cDNA. SYBR green qPCR was performed with specific primers for class IIa HDACs. Amplicons were similar in size and melting points. The ratio of the relative expression for each gene to the TATA-Box binding protein (*TBP*) gene was calculated by using the $2\Delta^{CT}$ formula. We could detect expression of *HDAC4*, *HDAC5*, and *HDAC7* but not *HDAC9*. Expression of *HDAC5* and *HDAC7* was similar in proliferating and differentiated Schwann cells. *HDAC4* mRNA was slightly up-regulated in differentiated Schwann cells. Data from three different experiments are given as mean ± SE. **(C–E)** Postnatal developmental expression profile of class IIa HDACs in the PNS: total RNA was extracted from sciatic nerves of P2, P5, P10, and P20 C57BL/6J mice and retrotranscribed to cDNA. SYBR green qPCR was performed with specific primers for class IIa HDACs. The ratio of the relative expression for each gene to *18S* was calculated by using the $2\Delta^{CT}$ formula. The mRNA for *HDAC4* is highly expressed at P2 and decreases as myelination proceeds (C and E). The mRNA for *HDAC5* and *HDAC7* is expressed at similar levels during postnatal development (D). Three animals per condition were used. Data are given as mean ± SE and analyzed with the *t* test (two-sided). *, $P < 0.05$.

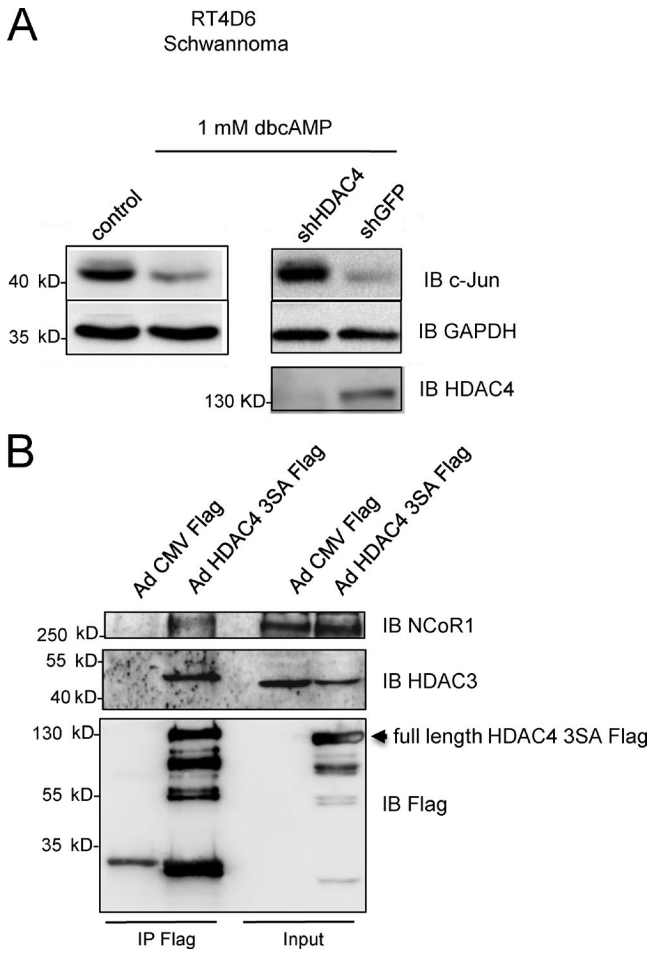

Figure S3. **On the role of HDAC4 in *c-Jun* down-regulation and the mechanism of action. (A)** Loss of HDAC4 prevents the down-regulation of *c-Jun* by cAMP in Schwanomma cells. Schwannoma RT4D6 cells were transfected with pENTR/U6 HDAC4 shRNA vector (shHDAC4) or pENTR/pTER shEGFP (shGFP) and incubated for 24 h with 1 mM dbcAMP. Nontransfected cells with and without dbcAMP (control) are also shown. Protein extracts were submitted to SDS-PAGE and immunobloted against c-Jun. Anti-HDAC4 immunoblot shows the loss of HDAC4. GAPDH was used as a loading control. **(B)** HDAC4 interacts with the NCoR1/HDAC3 complex in Schwann cells. Schwann cells were infected with Ad HDAC4 3SA Flag or Ad CMV Flag, lysed, and extracts pulled down with anti-Flag agarose beads. Immunoprecipitates and inputs were immunobloted with anti-NCoR1 or anti-HDAC3. NCoR1 and HDAC3 were recovered exclusively from Ad HDAC43SA Flag–infected cells. Expression and immunoprecipitation of the HDAC4 3SA Flag were checked by immunoblotting with anti-Flag monoclonal antibody.

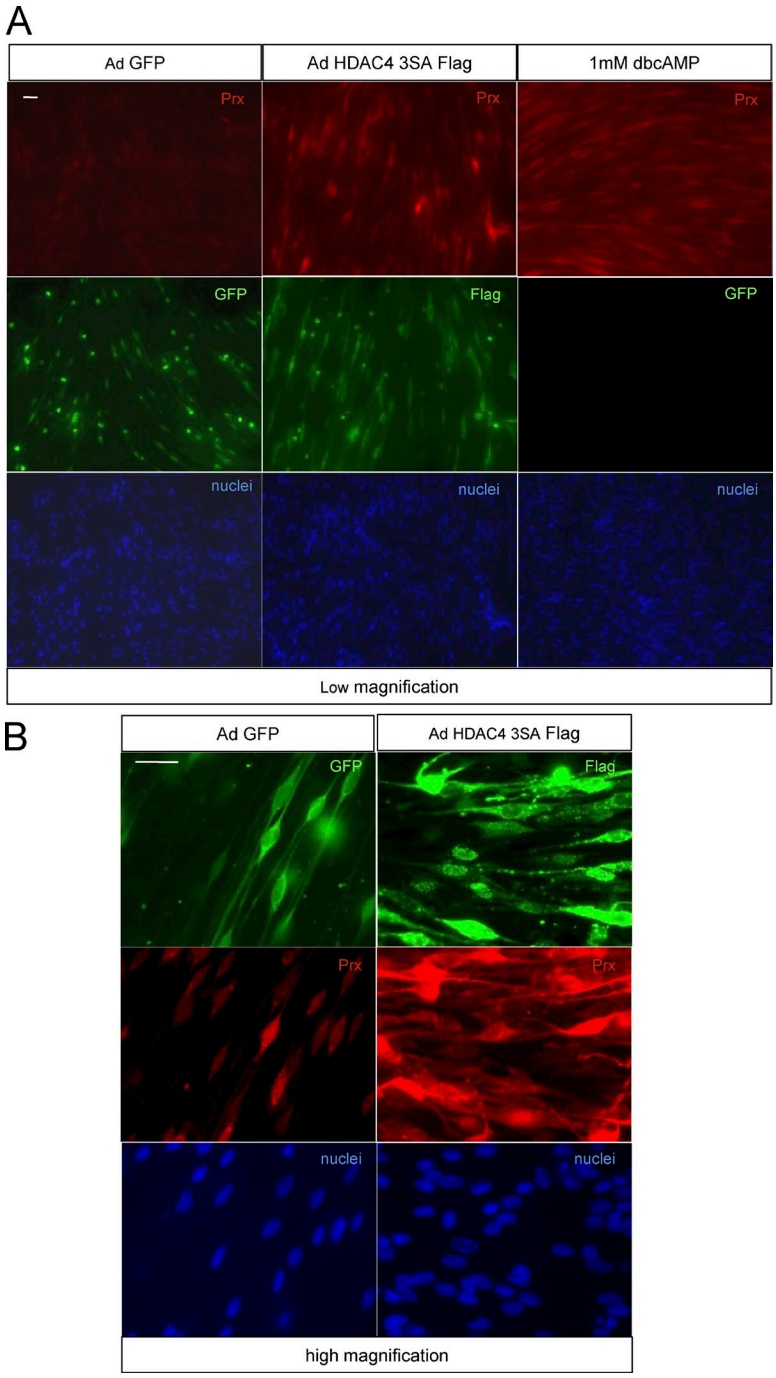

Figure S4. **Nuclear HDAC4 induces expression of Periaxin in Schwann cells.** Schwann cells were infected with Ad HDAC4 3SA Flag or Ad GFP. 72 h after infection, cells were incubated in SATO medium for 24 h, fixed, and submitted to anti-Periaxin immunofluorescence. Infected cells were identified with anti-GFP and anti-Flag antibodies. Nuclei were counterstained with Hoechst. **(A)** Low-magnifcation images with a panel of 1 mM dbcAMP–treated Schwann cells shown as a positive control. **(B)** High-magnification images of Ad GFP and Ad HDAC4 3SA Flag–infected cells. Bars, 25 µm.

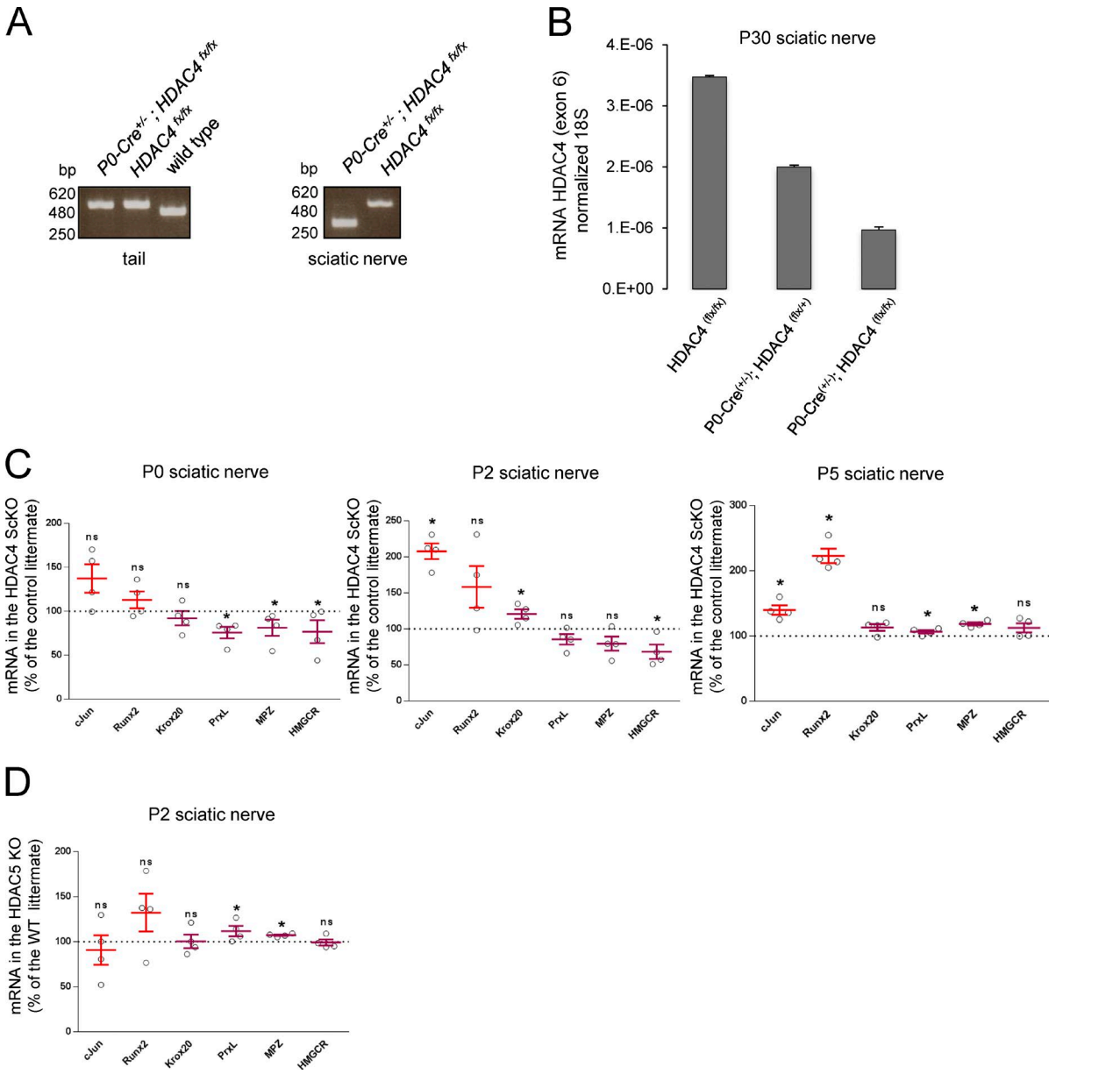

Figure S5. **In vivo elimination of class IIa HDACs in Schwann cells. (A)** Recombination of loxP sites of *HDAC4* in the sciatic nerves by the *P0-Cre* transgene. To detect exon deletion by recombination in Schwann cells in vivo, the sciatic nerves of a *P0-Cre*$^{(+/-)}$;*HDAC4*$^{(fx/fx)}$ and a *HDAC4*$^{(fx/fx)}$ littermate were removed and genomic DNA isolated. The tail DNA of these mice and one from a wild type were used as controls. PCR was performed and the products separated by agarose gel electrophoresis. As shown, a band of the expected size for the recombination (250 bp) was observed in the double mutant mouse and not in the floxed littermate. As expected, no recombination could be detected in the tail. **(B)** mRNA for *HDAC4* is decreased in the conditional KO. To explore if recombination causes a decrease in the expression of the *HDAC4* in conditional KO nerves, total RNA of the sciatic nerves of *P0-Cre*$^{(+/-)}$; *HDAC4*$^{(fx/fx)}$, *P0-Cre*$^{(+/-)}$; *HDAC4*$^{(fx/+)}$, and *HDAC4*$^{(fx/fx)}$ were obtained. mRNA was retrotranscribed to cDNA and SYBR green qPCR performed with specific primers for the *HDAC4* mRNA recombined sequence. The ratio of the relative expression for each gene to *18S* was calculated by using the $2\Delta^{CT}$ formula. As is shown, a relationship between gene dose and mRNA levels was observed. The residual expression of mRNA in the conditional KO probably comes from different nerve cell types not expected to express the Cre recombinase (such as fibroblasts, macrophages, vascular cells, and so forth) and/or Schwann cells with an incomplete recombination. Data are given as mean ± SE of six technical replicates. **(C)** Elimination of *HDAC4* in Schwann cells increases *c-Jun* and *Runx2* expression and has a partial impact in myelination markers. mRNA quantification for markers of nonmyelin- and myelin-forming cells in the PNS of *HDAC4* Schwann cell conditional KO. P0, P2, and P5 sciatic nerves were removed and total RNA extracted. RT-qPCR with mouse-specific primers for the indicated genes was performed and normalized to 18S rRNA. The graph shows the percentage of mRNA for each gene in the *P0-Cre*$^{(+/-)}$; *HDAC4*$^{(flx/flx)}$ normalized for the control *P0-Cre*$^{(-/-)}$; *HDAC4*$^{(flx/flx)}$ littermates. Mice from four different litters were evaluated per genotype. Data were analyzed with the Kolmogorov-Smirnov test. A scatter plot is shown with the results obtained in each experiment, which include the mean ± SE. **(D)** mRNA quantification for markers of nonmyelin- and myelin-forming cells in the PNS of *HDAC5*$^{(-/-)}$ mice. P2 sciatic nerves were removed and total RNA extracted. RT-qPCR with mouse-specific primers for the indicated genes was performed and normalized to *18S rRNA*. The graph shows the percentage of mRNA for each gene in the *HDAC5*$^{(-/-)}$ normalized for the control *HDAC5*$^{(+/+)}$ littermates. No changes in negative regulators of myelination were found. Also, no changes or a slight increase in myelin genes was observed. Mice from four different litters were evaluated per genotype. Data were analyzed with the Kolmogorov-Smirnov test. A scatter plot is shown with the results obtained in each experiment, which include the mean ± SE. *, $P < 0.05$.

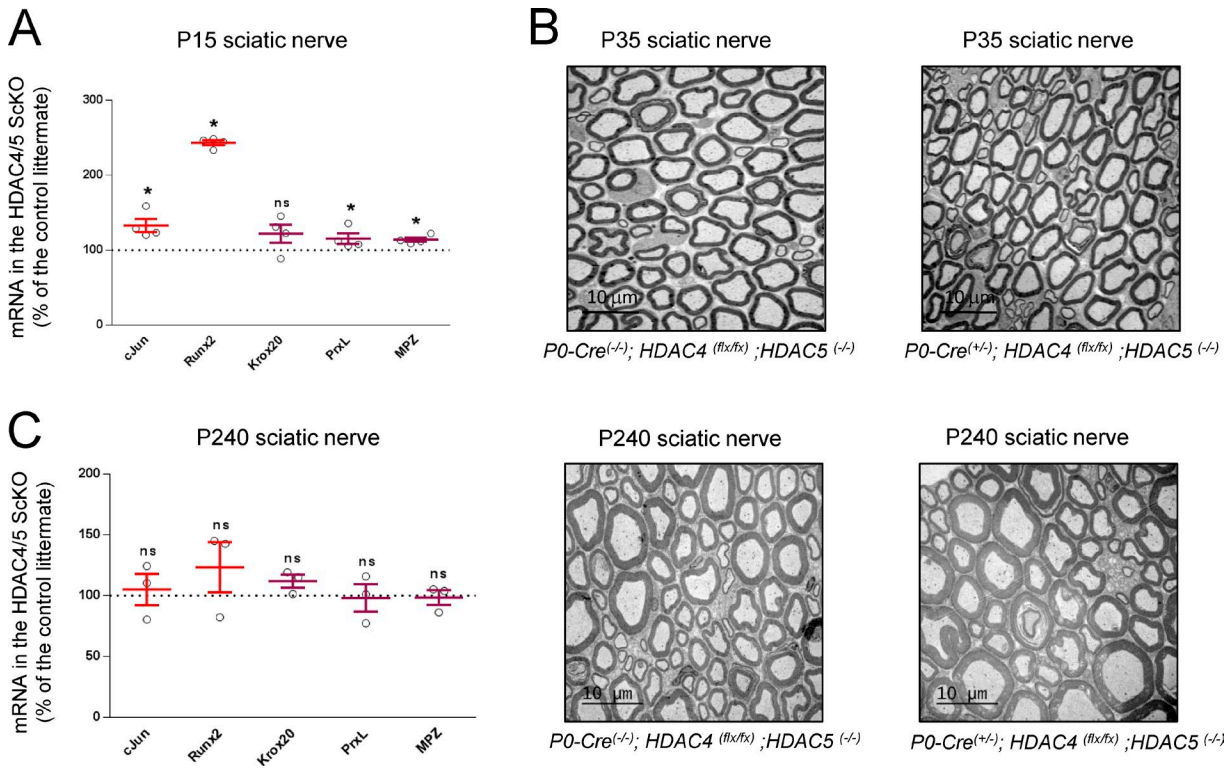

Figure S6. **Myelination in the *HDAC4/5 ScKO* is not changed at older ages. (A)** Although at P15, *c-Jun* and *Runx2* are still elevated, no change or a slight increase in the expression of myelin genes was observed in the nerves of the *HDAC4/5 ScKO*. **(B)** Myelination is morphologically indistinguishable at P35. **(C)** Older mice (P240) showed no change in either negative or positive regulators of myelination or myelin gross morphology. P15 and P240 sciatic nerves were removed and total RNA extracted. RT-qPCR with mouse-specific primers for the indicated genes was performed and normalized to *18S rRNA*. The graph shows the percentage of mRNA for each gene in the *P0-Cre*$^{(+/-)}$; *HDAC4*$^{(flx/flx)}$; *HDAC5*$^{(-/-)}$ normalized for the control *P0-Cre*$^{(-/-)}$; *HDAC4* $^{flx/flx}$; *HDAC5*$^{(-/-)}$ littermates. Mice from three to four different litters were evaluated per genotype. Data were analyzed with the Kolmogorov-Smirnov test. A scatter plot is shown with the results obtained in each experiment, which include the mean ± SE. *, P < 0.05. Transmission electron microscopy images from the sciatic nerves of different ages are also shown. Bars, 10 µm.

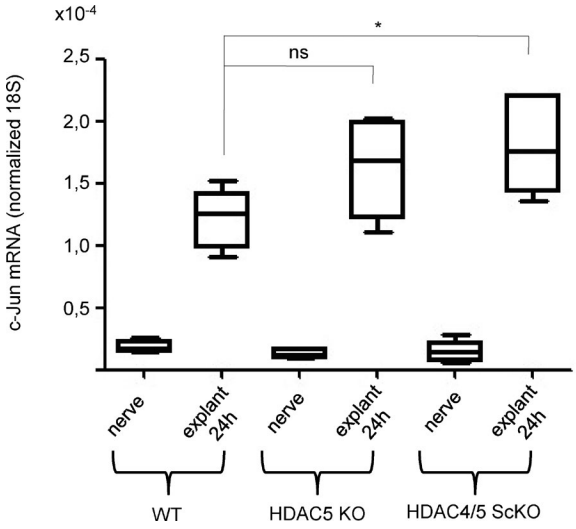

Figure S7. ***HDAC4*and *HDAC5* are rate-limiting for c-Jun induction after injury.** Nerve explants from *HDAC4/5 SCKO*, controls (*HDAC5 KO*), and C57BL/6J mice were incubated in DMEM with 5% FBS for 24 h. Total RNA was extracted and *c-Jun* mRNA determined by RT-qPCR. RNA from nonincubated contralateral sciatic nerve was also extracted to determine the mRNA for c-Jun in the noninjured nerve. A Tukey's box plot is shown. Data from five different experiments were analyzed with the one-way ANOVA, Tukey's multiple comparisons test. *, P < 0.05.

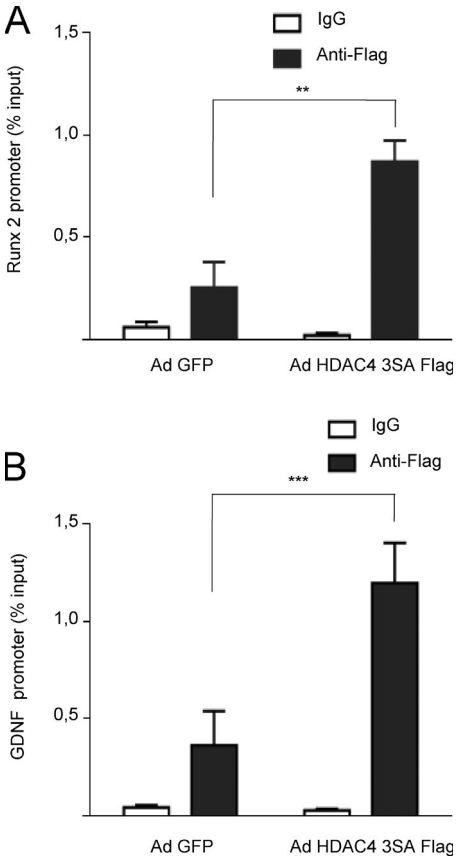

Figure S8. **HDAC4 binds to *Runx2* and *Gdnf* promoters in Schwann cells.** Schwann cells infected with Ad HDAC4 3SA Flag or Ad GFP were cross-linked with PFA. Chromatin was purified and immunoprecipitated with anti-Flag monoclonal antibody or a nonspecific mouse IgG (ChIP grade). qPCR was performed with specific primers for the promoter region of *Runx2* (A) and *Gdnf* (B). As shown, the recovery of *Runx2* and *Gdnf* promoter regions in the immunoprecipiates was enhanced in the HDAC4 3SA Flag–expressing Schwann cells. Nonsignificant recovery was obtained with the nonspecific IgG. Data from three different experiments are given as mean ± SE and analyzed with the paired *t* test (two-sided). **, P < 0.01; ***, P < 0.001.

Table S1. **List of antibodies and dilutions used**

Ab	Reference	Company	Species	Dilution	
				IF	WB
c-Jun	9165	Cell Signaling	Rabbit	1:300	1:1,000
Flag M2	F1804	Sigma-Aldrich	Mouse	1:500	1:500
GAPDH	G9545	Sigma-Aldrich	Rabbit	—	1:5,000
GFP	Ab13970	Abcam	Chicken	1:2,000	—
GFP	SC9996	Santa Cruz Biotechnology	Mouse	1:1,000	1:500
H3K9Ac	Ab12179	Abcam	Mouse	—	1:500
HDAC4	SC5245	Santa Cruz Biotechnology	Goat	—	1:500
HDAC4	H0163	Sigma-Aldrich	Mouse	—	1:500
HDAC4	SC-11418	Santa Cruz Biotechnology	Rabbit	1:200	—
IgG	M7769	Sigma-Aldrich	Mouse	—	—
Ki67	Ab15580	Abcam	Rabbit	1:100	—
Krox20	PRB-236P	Covance	Rabbit	1:100	—
Mef2	SC-313	Santa Cruz Biotechnology	Rabbit	1:200	—
Periaxin	Gillespie et al., 1994	Brophy's Laboratory	Rabbit	1:5,000	1:2,000
Sox-10	AF2864	R&D Systems	Goat	1:100	—
Alexa Fluor 488 Donkey anti–chicken	703-545-155	Jackson ImmunoResearch	Donkey	1:1,000	—
Alexa Fluor 488 goat anti–chicken	A11039	Molecular Probes	Goat	1:1,000	—
Alexa Fluor 488 goat anti–mouse	A11001	Molecular Probes	Goat	1:1,000	—
Alexa Fluor 488 donkey anti–mouse	A21202	Molecular Probes	Donkey	1:1,000	—
Alexa Fluor 594 goat anti–mouse	A11005	Molecular Probes	Goat	1:1,000	—
Alexa Fluor 488 goat anti–rabbit	A11008	Molecular Probes	Goat	1:1,000	—
Alexa Fluor 488 donkey anti–rabbit	A21206	Molecular Probes	Donkey	1:1,000	—
Alexa Fluor 546 goat anti–rabbit	A11010	Molecular Probes	Goat	1:1,000	—
Alexa Fluor 488 chicken anti–goat	A21467	Molecular Probes	Chicken	1:1,000	—
Alexa Fluor 555 donkey anti–goat	A21432	Molecular Probes	Donkey	1:1,000	—

IF, immunofluorescence; WB, Western blot; —, not applicable.

Table S2. **List of primers used**

Primers	Species	Sequence (5′–3′)	
18S	Mouse	Sense	CGGCTACCACATCCAAGGAA
		Antisense	GCTGGAATTACCGCGGCT
c-Jun	Mouse	Sense	CCTTCTACGACGATGCCCTC
		Antisense	GGTTCAAGGTCATGCTCTGTTT
DRP2	Mouse	Sense	TGTCCCCAGCCTCAGGTTA
		Antisense	CAACAGTCCCGTTCAAGAGC
HDAC4 deleted exon	Mouse	Sense	AATGCAGTGGTTCAGGTT
		Antisense	AGCACAGAGGTGAAGATG
GDNF	Mouse	Sense	GATTCGGGCCACTTGGAGTT
		Antisense	ATCTTAGAGTCCCGTCCGGC
HDAC4	Mouse	Sense	CATTGGAGGAGCTGCAGACA
		Antisense	GGAAGCCTGACGAACACTGA
HDAC5	Mouse	Sense	GGCATGAACTCTCCCAACGA
		Antisense	CTTCACCTCCACTGCCACAG
HDAC7	Mouse	Sense	CACCTGTCAGACCCAAGTCC
		Antisense	TGCTTGCTGTTGTCTCCACA
HDAC9	Mouse	Sense	TGCAGCAATAAGGAAAAGGCTG
		Antisense	ATGGTTGTTCACGTGGCAATG
HMGCR	Mouse	Sense	TGGATCGAAGGACGAGGAAAG
		Antisense	GAATTACGTCAACCATAGCTTCCG
Krox20	Mouse	Sense	ACCCCTGGATCTCCCGTATC
		Antisense	CAGGGTACTGTGGGTCAATGG
MAG	Mouse	Sense	GGAATCAGGAGACATCCCCAA
		Antisense	TTATCCAAAACAGCGGCAGG
MBP	Mouse	Sense	ATCCAAGTACCTGGCCACAG
		Antisense	CCTGTCACCGCTAAAGAAGC
MPZ	Mouse	Sense	ACCAGACATAGTGGGCAAGACCTC
		Antisense	AAGAGCAACAGCAGCAACAGCACC
PMP22	Mouse	Sense	GCTCTGTTCCTGTTCTTCTGCC
		Antisense	CACTGTGCCTCACTGTGTAGAT
Prx	Mouse	Sense	AGTGGCCAAGCTGAACATCC
		Antisense	AGAACTCGACGTCAACAGGG
Runx2	Mouse	Sense	GTCTTCCACACGGGGCAC
		Antisense	GCCAGAGGCAGAAGTCAGAG
18S	Rat	Sense	CTTAGAGGGACAAGTGGCG
		Antisense	GGACATCTAAGGGCATCACA
Artemin	Rat	Sense	ATCCATTTGAGCTTCGGGGG
		Antisense	CCACCCTCTTCTTGAGGCAG
ChIP cJun	Rat	Sense	TGAGTGCAAGCGGTGTCTTA
		Antisense	GTCCCCGCTTCAGTAACAAA
ChIP GDNF	Rat	Sense	CCATGAATCGGGAGTAGGAA
		Antisense	CCGGTCAAAGAGCACAAACT
ChIP Runx2	Rat	Sense	CCACCCAGCTGCTTGTACTT
		Antisense	TTCACATTCACTGCCCTCAG
cJun	Rat	Sense	AAGAACACAAAGCAGGGAGG

Table S2. **List of primers used *(Continued)***

Primers	Species	Sequence (5′–3′)	
		Antisense	GGGAGTTCATCCGCAATCTA
DRP2	Rat	Sense	GAGAAGATCCTGGCCCATTT
		Antisense	CCTCAGCTCTCCCTGAAGAA
GDNF	Rat	Sense	ACTGACTTGGGTTTGGGCTA
		Antisense	CCTGGCCTACCTTGTCACTT
HDAC4	Rat	Sense	GACAGCTCGCTGACCTCC
		Antisense	CCACTACACAGCCTACAGCC
HDAC5	Rat	Sense	GGTCGTAAAGCCACACTGGA
		Antisense	TCCAGCTTCTGCCGGTTAAG
HDAC7	Rat	Sense	CACCTGTCAGACCCAAGTCC
		Antisense	TGCTTGCTGTTGTCTCCACA
HDAC9	Rat	Sense	CCCAGCATCTGACCTCCAC
		Antisense	GAGCCAAGAGCTGCTCCC
HMGCR	Rat	Sense	TTGGTGGCCTCCATTGAGAT
		Antisense	AGAGGCCATGCATACGGAAA
Krox20	Rat	Sense	CCCAATGGTGAACTGGGAGG
		Antisense	TCCAAGGGCCTCTTCTCTCC
MBP	Rat	Sense	TCCATCCCAAGGAAAGGGGA
		Antisense	TCTGCCTCCGTAGCCAAATC
MPZ	Rat	Sense	TGCCCTGCTCTTCTCTTCTTT
		Antisense	CCATAGACTTCCCTGTCCGTG
Olig1	Rat	Sense	TGCGCGAAGTTATCCTACCC
		Antisense	CAGCGTAGCGATCTTGGAGA
PLP	Rat	Sense	GGCTAGGACATCCCGACAAG
		Antisense	TGTACACAGGCACAGCAGAG
PMP22	Rat	Sense	TTGCAAAGAAATCCAAGCGGA
		Antisense	AGAGTAGAAGCATGGTGGCTG
Prx	Rat	Sense	AATGTGCCGAGCCCTACAAG
		Antisense	AGGGGACAGACTCTGGATGT
Runx2	Rat	Sense	GCACCCAGCCCATAATAGAA
		Antisense	TGGAGATGTTGCTCTGTTCG
Sox10	Rat	Sense	GCAGAAAGTTAGCCGACCAG
		Antisense	GCGCTTGTCACTCTCGTTCA
P0 Cre genotyping	Mouse	Sense	CCACCACCTCTCTCCATTGCAC
		Antisense	GCTGGCCCAAATGTTGCTGG
HDAC4 floxed genotyping	Mouse	Sense	ATCTGCCCACCAGAGTATGTG
		Antisense	CTTGTTGAGAACAAACTCCTGCAGCT
		Reverse	CTCCAATTCTCCACAAGACAGC
HDAC5 KO genotyping	Mouse	Sense	CAAGGCCTTGTGCATGCTGGGCTGG
		Antisense	CTGCTCCCGTAGCGCAGGGTCCATG
		Reverse	GCCCGTTTGAGGGGACGACGACAGTATTCG
HDAC4 L175A mutagenesis	Human	Sense	GAAGTGAAGATGAAGGCACAAGAATTTGTCCTC
		Antisense	GAGGACAAATTCTTGTGCCTTCATCTTCACTTC
HDAC4 V179A mutagenesis	Human	Sense	GTTACAAGAATTTGCCCTCAATAAAAAGAAGG
		Antisense	CCTTCTTTTTATTGAGGGCAAATTCTTGTAAC

Table S2. **List of primers used *(Continued)***

Primers	Species	Sequence (5′–3′)	
HDAC4 D934N mutagenesis	Human	Sense	CCCCCTGGACACGCTGCGGAGGA
		Antisense	GCTCTCCTCCGCAGCGTGTCCAG
HDAC4 H803A mutagenesis	Human	Sense	GTGTCATCAGGCTTCAATGCCGTGGAGGGC
		Antisense	GCCCTCCACGGCATTGAAGCCTGATGACAC

References

Gillespie, C.S., D.L. Sherman, G.E. Blair, and P.J. Brophy. 1994. Periaxin, a novel protein of myelinating Schwann cells with a possible role in axonal ensheathment. *Neuron*. 12:497–508. https://doi.org/10.1016/0896-6273(94)90208-9