Zeb2 recruits HDAC–NuRD to inhibit Notch and controls Schwann cell differentiation and remyelination

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The mechanisms that coordinate and balance a complex network of opposing regulators to control Schwann cell (SC) differentiation remain elusive. Here we demonstrate that zinc-finger E-box-binding homeobox 2 (Zeb2, also called Sip1) transcription factor is a critical intrinsic timer that controls the onset of SC differentiation by recruiting histone deacetylases HDAC 1 and 2 (HDAC1/2) and nucleosome remodeling and deacetylase complex (NuRD) co-repressor complexes in mice. *Zeb2* deletion arrests SCs at an undifferentiated state during peripheral nerve development and inhibits remyelination after injury. Zeb2 antagonizes inhibitory effectors including Notch and Sox2. Importantly, genome-wide transcriptome analysis reveals a Zeb2 target gene encoding the Notch effector Hey2 as a potent inhibitor for Schwann cell differentiation. Strikingly, a genetic *Zeb2* variant associated with Mowat-Wilson syndrome disrupts the interaction with HDAC1/2–NuRD and abolishes Zeb2 activity for SC differentiation. Therefore, Zeb2 controls SC maturation by recruiting HDAC1/2–NuRD complexes and inhibiting a Notch–Hey2 signaling axis, pointing to the critical role of HDAC1/2–NuRD activity in peripheral neuropathies caused by *ZEB2* mutations.

Schwann cells of the peripheral nervous system (PNS) ensheath and wrap myelin membranes around axons to maximize the efficiency of rapid saltatory conduction of action potentials. Functional defects of SCs contribute to various forms of peripheral neuropathies^{1–3}. A recently defined autosomal dominant disorder, Mowat-Wilson syndrome (MOWS), is caused by mutations in *ZEB2* (also called *SIP1* or *ZFHX1B*), the gene that encodes zinc finger E-box-binding homeobox 2 protein. MOWS is characterized by a wide spectrum of congenital anomalies encompassing growth defects, intellectual disability, delayed motor development and neurocristopathies with defects in neural crest derivatives^{4–6}. In mice, Zeb2 is critical for neurogenesis and gliogenesis in the CNS^{7–9}. Recent studies indicate that Zeb2 can interact with NuRD, suggesting that NuRD association may be important to Zeb2 functions^{4,10}. At present, whether and how Zeb2 controls SC differentiation and myelination remain elusive. Elucidation of Zeb2 functions in peripheral nerve cell development in addition to CNS neurogenesis may provide a better understanding of the etiology of neurological diseases associated with MOWS caused by *ZEB2* mutations.

Development and myelination of neural-crest-derived SCs are coordinately regulated by a spectrum of extracellular signals and intrinsic regulators. These comprise positive cues including neuregulin 1 (NRG1)^{11,12} and laminin–integrin signaling^{13,14},

G-protein-coupled receptors (GPCRs) such as Gpr126 (ref. 15) and negative cues including Notch signaling and Sox2 (ref. 16). Timely initiation of SC myelination not only demands a strict interplay between extrinsic cues and intracellular inputs, but also requires the precise balance of the activity of an intricate network of transcriptional regulators. Transcription factors such as the high mobility group (HMG)-type protein Sox10, the Pit-Oct-Unc (POU)-homeodomain proteins Oct6 (also called Pou3f1) and Pou3f2 (also called Brn2), and the zinc-finger proteins Krox20 (also called Egr2) and YY1 are crucial for promoting SC myelination^{17–20}. How these multiple signaling pathways converge and coordinate the myelination program and, more importantly, what orchestrates the fine-tuning of proper transcriptional outputs are not fully understood.

Here, using SC-lineage-specific *in vivo* mutagenesis, we identified Zeb2 as a critical intrinsic timer controlling the onset of SC differentiation during normal myelination and also in remyelination after peripheral nerve injury. The predominant effects of Zeb2 during SC development are to directly repress a network of inhibitory pathways including Sox2 and Notch–Hey2 signaling. We further find that a single amino acid variant in ZEB2 seen in a patient with MOWS disrupts interaction with the HDAC1/2–NuRD co-repressor complex and abolishes the ability of ZEB2 to effect SC differentiation. Our data thus establish a critical role for HDAC1/2–NuRD-dependent

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Zeb2 activity in initiating SC differentiation and myelination through opposing Notch- and Sox2-mediated negative regulatory circuitry.

RESULTS

Dynamic Zeb2 expression during SC lineage development

To examine Zeb2 expression in SCs, we coimmunolabeled for Zeb2 and for SC lineage marker Sox10 in developing sciatic nerves (Fig. 1a). The vast majority of Zeb2-positive cells were also Sox10-positive at postnatal day (P) 7. In keeping with its function as transcription regulator, Zeb2 was detected in the nuclei of Sox10⁺ SCs. Concurrent with the onset of myelination in the developing nerves, Zeb2 was highly expressed in SCs during the first 2 postnatal weeks, and the signal gradually declined as the animal reaches adulthood at 10 weeks (Fig. 1a).

To further define Zeb2 levels during SC lineage progression, we examined Zeb2 mRNA expression using SC-enriched sciatic nerves at different stages by real-time quantitative PCR (qPCR). Consistently, Zeb2 transcripts were detected at the neonatal stage P0 and peaked at the perinatal stage P10, but then were reduced in adulthood (Fig. 1b).

Zeb2 mutant mice display severe motor deficits

To determine the cell-autonomous function of Zeb2 in SC development, we generated conditional mutant mice lacking functional Zeb2 in SCs by breeding mice carrying a Zeb2 loxP-flanked allele (Zeb2^{loxP/loxP})^{8,21} with a SC-expressing Cre line driven by the Desert

hedgehog (Dhh) promoter¹⁷ (Fig. 1c). We used Zeb2^{loxP/+};Dhh-cre or Zeb2^{loxP/loxP} littermates as controls since they were phenotypically normal in SC development (Supplementary Fig. 1a–g).

Cre-mediated inactivation of Zeb2 efficiently abrogated Zeb2 presence in Sox10⁺ SCs in peripheral nerves (Fig. 1d). Zeb2^{loxP/loxP}; Dhh-cre (hereafter referred to as Zeb2 cKO) mice were born in numbers predicted by Mendelian ratios. During the first postnatal week, mutant mice were essentially indistinguishable from littermate controls. Starting in the second week, however, all Zeb2 cKO mice began to develop severe tremor, unsteady gait and hindlimb paralysis, and the majority died by 8 weeks (Supplementary Fig. 2a).

Upon tail suspension, mutant mice at P21 showed abnormal hindlimb clasp (Fig. 1e). Zeb2 cKO sciatic nerves at P7 exhibited a thin and translucent appearance, in stark contrast to the thick, opaque nerves of control mice, suggesting a severe deficit at the onset of myelogenesis (Fig. 1f). Consistent with the overt clinical signs of hypomyelination in the Zeb2 cKO mutants, the motor unit function of mutant mice was severely impaired, as reflected by a marked reduction in conduction velocity (Fig. 1g) measured by electrophysiological recordings of compound muscle action potentials (CMAPs) during electrical stimulation of the sciatic nerve *in vivo*. The nerve conduction velocity was reduced from 27.7 ± 2.7 m s⁻¹ in littermate controls to 1.8 ± 0.1 m s⁻¹ in the mutants at the young adult stage (P42), which is close to the level of the slower, thinly myelinated Aδ or unmyelinated C fiber conduction velocity in mice²² (Fig. 1h). Furthermore, the mean peak amplitudes and duration of CMAPs

Figure 1 SC-specific deletion of Zeb2 results in peripheral nerve hypomyelination. (a) Co-localization of Zeb2 (green) with Sox10 (red) in SC nuclei from 1 to 10 weeks (wk) by immunofluorescence labeling. Scale bar, 100 μm. The experiment was repeated 3 times on 3 control and mutant tissues. (b) qPCR showing Zeb2 transcript levels in mouse sciatic nerves at various developmental age. $n = 3$ control tissues at each time point; mean $\pm$ s.e.m.; *** $P < 0.001$; one-way ANOVA with multiple comparisons test; $P_{(P10)} = 0.0003$, $P_{(P20)} = 0.0009$, $P_{(P60)} < 0.0001$; $F_{3,12} = 61.91$. (c) Exon 7 of the loxP-flanked Zeb2 allele is excised by Dhh-cre. (d) Colabeling of Zeb2 (green) with Sox10⁺ (red) SC nuclei in mutant sciatic nerves at P7. Scale bar, 50 μm; $n = 3$ control and mutant tissues. (e) Tail suspension test showing abnormal hindlimb reflex in Zeb2 cKO (Zeb2^{loxP/loxP}; Dhh-cre^{+/+}) but not in a control littermate (Zeb2^{loxP/+}; Dhh-cre^{+/+}) at P21. (f) Sciatic nerves from control and Zeb2 cKO mice at P7. (g) CMAPs from P42 control and Zeb2 cKO. S, stimulus (black arrows); R, initiation of CMAP response (red arrows). (h) Nerve conduction velocities in Zeb2 cKO (mutant) and control littermates at P42. Data are presented as mean $\pm$ s.e.m. $n = 4$ control and mutant tissues; *** $P < 0.001$, $t = 9.538$, d.f. = 6; two-tailed unpaired Student's t -test. (i) Mean peak amplitudes of CMAPs in sciatic nerves of control and Zeb2 cKO (mutant) mice at P42. Data are presented as mean $\pm$ s.e.m. $n = 4$ control and mutant tissues; * $P < 0.05$; $P = 0.0417$, $t = 2.582$, d.f. = 6; two-tailed unpaired Student's t -test. (j) Average durations of CMAPs in sciatic nerves of control and Zeb2 cKO (mutant) mice at P42. Data are presented as mean $\pm$ s.e.m. ($n = 4$ control and mutant tissues; * $P < 0.05$; $P = 0.0155$, $t = 3.348$, d.f. = 6; two-tailed unpaired Student's t -test).

were also severely affected in *Zeb2* cKO mice (Fig. 1i,j), accounting for the defect in motor function in *Zeb2* mutants.

Zeb2-deficient SCs fail to myelinate in peripheral nerves

We evaluated myelinogenesis in *Zeb2* cKO peripheral nerves by myelin basic protein (MBP) immunolabeling. In contrast to the robust expression in control nerves, MBP was scarcely expressed in the mutant sciatic nerves at P14 (Fig. 2a). Accordingly, expression of the major myelin genes *Mbp* and *Mpz* in sciatic nerves was virtually undetectable in mutants by *in situ* hybridization (Fig. 2b) and qPCR (Fig. 2c). Ultrastructural examination by electron microscopy (EM) revealed that, whereas by P4 control SCs had established a 1:1 relationship with large-caliber axons and begun wrapping myelin membrane around axons, the majority of mutant SCs were associated with multiple axons and failed to initiate myelination, indicative of incomplete axonal sorting and a dysmyelination phenotype (Fig. 2d). At P21, axon bundles ensheathed by *Zeb2* cKO SCs remained essentially unsorted and unmyelinated (Fig. 2e,f), despite apparent normal SC process extension around associated axons (Fig. 2f). Moreover, small-diameter axons remained unsegregated in axon bundles with large diameter axons, further confirming sorting defects in *Zeb2* cKO (Supplementary Fig. 1c). However, in testing for cutaneous thermal hypersensitivity, *Zeb2* cKO showed a similar decrease in withdrawal latency to 50 °C water following tissue inflammation compared to their baseline responsiveness, as was seen with littermate controls (Supplementary Fig. 1h).

Figure 2 *Zeb2* is required for SC myelination. (a) MBP staining in P14 control and *Zeb2* cKO longitudinal sciatic nerves ($n = 3$ control and mutant tissues). Scale bars, 50 μ m. (b) Expression of *Mbp* and *Mpz* in transverse sections of sciatic nerves from control and *Zeb2* cKO mice ($n = 3$ control and mutant tissues) at P21 by *in situ* hybridization. Scale bars, 200 μ m. (c) qPCR showing the levels of myelin-related genes in sciatic nerves of P7 control and *Zeb2* cKO mice. Data are presented as mean $\pm$ s.e.m. *** $P < 0.0001$, $n = 3$ control and 3 mutant tissues, $P_{(Prx)} < 0.0001$, $t = 2,750$, d.f. = 4; $P_{(Mbp)} < 0.0001$, $t = 512.5$, d.f. = 4; $P_{(Mpz)} < 0.0001$, $t = 1,088$, d.f. = 4; two-tailed unpaired Student's t -test. (d,e) Representative EM images showing ultrastructure of control and *Zeb2* cKO sciatic nerves at P4 (d) and P21 (e). $n = 3$ control and mutant tissues. Arrowheads indicate axons and SC-axon units, respectively. Scale bars, 2 μ m. (f) EM images showing a 1:1 SC-axon unit in a P21 control sciatic nerve (top) and a SC (S) associating with multiple axons in a mutant nerve (bottom). $n = 3$ control and mutant tissues. Arrow indicates mutant SC process surrounding an axon. Scale bars, 500 nm. (g) Representative EM images of P60 control and *Zeb2* cKO sciatic nerves. $n = 3$ control and mutant tissues. Scale bars, 2 μ m. (h) Tamoxifen (TAM) administration scheme: pups were injected with TAM via intraperitoneal injections (i.p.) into lactating dams once per day from P0 to P4, and sciatic nerves were collected on P14. (i) EM images of cross sections of P14 sciatic nerves from control (*Zeb2*^{loxP/+}; *Plp1*-cre^{ERT}; $n = 3$) and *Zeb2* iKO mice (*Zeb2*^{loxP/loxP}; *Plp1*-cre^{ERT}; $n = 4$) induced by TAM from P0–4. Arrows denote incompletely sorted axon bundles. Scale bars, 2 μ m. (j) Myelinated axons per cross section of P14 sciatic nerves from control and TAM-treated *Zeb2* iKO. Data are presented as mean $\pm$ s.e.m.; $n = 3$ controls and 4 mutants; two-tailed unpaired Student's t -test. ** $P < 0.01$; $P = 0.0062$, $t = 4.529$; d.f. = 5.

Very few mutant mice survived to adulthood (Supplementary Fig. 2a). The gross structure of peripheral nerves in rare *Zeb2* cKO survivors at 2 months showed minifascicles of SCs and axons with no apparent sign of myelination, suggesting that *Zeb2* loss in SCs leads to a sustained myelination block in peripheral nerves (Fig. 2g).

To bypass the possible influence of the *Zeb2* loss during early SC development, we inactivated *Zeb2* in the peripheral nerves of neonates using a tamoxifen-inducible *Plp1*-cre^{ERT} driver²³ with tamoxifen administration from P0 to P4 (Fig. 2h). By P14, more than half of the large axons were not properly sorted and remained unmyelinated in *Zeb2* inducible knockout (iKO; *Zeb2*^{loxP/loxP}; *Plp1*-cre^{ERT}) mutant nerves (Fig. 2i,j). The ultrastructural observations seen upon *Zeb2* deletion in immature SCs suggest that *Zeb2* is required for proper initiation of SC differentiation and myelination.

The diminished expression of *Zeb2* in mature nerves (Fig. 1a,b) suggested a nonobligatory function in myelin maintenance. Accordingly, tamoxifen-induced deletion of *Zeb2* in adult mice altered neither the myelin sheath's thickness nor its integrity in peripheral nerves (Supplementary Fig. 2b), suggesting a crucial role for *Zeb2* in initiating or priming SC differentiation rather than maintaining myelin sheaths.

Zeb2 controls myelinogenic programs for SC differentiation

In *Zeb2* cKO sciatic nerves at P7, we detected reduced levels of mRNAs encoding SC prodifferentiation regulators including Sox10, Krox20 and Oct6 (Fig. 3a). In contrast, among known negative regulators, we

Figure 3 Zeb2 deletion in SCs inhibits SC differentiation and myelination. (a) qPCR showing levels of promyelinating transcriptional regulators in sciatic nerves of P7 control and *Zeb2* cKO mice. Data are presented as mean $\pm$ s.e.m. *** $P < 0.001$, $n = 3$ control and mutant tissues, $P_{(Sox10)} < 0.0001$, $t = 101.4$, d.f. = 4; $P_{(Krox20)} < 0.0001$, $t = 291.8$, d.f. = 4; $P_{(Oct6)} < 0.0001$, $t = 14,865$, d.f. = 4; two-tailed unpaired Student's t -test. (b) qPCR showing the levels of negative myelination regulators in control and mutant sciatic nerves at P7. Data are presented as mean $\pm$ s.e.m. * $P < 0.05$, $n = 3$ control and mutant tissues; $P_{(Hes1)} = 0.0308$, $t = 2.617$, d.f. = 8; $P_{(Hes5)} = 0.0081$, $t = 4.885$, d.f. = 4; $P_{(Id4)} = 0.0466$, $t = 2.846$, d.f. = 8; $P_{(Notch1)} = 0.2181$, $t = 1.376$, d.f. = 6; $P_{(Jag1)} = 0.0496$, $t = 2.784$, d.f. = 4; two-tailed unpaired Student's t -test. (c) Immunolabeling of promyelinating transcription factors Sox10, Krox20 and Oct6 in longitudinal sections of control and *Zeb2* cKO sciatic nerves ($n = 3$ animals) at P7. Scale bars, 100 μ m. (d) Quantification of Sox10⁺, Krox20⁺ and Oct6⁺ cells relative to DAPI⁺ nuclei in control and *Zeb2* cKO sciatic nerves (longitudinal sections) at P1. Data are presented as mean $\pm$ s.e.m. ** $P < 0.01$; *** $P < 0.001$, $n = 4$ control and 6 mutant tissues for Sox10; $n = 3$ control and mutant tissues for Krox20 and Oct6, $P_{(Sox10)} = 0.4801$, $t = 0.7406$, d.f. = 8; $P_{(Krox20)} = 0.0072$, $t = 5.052$, d.f. = 4; $P_{(Oct6)} < 0.0001$, $t = 27.75$, d.f. = 4; two-tailed unpaired Student's t -test. (e) Quantification of Sox10⁺, Krox20⁺ and Oct6⁺ cells relative to DAPI⁺ nuclei in control and *Zeb2* cKO sciatic nerves at P7. Data are presented as mean $\pm$ s.e.m. * $P < 0.05$; *** $P < 0.001$, $n = 4$ control and mutant tissues for Sox10; $n = 3$ control and mutant tissues for Krox20 and Oct6; $P_{(Sox10)} = 0.0184$, $t = 3.120$, d.f. = 6; $P_{(Krox20)} < 0.0001$, $t = 23.3$, d.f. = 4; $P_{(Oct6)} < 0.0001$, $t = 16.4$, d.f. = 4; two-tailed unpaired Student's t -test. (f) Immunofluorescence labeling for Ki67 in control and *Zeb2* cKO sciatic nerves ($n = 3$ animals per genotype) at P28. Ki67, green; Sox10, red. Scale bars, 50 μ m. (g) Quantification of cell proliferation by Ki67 positive nuclei in control and mutant sciatic nerves at P28. Data are presented as mean $\pm$ s.e.m.; ** $P < 0.01$, $n = 3$ control and mutant tissues; $P = 0.0013$, $t = 11.82$, d.f. = 4, two-tailed unpaired Student's t -test. (h) Quantification of the proliferation rate by BrdU-incorporated cells per Sox10⁺ SC in control and mutant sciatic nerves P7. $n = 3$ control and mutant tissues; Data are presented as mean $\pm$ s.e.m.; ** $P < 0.01$, $P = 0.0052$, $t = 5.527$, d.f. = 4; two-tailed unpaired Student's t -test. (i) Immunofluorescence labeling for cleaved caspase 3 in P1 control and *Zeb2* cKO sciatic nerves ($n = 3$ control and mutant tissues). Cleaved caspase 3 (cl. caspase 3), white arrows; DAPI, blue. Scale bars, 100 μ m. (j) Quantification of apoptosis by cl. caspase 3-labeled cells per 0.01 mm² in P1 control and mutant sciatic nerves. Data are presented as mean $\pm$ s.e.m., $n = 3$ control and mutant tissues, $P = 0.6334$, $t = 0.5155$, d.f. = 4; two-tailed unpaired Student's t -test. (k) qPCR showing *Sox10*, *Oct6* and *Krox20* expression in rat SCs induced to differentiate post-transfection with control and *Zeb2* vectors. Data are presented as mean $\pm$ s.e.m. * $P < 0.05$, ** $P < 0.01$; $n = 3$ independent experiments, $P_{(Sox10)} = 0.1826$, $t = 1.610$, d.f. = 4; $P_{(Krox20)} = 0.0011$, $t = 8.396$, d.f. = 4; $P_{(Oct6)} = 0.04811$, $t = 2.814$, d.f. = 4; two-tailed unpaired Student's t -test. (l) qPCR showing myelin gene expression in rat SCs transfected with control and *Zeb2* vectors. Data are presented as mean $\pm$ s.e.m. * $P < 0.05$, ** $P < 0.01$; $n = 3$ independent experiments, $P_{(Mbp)} = 0.0015$, $t = 7.775$, d.f. = 4; $P_{(Mbp)} = 0.0049$, $t = 5.626$, d.f. = 4; $P_{(Mag)} = 0.0184$, $t = 3.843$, d.f. = 4; $P_{(Cnp)} = 0.0169$, $t = 3.942$, d.f. = 4; two-tailed unpaired Student's t -test. (m) qPCR showing expression of negative regulatory genes in rat SCs transfected with control and *Zeb2* vectors. Data are presented as mean $\pm$ s.e.m. * $P < 0.05$, ** $P < 0.01$; $n = 3$ independent experiments; $P_{(Id4)} = 0.9738$, $t = 0.06492$, d.f. = 4; $P_{(Jag1)} = 0.9568$, $t = 0.05771$, d.f. = 4; $P_{(Hes1)} = 0.8649$, $t = 0.1814$, d.f. = 4; $P_{(Rbp1)} = 0.0105$, $t = 4.538$, d.f. = 4; $P_{(Notch1)} = 0.8643$, $t = 0.1822$, d.f. = 4; two-tailed unpaired Student's t -test.

observed an upregulation of *Jag1*, encoding a ligand for Notch, and *Hes1* (Fig. 3b). In step with their transcript levels, Oct6 and Krox20 proteins were essentially absent in mutant SCs in these *Zeb2* cKO sciatic nerves (Fig. 3c–e). We further investigated Zeb2 functions in SC differentiation by *Zeb2* siRNA knockdown in rat SC cultures, either in proliferation or differentiation medium. The expression of *Oct6*, *Krox20* and major myelin genes, as well as their corresponding proteins, was markedly reduced in SCs that underwent *Zeb2* knockdown (Supplementary Fig. 3a–d), suggesting a cell-autonomous requirement for Zeb2 in SC differentiation. In seeking to determine whether Zeb2 could regulate *Oct6* directly, we

identified two Zeb2 consensus-binding sites (CACCTG) (ref. 24) in the *Oct6* promoter and detected genomic occupancy of Zeb2 on the *Oct6* promoter in differentiating SCs but not in proliferating cells (Supplementary Fig. 3e).

The proportion of Sox10⁺ SCs among DAPI⁺ cells in mutant nerves at P1 was comparable to that in control nerves (Fig. 3d), despite a slight reduction in SC number at P7 (Fig. 3e), which is likely due to the overall reduction in mature SCs. Accordingly, the SC proliferation rate revealed by proliferative marker Ki67 staining and 5-bromo-2'-deoxyuridine (BrdU) incorporation appeared to increase substantially in *Zeb2* mutant sciatic nerves (Fig. 3f–h). Apoptosis of mutant SCs,

as determined by immunolabeling for cleaved caspase-3 in P1 sciatic nerves, was not significantly altered (Fig. 3i,j). This indicates that *Zeb2* deletion arrests SCs at their immature stage by blocking the transition from immature SCs to Oct6⁺Krox20⁺ differentiating SCs.

Conversely, *Zeb2* overexpression in SCs *in vitro* enhanced the induction of *Oct6* and *Krox20* (Fig. 3k) and myelin gene expression (Fig. 3l). Notably, we detected a substantial downregulation of the Notch effector *Rbpj* upon enforced *Zeb2* expression in SCs (Fig. 3m). Thus, our data suggests that *Zeb2* controls SC myelination by priming the transcriptional program that promotes SC differentiation, while attenuating the *Rbpj*-mediated Notch inhibitory pathway.

Zeb2 is required for SC differentiation during nerve repair

Although *Zeb2* was maintained at minimal levels in mature SCs, it was highly upregulated in SCs in regenerating sciatic nerves after injury (Fig. 4a,b). We hypothesized that *Zeb2* is required for SC differentiation and ultimately remyelination during nerve repair. To test this, we employed the peripheral nerve transection model (Fig. 4a). After transection, SCs dedifferentiate, proliferate, establish a tissue bridge between the proximal and distal nerve stumps, and then guide regenerating axons across the bridge to reach the distal stump²⁵, with subsequent SC differentiation and remyelination. To determine the role of *Zeb2* in SC differentiation following nerve injury, we treated control (*Zeb2^{loxP/loxP}* or *Zeb2^{loxP/+}*; *Plp1-cre^{ERT}*) or *Zeb2^{loxP/loxP}*; *Plp1-cre^{ERT}* (*Zeb2* iKO) mice at 4–6 weeks with tamoxifen before and after injury, which efficiently removed *Zeb2* from transected nerves in the *Zeb2* iKO mice (Fig. 4c). Sciatic nerves were removed for analysis 14 d after axotomy

to assess SC differentiation, by which time a tissue bridge would have formed and axons guided by associated SCs as regeneration units would have proceeded to grow into the distal stump^{25,26}. Here we focused our analysis on the regenerating site, defined as the SC-axon growth tip just proximal to the injury in the tissue bridge. In the regenerating region of *Zeb2* iKO nerves, proliferation of mutant SCs (Ki67⁺Sox10⁺ cells) was indistinguishable from that of control SCs, but the number of Sox10⁺ SCs was significantly reduced (Fig. 4d–f). Proliferating SCs were not observed in either control or mutant contralateral sciatic nerves (data not shown). In the regenerating nerve region, mutant SCs failed to differentiate properly, as revealed by a substantial decrease in Oct6⁺ or Krox20⁺ promyelinating SCs in mutant nerves (Fig. 4g–i).

We further assessed the extent of remyelination in control and *Zeb2* iKO sciatic nerves by EM 8 weeks after surgery. During nerve regeneration, little, if any, radial sorting is required. In line with this, SCs lacking *Zeb2* displayed normal association with axons in 1:1 ratios. While a majority of axons were remyelinated 8 weeks post injury in control nerves, *Zeb2* iKO SCs were blocked at the promyelinating stage and failed to remyelinate (Fig. 4j,k). Myelin profiles in *Zeb2* iKO nerves were scarce and axons that did show myelin profiles were primarily thinly myelinated. Together, our data suggest that *Zeb2* is essential for SC myelination both in development and nerve regeneration.

Zeb2 represses SC differentiation inhibitor Sox2

Given severe SC myelination defects in both developing and regenerating nerves in *Zeb2* mutants, we then sought to examine the expression of classic negative myelination regulators, such as Sox2 and c-Jun²⁷.

Figure 4 *Zeb2* is required for SC differentiation during nerve repair. (a) Nerve transection scheme in control and *Zeb2* iKO. Mice aged 4– to 6 weeks were treated with TAM via i.p. for 10 d, followed by nerve cut injury. Mice were then given TAM for 8 d, and nerves were analyzed at indicated days post injury (dpi).

(b) Immunofluorescence labeling for *Zeb2* (red) and Sox10 (green) in lesions of control sciatic nerves at 14 dpi ($n = 3$ control and mutant tissues). SCs in the intact proximal nerves show negligible *Zeb2* immunoreactivity. Scale bar, 50 μ m. (c) Immunofluorescence labeling for *Zeb2* (red) and Sox10 (green) in the regenerating site of control and *Zeb2* iKO sciatic nerves 14 dpi. Arrows: Sox10⁺*Zeb2*⁺ SCs in control and *Zeb2* iKO mutant nerves. Scale bar, 50 μ m. (d) Immunofluorescence labeling for Ki67 (red) and Sox10 (green) in the regenerating region of control and *Zeb2* iKO sciatic nerves 14 dpi ($n = 5$ control and mutant tissues). Scale bar, 50 μ m. (e) Quantification of Sox10⁺ SCs in the regenerating area in control and *Zeb2* iKO sciatic nerves 14 dpi. * $P < 0.05$; $n = 5$ control and mutant tissues; $P = 0.0128$, $t = 3.192$, d.f. = 8; two-tailed unpaired Student's *t*-test. (f) Quantification of Ki67⁺ proliferative SCs in the regenerating area in control and *Zeb2*-iKO sciatic nerves 14 dpi. n.s., not significant; $n = 5$ control and mutant tissues; $P = 0.8308$ $t = 0.2207$ d.f. = 8; two-tailed unpaired Student's *t*-test.

(g) Immunofluorescence labeling of Sox10 (green) and Oct6 (red, upper) or Krox20 (red, bottom) in the regenerating region of 14 dpi control and *Zeb2* iKO sciatic nerves ($n = 5$ control and mutant tissues). Scale bars, 50 μ m. (h) Quantification of the proportion of Oct6⁺ SCs in 14 dpi control and *Zeb2* iKO sciatic nerves in the regenerating region ($n = 4$ control and 5 mutant tissues). ** $P < 0.01$; $P = 0.0087$, $t = 3.601$, d.f. = 7; two-tailed unpaired Student's *t*-test. (i) Quantification of the proportion of Krox20⁺ SCs (right) in the regenerating region of control and *Zeb2* iKO sciatic nerves 14 dpi ($n = 4$ control and 5 mutant tissues). *** $P < 0.001$; $P = 0.0004$, $t = 6.402$, d.f. = 7; two-tailed unpaired Student's *t*-test. (j) EM images of transverse sections of control and *Zeb2* iKO 8 weeks after transection ($n = 3$ control and mutant tissues). Scale bar, 4 μ m. (k) Quantification of the proportion of myelinated axons per field from EM images of control vs. *Zeb2* iKO 8 weeks after cut injury ($n = 3$ control and mutant tissues). Data are presented as mean $\pm$ s.e.m.; *** $P < 0.0001$; $P < 0.0001$, $t = 37.64$, d.f. = 4; two-tailed unpaired Student's *t*-test. For box-and-whisker plots, whiskers show minima and maxima; boxes extend from the first to the third quartiles with cross lines at the medians.

At perinatal stages, in keeping with the severe reduction of myelinating SCs marked by Krox20, expression of the immature SC marker Sox2, which maintains SCs in an undifferentiated state²⁷, persisted even at P14, when Sox2 was rarely detectable in control nerves (Fig. 5a,b). Western blotting analysis corroborated Sox2 upregulation in postnatal mutant nerves (Fig. 5c). By contrast, c-Jun, another myelination inhibitor²⁸, appeared normally expressed in mutant sciatic nerves compared with controls (Fig. 5d,e).

Sox2 overexpression in SCs *in vitro* markedly suppressed expression of Krox20 upon induction of differentiation by dibutyryl cyclic AMP (Fig. 5f,g), though no apparent alterations of SC proliferation were detected (Fig. 5h). These observations suggest that upregulation of the differentiation inhibitor Sox2 caused by *Zeb2* deletion inhibits SC differentiation.

Since *Zeb2* can function as a transcriptional repressor²⁴, we then hypothesized that it might directly target the promoter-proximal regulatory elements of *Sox2* and inhibit its expression. We identified three conserved *Zeb2* consensus binding sites in the promoter region of *Sox2* within 5 kb upstream of the transcription start site (TSS) (Fig. 5i). Chromatin immunoprecipitation (ChIP) assays with a

Zeb2-specific antibody showed a strong enrichment of endogenous *Zeb2* binding to these sites in the *Sox2* promoter region in differentiating SCs, but not to that of cells in their proliferating immature state (Fig. 5i). In contrast, no occupancy of *Zeb2* to its candidate binding site in the *Jun* promoter was detected (Supplementary Fig. 4a). We also detected no enrichment of *Zeb2* binding to *Sox2* distal control regions (ChIP-PCR; Supplementary Fig. 4b). Furthermore, *Zeb2* overexpression in SCs significantly reduced Sox2 (Fig. 5j). In accord, luciferase reporter assays showed that enforced *Zeb2* expression in SCs effectively repressed *Sox2* promoter activity (Fig. 5k). To address the specificity of *Zeb2* action, we cotransfected the *Zeb2* vector and a *Sox2* promoter in which the *Zeb2* binding site was mutated. *Zeb2* repressive activity was significantly diminished, and luciferase reporter activity was restored to control levels (Fig. 5k). Together, our data indicate that *Zeb2* directly targets the *Sox2* promoter and inhibits *Sox2* expression to promote terminal SC differentiation.

Zeb2 regulates multiple SC differentiation pathways

To identify potential additional *Zeb2* targets that regulate SC differentiation, we performed RNA-seq of control and *Zeb2*

cKO sciatic nerves at P7. Gene ontology analysis of differentially regulated genes indicated that the functions of downregulated genes were particularly pertinent to lipid biosynthesis, myelination and SC differentiation (Fig. 6a), whereas those of upregulated genes could be classified into categories involving cell proliferation control, adhesion and extracellular matrix organization (Fig. 6b). Consistent with our *in situ* hybridization and qPCR results, we detected a strong downregulation of major myelin genes and differentiation regulatory genes such as *Mbp*, *Mag*, *Mpz*, *Krox20* and *Oct6*, with a concomitant increase in genes encoding myelination inhibitors, including *Sox2*, *Jag1* and *Egr1* in *Zeb2* cKO sciatic nerves (Fig. 6c,d). In keeping with the dramatic sorting defect in *Zeb2* cKO nerves, we detected differential expression of selected genes involved in the regulation of radial sorting, including laminin $\alpha 2$ (*Lama2*), *Cdc42* and integrin $\alpha 6$ (*Itga6*) (ref. 29; Supplementary Fig. 5a,b). The RNA-seq data demonstrated an upregulation of the transforming growth factor- β (TGF β) signaling genes including *Tgfb2*, *Bmper*, *Smad3* and *Bmp7* in *Zeb2* cKO sciatic nerves (Fig. 6c); however, in contrast to the activation of bone morphogenetic protein (BMP)-receptor-activated Smads (*Smad1*, *Smad5* and *Smad8*) and downregulation of *Smad7* observed in oligodendrocytes lacking *Zeb2* (ref. 8), we did not detect *Smad* activation or BMP signaling inhibition, assayed by phosphorylation of *Smad1*, *Smad5* and *Smad8* and gross *Smad7* levels, respectively (Supplementary Fig. 6). These observations indicate that overall BMP–Smad signaling is not substantially altered in *Zeb2* mutant SCs, suggesting that divergent mechanisms from those in CNS myelinogenesis⁸ are at work by *Zeb2* in SCs for the control of PNS myelination.

Zeb2 inhibits a Notch–Hey2 axis for SC differentiation

We next set out to investigate other potential *Zeb2*-regulated signaling pathways for SC differentiation on the basis of transcriptome profiling analysis. We detected a cohort of signaling components in the Notch pathway that were significantly upregulated in *Zeb2* cKO sciatic nerves (Fig. 6c). Among the upregulated genes, *Hey2*, a *Hes*-related family basic helix-loop-helix effector of Notch³⁰, displayed the most substantial increase in mRNA level compared to control nerves (Fig. 6d). Because *Hey2* functions as a transcriptional repressor in other systems, such as in cardiac and vascular development^{30,31}, we postulated that *Hey2* might act as a blocker of SC differentiation. *Hey2* transcript levels peaked at P5 and then rapidly declined to near zero at P15, which closely paralleled the expression profile of myelination inhibitors, such as *Sox2* (Fig. 6e)^{27,32}. We validated the increase in *Hey2* mRNA and protein levels in *Zeb2* cKO nerves by qPCR (Fig. 6f) and western blotting (Fig. 6g), respectively.

In seeking to determine whether *Zeb2* could regulate *Hey2* directly, we identified an evolutionarily conserved *Zeb2* binding site upstream of the TSS and found that *Zeb2* binding was enriched on the *Hey2* promoter in differentiating SCs, but not in proliferating cells (Fig. 6h). ChIP of the distal control region of *Hey2* showed no *Zeb2* binding (Supplementary Fig. 4b). Moreover, enforced *Zeb2* expression in SCs significantly downregulated *Hey2* (Fig. 6i). *Zeb2* overexpression in SCs repressed *Hey2* promoter activity, and this repression was partially abolished when the *Zeb2*-binding site in this promoter was mutated (Fig. 6j). Overexpression of *Hey2* inhibited *Krox20* induction in SCs cultured under differentiation conditions (Fig. 6k,l). In contrast, the proliferative capacity of SCs

Figure 6 *Zeb2* regulates the SC differentiation program and inhibits Notch effector *Hey2* expression. **(a,b)** Gene ontology analysis of downregulated **(a)** or upregulated **(b)** genes in P7 *Zeb2* cKO sciatic nerves compared with controls. Each dot in the connecting lines represents the gene count of the corresponding biological function categories. **(c)** Heat map showing representative differentially expressed genes and their categories in control vs. *Zeb2* cKO sciatic nerves at P7; $n = 2$ independent experiments. **(d)** qPCR analysis of genes related to SC differentiation, neural crest, Notch and TGF β –BMP pathways in *Zeb2* cKO sciatic nerves relative to controls at P7. $n = 3$ independent experiments. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; $P_{(Pxx)} < 0.0001$, $t = 2.750$, d.f. = 4; $P_{(Mbp)} < 0.0001$, $t = 512.5$, d.f. = 4; $P_{(Sox10)} < 0.0001$, $t = 101.4$, d.f. = 4; $P_{(Krox20)} < 0.0001$, $t = 291.8$, d.f. = 4; $P_{(Oct6)} < 0.0001$, $t = 14.865$, d.f. = 4; $P_{(Twist1)} = 0.0003$, $t = 11.99$, d.f. = 4; $P_{(Snail)} = 0.0016$, $t = 4.45$, d.f. = 9; $P_{(Slug)} = 0.0007$, $t = 9.39$, d.f. = 4; $P_{(Hes1)} = 0.0974$, $t = 1.962$, d.f. = 6; $P_{(Notch1)} = 0.2186$, $t = 1.376$, d.f. = 6; $P_{(Jag1)} = 0.0496$, $t = 2.784$, d.f. = 4; $P_{(Jag2)} = 0.0179$, $t = 3.879$, d.f. = 4; $P_{(Hey2)} = 0.0284$, $t = 3.355$, d.f. = 4; $P_{(Tgfb2)} = 0.001$, $t = 6.000$, d.f. = 6; $P_{(Bmper)} = 0.0313$, $t = 3.254$, d.f. = 4; $P_{(Bmp7)} = 0.0013$, $t = 5.703$, d.f. = 6; two-tailed unpaired Student's *t*-test. Whiskers show minima and maxima; boxes extend from the first to the third quartiles with cross lines at the medians. **(e)** qPCR analysis of *Hey2* levels in mouse sciatic nerves during development ($n = 3$ control and mutant tissues for each time point). Data are presented as mean $\pm$ s.e.m. **(f)** Transcript levels of *Hey2* in *Zeb2* cKO sciatic nerves relative to those in control nerves at P7, 15 and 30. Data are presented as mean $\pm$ s.e.m. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$, $n = 3$ control and mutant tissues for each time point, $P_{(P7)} = 0.0136$, $t = 5.232$, d.f. = 4; $P_{(P15)} = 0.0101$, $t = 4.590$, d.f. = 4; $P_{(P30)} = 0.0097$, $t = 4.641$, d.f. = 4; two-tailed unpaired Student's *t*-test. **(g)** Western blot showing *Hey2* expression in control and *Zeb2* cKO sciatic nerves at P14. GAPDH was the loading control. Data are from 3 independent experiments. Full-length blots are presented in Supplementary Figure 9b. **(h)** ChIP analysis of *Zeb2* recruitment to the consensus *Zeb2* binding site on the *Hey2* promoter (red bar, upper panel) in primary SCs under proliferation (pro) or differentiation (diff, 9 h) media. IgG IP was used as control. Data are mean $\pm$ s.e.m.; *** $P < 0.001$; proliferation: $P = 0.9367$, $t = 0.08283$, d.f. = 6 $n = 4$; differentiation: $P = 0.0005$, $t = 10.27$, d.f. = 4 $n = 3$; two-tailed unpaired Student's *t*-test; n : independent experiments. **(i)** qPCR analysis showing *Hey2* gene expression in *Zeb2*-overexpressing SCs under differentiation media for 18 h, compared with vector-expressing control (* $P < 0.05$; $n = 3$ independent experiments, $P = 0.0272$, $t = 3.404$, d.f. = 4; two-tailed unpaired Student's *t*-test). Data are presented as mean $\pm$ s.e.m. **(j)** Percentages of normalized luciferase activity in rat SCs transfected with luciferase reporter driven by wild-type or mutant *Hey2* promoter together with *Zeb2*-expressing vector. Data are presented as mean $\pm$ s.e.m., $n = 3$ independent experiments. ** $P < 0.01$, $P = 0.0025$, $t = 6.747$, d.f. = 4; two-tailed unpaired Student's *t*-test. **(k)** *Krox20* expression in rat SCs transfected with control (pGFP) and *Hey2*-expressing (pHey2) vectors under differentiation conditions for 3 d. The experiment was repeated 3 times. Scale bars, 20 μ m. **(l)** Quantification of the percentage of *Krox20*⁺ among transfected cells ($n = 3$ independent experiments). Data are presented as mean $\pm$ s.e.m.; * $P < 0.05$; $P = 0.0411$, $t = 2.733$, d.f. = 4; two-tailed unpaired Student's *t*-test. **(m)** Ki67 expression in rat SCs transfected with control (pGFP) and *Hey2*-expressing (pHey2) vectors for 48 h in proliferation medium. Scale bars, 20 μ m. Data are from 5 independent experiments. **(n)** Quantification of the percentage of Ki67⁺ among transfected cells ($n = 5$ independent experiments). Data are presented as mean $\pm$ s.e.m.; ** $P < 0.01$; $P = 0.0052$, $t = 3.800$, d.f. = 8; two-tailed unpaired Student's *t*-test. **(o)** qPCR showing the efficiency of siRNA-mediated knockdown of *Zeb2*, *Sox2* and *Hey2*. Data are presented as mean $\pm$ s.e.m.; *** $P < 0.001$; $n = 5$ independent experiments; $P_{(Zeb2)} < 0.0001$, $t = 23.37$, d.f. = 9; $P_{(Hey2)} < 0.0001$, $t = 11.43$, d.f. = 8; $P_{(Sox2)} < 0.0001$, $t = 11.47$, d.f. = 9; two-tailed unpaired Student's *t*-test. Whiskers show minima and maxima, boxes extend from the first to the third quartiles with cross lines at the medians. **(p)** qPCR showing myelin-associated gene expression in rat SCs with siRNA knockdown of *Zeb2* and *Sox2* or *Hey2* compared with single *Zeb2* siRNA knockdown. Data are presented as mean $\pm$ s.e.m.; ** $P < 0.01$; *** $P < 0.001$; $n = 5$ independent experiments; one-way ANOVA with Tukey's multiple comparisons test; $P_{(Krox20)} < 0.001$: scrambled vs. si-*Zeb2*, si-*Zeb2* vs. si-*Zeb2*, si-*Hey2*; $P_{(Krox20)} < 0.01$: si-*Zeb2* vs. si-*Zeb2*, si-*Sox2*; $F_{3,16} = 345.8$; $P_{(Mpz)} < 0.001$: scrambled vs. si-*Zeb2*, si-*Zeb2* vs. si-*Zeb2*, si-*Sox2*; $P_{(Mpz)} < 0.01$: si-*Zeb2* vs. si-*Zeb2*, si-*Hey2*; $F_{3,10} = 29.67$; $P_{(Mbp)} < 0.001$: scrambled vs. si-*Zeb2*, si-*Zeb2* vs. si-*Zeb2*, si-*Hey2*; $P_{(Mbp)} < 0.05$: si-*Zeb2* vs. si-*Zeb2*, si-*Sox2*; $F_{3,10} = 20.59$.

assessed by Ki67 expression with constitutive Hey2 expression was significantly higher than that of control GFP-transfected SCs (Fig. 6m,n). These observations suggest that Hey2 inhibits SC differentiation by arresting SCs in an undifferentiated state and driving their proliferation and that Zeb2 directly represses *Hey2*, thereby promoting SC myelination.

To further confirm that Zeb2 downregulates *Hey2* or *Sox2* transcription in order to promote SC differentiation, we performed sequential siRNA-mediated knockdown experiments, first by Zeb2 knockdown in SCs and then followed 24 h later by either knockdown of *Sox2* or *Hey2* using siRNA. Subsequent knockdown of *Sox2* or *Hey2* in Zeb2-deficient SCs partially restored myelin-associated gene expression (Fig. 6o,p), indicating that Zeb2 drives SC differentiation via repression of inhibitory factor genes such as *Sox2* and *Hey2*.

As Notch signaling negatively controls SC myelination¹⁶, we explored the possibility that Zeb2 promotes SC differentiation and myelination by opposing a Notch-pathway-mediated differentiation block. To this end, we generated Zeb2 cKO mice carrying a Notch-responsive GFP reporter transgene (Supplementary Fig. 7a)³³. There was a striking induction of Notch-dependent GFP expression in Zeb2 mutant nerves (Fig. 7a). Furthermore, overexpression of Zeb2

significantly repressed the reporter activity of a *TPI* (the Epstein Barr virus terminal protein 1) promoter carrying RBP-Jκ (recombination signal-binding protein 1 for Jκ) binding sites (Fig. 7b), which are highly specific to Notch signaling activation³⁴, suggesting that Zeb2 inhibits Notch signaling in SCs.

Notch inhibition restores differentiation of Zeb2-mutant SCs

To test whether activation of Notch signaling is responsible for the differentiation failure in Zeb2-deficient SCs, we treated scrambled-siRNA and Zeb2-knockdown SCs over 4 d with the Notch inhibitor DAPT (5 μM), which inhibits γ-secretase, a protease required for Notch1 activation³⁵. The addition of DAPT efficiently inhibited gene expression of *Jag1* and *Hey2* in scramble-transfected SCs (Fig. 7c), whereas this treatment did not alter Zeb2 levels. In accord, SCs with Zeb2 knockdown showed increased levels of the Notch signaling genes *Jag1* and *Hey2*. DAPT treatment effectively reduced expression of these genes (Fig. 7c) and restored expression of major myelin genes (for example, *Mpz*, *Cnp*) to normal levels in Zeb2-knockdown SCs (Fig. 7d). Notch inhibition by DAPT also partially rescued the otherwise perturbed induction of *Oct6* in differentiating SCs with Zeb2 knockdown (Fig. 7d-f). Strikingly, treatment of Notch inhibitor LY411575 *in vivo* led to a robust increase in Krox20+ SCs and appearance of MBP+ or

MAG⁺ (myelin-associated glycoprotein) myelin segments in neonatal *Zeb2* cKO mice (Fig. 7g,h). Furthermore, blocking Notch signaling transduction by delivering a lentivirus expressing a dominant-negative form of the Notch coactivator Mastermind (DN-MAML)³⁶ reduced *Hey2* or *Jag1* expression and partially restored expression of *Krox20* in *Zeb2*-knockdown SCs (Fig. 7i,j). Collectively, our data suggest that *Zeb2* inhibits Notch activation, at least in part, by suppressing its downstream effector *Hey2*, thereby promoting differentiation of SCs from their undifferentiated state.

Zeb2 recruits HDAC to antagonize differentiation inhibition

The observation that *Zeb2* could effectively suppress multiple differentiation inhibitory pathways prompted us to postulate that *Zeb2* functions by forming a co-repressor complex. A previous study of the functional domain of *Zeb2* identified a NuRD-interacting

motif (NIM) close to the N terminus of *Zeb2*, which is similar to the minimal domain needed for NuRD recruitment identified in the transcription factor *Sall1* (refs. 10,37). *Zeb2* associates with NuRD components that include histone deacetylases HDAC1 and HDAC2 (ref. 10). HDAC1 and HDAC2 control various stages of SC development; conditional inactivation of *HDAC1* and *HDAC2* in the SC lineage impairs radial sorting, SC development and myelination^{38,39}, a result that closely resembles the *Zeb2* cKO phenotype. We therefore investigated the possibility that *Zeb2* may interact with HDAC in the context of a NuRD co-repressor complex to suppress negative myelination regulators. Co-expression of *Zeb2* together with HDAC1 or HDAC2 enhanced the repression of *Hey2* and *Sox2*, to an extent much greater than that of *Zeb2* overexpression alone, in SCs (Fig. 8a). By coimmunoprecipitation, we determined that HDAC1 and HDAC2 were present in a complex with *Zeb2* (Fig. 8b).

Figure 7 *Zeb2* represses a Notch–*Hey2* signaling axis. (a) Representative immunolabeling of GFP and Sox10 in P7 sciatic nerves of control and *Zeb2* cKO mice carrying a Notch–EGFP reporter ($n = 3$ for each group). Scale bars, 50 μm . (b) Luciferase activity in HEK 293T cells transfected with a luciferase reporter driven by Notch-responsive TP-1 promoter (vector) and *Zeb2*- or Notch ΔE -expressing vector. Data are presented as mean $\pm$ s.e.m., $^{**}P < 0.01$; $^{***}P < 0.001$; $P_{(\text{Zeb2})} < 0.001$; $P_{(\text{Notch}\Delta\text{E})} < 0.0001$; $F_{2,6} = 890.1$; $n = 3$ independent experiments; one-way ANOVA with Tukey's multiple comparison tests. (c) qPCR showing *Jag1* and *Hey2* expression in *Zeb2* knockdown in SCs treated with 5 μM DAPT over 4 d. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$, one-way ANOVA with Tukey's multiple comparisons test; *Zeb2*: $P_{(\text{DMSO})} < 0.0001$, $P_{(\text{DAPT})} < 0.0001$; $F_{3,20} = 79.78$; *Jag1*: $P_{(\text{DMSO})} < 0.001$, $P_{(\text{DAPT})} < 0.01$; $P_{(\text{si-Zeb2, DMSO vs. DAPT})} < 0.01$; $F_{3,19} = 22.75$; *Hey2*: $P_{(\text{DMSO})} < 0.0001$, $P_{(\text{DAPT})} < 0.0001$; $P_{(\text{scrambled, DMSO vs. DAPT})} < 0.05$; $P_{(\text{si-Zeb2, DMSO vs. DAPT})} < 0.0001$; $F_{3,20} = 119.5$; data are from 6 independent experiments. (d) qPCR showing *Oct6*, *Mpz* and *Cnp* expression in *Zeb2*-knockdown SCs treated with DAPT. $^{**}P < 0.01$; $^{***}P < 0.001$; one-way ANOVA with Tukey's multiple comparison's test; *Oct6*: $P_{(\text{DMSO})} < 0.0001$; $P_{(\text{si-Zeb2, DMSO vs. DAPT})} < 0.01$; $F_{3,18} = 33.29$; *Mpz*: $P_{(\text{DMSO})} < 0.01$; $P_{(\text{si-Zeb2, DMSO vs. DAPT})} < 0.01$; $F_{3,19} = 6.745$; *Cnp*: $P_{(\text{DMSO})} < 0.05$; $P_{(\text{si-Zeb2, DMSO vs. DAPT})} < 0.001$; $F_{3,19} = 10.16$; data are from 5 independent experiments. For c and d, whiskers show minima and maxima; boxes extend from the first to the third quartiles with cross lines at the medians. (e) Immunostaining of Oct6 (red) in scrambled or si-*Zeb2*-knockdown SCs treated with DMSO or DAPT for 4 d in differentiation medium. DAPI, blue. The experiment was repeated 3 times. Scale bars, 50 μm . (f) Quantification of Oct6-expressing cells among scrambled or si-*Zeb2*-knockdown SCs treated with DMSO or DAPT for 4 d in differentiation medium. Data are presented as mean $\pm$ s.e.m.; $^{**}P < 0.01$; $^{***}P < 0.001$; one-way ANOVA with Tukey's multiple comparison's test; $P_{(\text{DMSO})} < 0.001$, $P_{(\text{DAPT})} < 0.001$; $P_{(\text{si-Zeb2, DMSO vs. DAPT})} < 0.01$; $F_{3,8} = 134.6$. Data are from 3 independent experiments. (g) Immunostaining of Krox20, MBP or MAG in sciatic nerves of *Zeb2* cKO mice treated with vehicle or Notch-inhibitor LY411575 from P2 to P5 and killed on P8. Arrows indicate MBP⁺ or MAG⁺ myelin segments. Data are from 3 individual mice from each group. Scale bars, 50 μm . (h) Quantification of Krox20⁺ nuclei in *Zeb2* cKO sciatic nerves treated with vehicle or LY411575. Data are presented as mean $\pm$ s.e.m.; $n = 3$ individual mice from each group; $^{*}P < 0.05$; $P = 0.0135$, $t = 4.221$, d.f. = 4; two-tailed unpaired Student's *t*-test. (i) qPCR showing *Jag1* and *Hey2* expression in *Zeb2*-knockdown SCs transduced with GFP- or DN-MAML-GFP-expressing lentivirus for 48 h in proliferation medium. Data are presented as mean $\pm$ s.e.m.; $^{**}P < 0.01$; $^{***}P < 0.001$; one-way ANOVA with Tukey's multiple comparison test, *Jag1*: $P_{(\text{scrambled GFP vs. si-Zeb2 GFP})} < 0.01$, $P_{(\text{si-Zeb2 GFP vs. si-Zeb2 DN-MAML GFP})} < 0.01$; $F_{2,6} = 18.91$; *Hey2*: $P_{(\text{scrambled GFP vs. si-Zeb2 GFP})} < 0.001$, $P_{(\text{si-Zeb2 GFP vs. si-Zeb2 DN-MAML GFP})} < 0.001$; $F_{2,9} = 30.74$. Data are from 3 independent experiments. (j) qPCR showing *Krox20* levels in *Zeb2*-knockdown SCs transduced with GFP- or DN-MAML-GFP-expressing lentivirus. Data are presented as mean $\pm$ s.e.m.; $^{***}P < 0.001$; one-way ANOVA with Tukey's multiple comparison test $P_{(\text{scrambled GFP vs. si-Zeb2 GFP})} < 0.0001$, $P_{(\text{si-Zeb2 GFP vs. si-Zeb2 DN-MAML GFP})} < 0.0001$; $F_{2,16} = 244.8$. Data are from 5 independent experiments.

We identified a human ZEB2 variant [c.64A>G; Arg22Gly] carrying a single residue ZEB2 mutation (R22G) in an individual with mild MOWS in the NuRD-interacting domain of ZEB2 (ZEB2^{R22G}; **Fig. 8c**). Strikingly, we found that ZEB2^{R22G} was sufficient to abolish HDAC1 and HDAC2 recruitment to the ZEB2 complex (**Fig. 8d**). Moreover, this missense mutation in ZEB2 markedly disrupted ZEB2 interaction with other NuRD subunits as determined by coimmunoprecipitation,

whereas CtBP (C-terminal-binding protein 1) binding was unaffected (**Fig. 8e** and **Supplementary Fig. 7b**). We further confirmed the endogenous interaction of Zeb2 with NuRD complex components, including HDAC1, HDAC2 and Mi2/Chd4, in rat SCs (**Fig. 8f**). Most notably, exogenous expression of ZEB2^{R22G} markedly diminished the induction of *Oct6* and *Krox20* in SCs (**Fig. 8g–i**). However, overexpressing a Zeb2 mutant protein with deletion of CtBP-interacting

Figure 8 Zeb2 associates with HDAC1 and HDAC2 to repress SC differentiation inhibitor expression. **(a)** qPCR showing the fold change in *Hey2* and *Sox2* expression in SCs overexpressing Zeb2 or coexpressing Zeb2, HDAC1 and HDAC2 relative to vector-expressing cells. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; one-way ANOVA with Tukey's multiple comparison tests; *Hey2*: $P_{(\text{vector vs. Zeb2})} < 0.05$, $P_{(\text{Zeb2 vs. Zeb2 + Hdac1/2})} < 0.05$; $P_{(\text{vector vs. Zeb2 + Hdac1/2})} < 0.0001$; $F_{2,20} = 15.72$; *Sox2*: $P_{(\text{vector vs. Zeb2})} < 0.001$, $P_{(\text{Zeb2 vs. Zeb2 + Hdac1/2})} < 0.001$; $P_{(\text{vector vs. Zeb2 + Hdac1/2})} < 0.0001$; $F_{2,10} = 40.59$. Data are from 3 independent experiments. **(b)** Co-immunoprecipitation with Flag-tagged HDAC1 or HDAC2 in HEK 293T cells cotransfected with Myc-tagged Zeb2. IP, immunoprecipitation; GAPDH, loading control. Full-length blots are presented in **Supplementary Figure 9c**. The experiment was repeated 3 times. **(c)** Alignment of SALL1, human wild-type and mutant ZEB2^{R22G} in NuRD binding domain (underline). Amino acids in bold are critical for NuRD binding. The red box highlights the amino acid change in the ZEB2^{R22G} mutant. **(d)** Top: schematic domains of ZEB2^{WT} and ZEB2^{R22G} proteins. NIM, N- and C-terminal zinc finger domains, respectively; SBD, Smad-binding domain; HD, homeodomain-like domain; CID, CtBP interacting domain. Bottom: coimmunoprecipitation of Zeb2 in HEK 293T cells transfected with Flag-tagged HDAC1 and HDAC2 together with Zeb2 or Zeb2^{R22G} expressing vectors. IgG was used as the IP control. The experiment was repeated 3 times. Full-length blots are presented in **Supplementary Figure 9d**. **(e)** Western blot showing coimmunoprecipitation of endogenous NuRD subunits with ZEB2^{WT} but not ZEB2^{R22G} in HEK293T cells transiently expressing Flag-ZEB2^{WT} or Flag-ZEB2^{R22G}. The experiment was repeated 3 times. Full-length blots are presented in **Supplementary Figure 10b**. **(f)** Western blot showing coimmunoprecipitation with an antibody to Zeb2 and control IgG in rat SCs using antibodies to HDAC1, HDAC2 and Mi-2. The experiment was repeated 3 times. Full-length blots are presented in **Supplementary Figure 10a**. **(g)** Immunolabeling of Oct6 or Krox20 in rat SCs transfected with Zeb2^{WT}, Zeb2^{R22G} or Zeb2^{ΔCID} vectors carrying a GFP reporter under differentiation conditions for 48 h. Arrows, transfected cells. Scale bar, 50 μm . Data are from 5 independent experiments. **(h)** Quantification of the proportion of Oct6⁺ cells among transfected GFP⁺ cells ($n = 5$ independent experiments). *** $P < 0.001$. One-way ANOVA with Tukey's multiple comparison tests; $P_{(\text{Zeb2 vs. Zeb2R22G})} < 0.0001$; $P_{(\text{Zeb2 vs. Zeb2CID})} = 0.5330$; $F_{2,11} = 36.46$. **(i)** Quantification of the proportion of Krox20⁺ cells among transfected cells ($n = 5$ independent experiments). * $P < 0.05$. One-way ANOVA with Tukey's multiple comparison tests; $P_{(\text{Zeb2 vs. Zeb2R22G})} < 0.05$; $P_{(\text{Zeb2 vs. Zeb2CID})} = 0.2225$; $F_{2,12} = 3.497$. **(j)** qPCR analysis of Zeb2 targets, *Jag1*, *Hey2* and *Sox2* in rat SCs transfected with Zeb2 or Zeb2^{R22G} mutant expression vectors. * $P < 0.05$; ** $P < 0.01$; one-way ANOVA with Tukey's multiple comparisons test; *Hey2*: $P_{(\text{vector vs. Zeb2})} < 0.01$, $P_{(\text{Zeb2 vs. Zeb2R22G})} < 0.05$; $F_{2,21} = 6.884$; $n = 7$ independent experiments; *Jag1*: $P_{(\text{vector vs. Zeb2})} < 0.01$, $P_{(\text{Zeb2 vs. Zeb2R22G})} < 0.01$; $F_{2,10} = 16.24$; $n = 4$ independent experiments; *Sox2*: $P_{(\text{vector vs. Zeb2})} < 0.01$, $P_{(\text{Zeb2 vs. Zeb2R22G})} < 0.05$; $F_{2,13} = 7.584$; $n = 5$ independent experiments. **(k)** qPCR analysis of myelin-related genes *Oct6*, *Krox20* and *Cnp* in rat SCs transfected with Zeb2 or Zeb2^{R22G} mutant expression vectors. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; one-way ANOVA with Tukey's multiple comparisons tests; *Oct6*: $P_{(\text{vector vs. Zeb2})} < 0.05$, $P_{(\text{Zeb2 vs. Zeb2R22G})} < 0.001$; $F_{2,15} = 20.24$; $n = 5$ independent experiments; *Krox20*: $P_{(\text{vector vs. Zeb2})} < 0.01$, $P_{(\text{Zeb2 vs. Zeb2R22G})} < 0.001$; $F_{2,14} = 12.31$; $n = 6$ independent experiments; *Cnp*: $P_{(\text{vector vs. Zeb2})} < 0.05$, $P_{(\text{Zeb2 vs. Zeb2R22G})} < 0.01$; $F_{2,9} = 11.25$; $n = 4$ independent experiments. **(l)** HDAC1 occupancy by ChIP-PCR in rat SCs knocked down with control or Zeb2 siRNA on the promoters of Zeb2 target genes. Chromatin was from SCs induced to differentiate by cAMP-containing medium for 9 h. IgG IP was used as a negative control. Data are mean $\pm$ s.e.m.; *** $P < 0.001$; $n =$ at least 3 independent experiments; $P_{(\text{negative control})} = 0.4683$, $t = 0.7741$, d.f. = 6; $P_{(\text{Sox2, 1.1 kb})} < 0.0001$, $t = 32.85$, d.f. = 8; $P_{(\text{Hey2, 3.9 kb})} < 0.0001$, $t = 24.63$, d.f. = 8; $P_{(\text{Oct6, 3.9 kb})} < 0.0001$, $t = 11.26$, d.f. = 6; $P_{(\text{Jnu, 6 kb})} = 0.6954$, $t = 0.4209$, d.f. = 4; two-tailed unpaired Student's t -test. For box-and-whisker plots, whiskers show minima and maxima; boxes extend from the first to the third quartiles with cross lines at the medians.

domains (Zeb2^{ΔCID})⁴⁰ did not alter induction of either *Oct6* (Fig. 8g–h) or *Krox20* (Fig. 8i) in SCs. Accordingly, enforced ZEB2^{R22G} expression in SCs also lost the capacity to repress Zeb2 target genes, such as *Hey2*, *Sox2* and *Jag1*, or to activate the transcription of the myelin-related genes *Oct6*, *Krox20* and *Cnp* (Fig. 8j–k).

To determine whether recruitment of HDAC1 and HDAC2 by Zeb2 is required for target expression, we performed ChIP-qPCR in Zeb2-deficient SCs. The reduction of Zeb2 in SCs significantly compromised the enrichment of HDAC1 on the Zeb2-consensus binding regions in the promoters of Zeb2 target genes, such as *Sox2*, *Oct6* and *Hey2*, in Zeb2-knockdown SCs (Fig. 8l). In contrast, HDAC1 occupancy on the promoter of a non-Zeb2 target, *Jun*, in Zeb2-knockdown SCs was comparable with that in IgG controls (Fig. 8l). Similar results were also obtained for HDAC2 recruitment (data not shown). This suggests that the Zeb2–HDAC1–HDAC2 complex is required for the regulation of target gene transcription in SCs. Thus, our findings indicate that Zeb2 represses SC differentiation inhibitors by associating with the HDAC1–HDAC2–NuRD co-repressor complex to promote SC myelination.

DISCUSSION

In this study, we provided *in vivo* genetic evidence for the critical function of Zeb2 in SC precursor differentiation and peripheral nerve myelination, as well as remyelination during nerve regeneration, consistent with the observations by Quintes *et al.*⁴¹ in this issue. We establish Zeb2 as a key regulatory switch that controls the timing for the onset of normal SC differentiation and remyelination after injury. Furthermore, our findings reveal that Zeb2 cooperates with a HDAC1–HDAC2–NuRD chromatin-remodeling complex as a repressor targeting a network of inhibitory pathways, including *Sox2* and Notch signaling, to drive SC differentiation, in conjunction with acting as a transcriptional activator of other target genes such as *Oct6* (ref. 42). Moreover, we identified a Zeb2-modulated Notch–Hey2 signaling axis that blocks SC differentiation. Inhibition of Notch signaling partially rescues the SC differentiation defect caused by Zeb2 deficiency. Thus, Zeb2 regulates SC differentiation and myelination at least in part through de-repression of Notch signaling (Supplementary Fig. 7c,d), pointing to the importance of HDAC1–HDAC2–NuRD-dependent ZEB2 activity in MOWS-associated neuropathies.

Zeb2 recruits HDAC1/2–NuRD to control SC differentiation

Delineation of differentially expressed genes by transcriptome analysis reveals that Zeb2 modulates inhibitory pathways, namely *Sox2* effects and Notch–Hey2 signaling, during SC differentiation. Whether Zeb2 regulation of these individual inhibitory signals exists in parallel, and whether Zeb2 mediates its actions through other unidentified cofactors of these or other pathways to control SC myelination, would require further investigation. Nonetheless, we find that Zeb2 engages the HDAC1/2–NuRD complex to exert repressive activity on myelination inhibitory factors. Several studies have reported radial sorting and myelination impairment in mouse mutants with SC-specific inactivation of NuRD subunits or associated factors including Mi-2/Chd4 and HDAC1/2^{38,39,43} as well as interacting partners such as NAB proteins^{32,44}. Co-expression of Zeb2, HDAC1 and HDAC2 in SCs further augments repression in expression of inhibitory genes *Sox2* and *Hey2*. Remarkably, a single missense mutation, Zeb2^{R22G}, in the Zeb2 N-terminal NuRD-interacting domain completely abolishes its association with the HDAC1/2–NuRD and inhibits SC differentiation, likely due to inefficient repression of the aforementioned differentiation-inhibitory signals. Thus, by interacting with the HDAC1/2–NuRD complex⁴⁵, Zeb2 may mediate its

repressive activity to fine-tune transcription of the genes that negatively impact myelination. Taken together, our data indicate that Zeb2 acts as a key transcriptional repressor that effectively extinguishes multiple inhibitory pathways to direct the onset of SC differentiation and initiation of myelination.

A Notch–Hey2 axis for inhibiting SC differentiation

Notch signaling regulates multiple stages of SC development, from promoting SC formation and proliferation to negatively regulating myelination¹⁶. Strikingly, in the absence of Zeb2, Notch signaling is turned on constitutively in sciatic nerves, exerting a negative regulatory effect on SC differentiation and myelination. We observed a substantial increase of proliferating immature SCs in Zeb2 mutants, which is consistent with the notion that activation of Notch signaling leads to increased SC proliferation¹⁶. The transcriptome profiling analysis further identified *Hey2*, a transcriptional repressor downstream of Notch⁴⁶, as a Zeb2 target that suppresses *Krox20* expression and blocks SC differentiation.

Nonetheless, unlike the sustained block of SC maturation in Zeb2 cKO nerves, genetically enforced Notch (i.e., NCID) expression in SCs does not completely prevent the initiation of myelogenesis¹⁶. Therefore, we cannot rule out the possibility that Hey2 regulation of SC development may also be independent of canonical Notch signaling, as reported in other contexts^{47,48}. If such is the case, Zeb2-regulated Hey2 would introduce an additional layer of repression to the already existing Notch inhibition on SC myelination.

Zeb2-deficient SCs exhibit persistent upregulation of *Sox2* and a substantial increase in *Sox2*⁺ immature SCs in peripheral nerves. *Sox2* has been shown to act downstream of Notch signaling in certain contexts. For instance, Notch activation induces *Sox2* in sensory progenitors of the inner ear⁴⁹. Elevation of *Sox2* levels maintains SCs in the undifferentiated state by suppressing expression of SC differentiation markers such as *Krox20*³². Given that Hey2 overexpression inhibits *Krox20* and increases the proliferation of immature SCs (Fig. 6k–n), Notch–Hey2 activation may induce or maintain *Sox2* expression to inhibit SC differentiation.

Distinct mechanisms of Zeb2 in CNS and PNS myelination

Recent studies of Zeb2 revealed that it activates Smad7 and inhibits the action of activated BMP–Smads on genes that would otherwise be inhibitory for oligodendrocyte myelination and hence promotes myelination in the CNS⁸. In contrast, the present study shows that Zeb2 does not appear to control SC differentiation via regulation of BMP–Smad signaling or Smad7 expression in the PNS, suggesting that Zeb2 controls SC myelination via a different mechanism. Here we find that Zeb2 directs SC differentiation by a disinhibition mechanism via antagonizing Notch–Hey2 signaling and *Sox2* in the PNS, which was not observed in the CNS⁸. Furthermore, we found that in the PNS Zeb2, through recruitment of the HDAC1/2–NuRD, negatively regulates *Sox2* and the Notch effector Hey2 to guard against the blockade of SC myelination mediated by these inhibitors.

In contrast to substantial downregulation of *Sox10* in oligodendrocytes of the Zeb2 mutant spinal cord⁸, Zeb2 does not seem to regulate *Sox10* (Fig. 3d,k). However, we cannot exclude the possibility that *Sox10* and Zeb2 coordinate to drive SC differentiation, similarly to the cooperative role they play in the enteric nervous system⁵⁰. Alternatively, we observed a preferential recruitment of Zeb2 to the *Oct6* promoter when SCs begin to differentiate (Supplementary Fig. 3e). Given the downregulation of key promyelination regulators *Oct6* and *Krox20* in Zeb2-ablated sciatic nerves, our data suggests that Zeb2 may function as a dual-level switch

control of SC differentiation through inactivating multiple differentiation inhibitors, including Notch signaling and Sox2, while promoting pro-myelinating factors Oct6 and Krox20 during SC development.

Zeb2 inactivation in SCs during nerve repair inhibits SC redifferentiation and thus remyelination, supporting the crucial role of Zeb2 in myelin regeneration in the PNS. However, we did not detect any difference in Sox2 or Hey2 mRNA expression in the mutant injured nerves 7 d post-transection, at which time regenerating mouse nerves would have reached the midpoint of axonal regrowth, as compared to the control injured sciatic nerves (Supplementary Fig. 8). Thus, we cannot rule out the possibility that Zeb2 may utilize other mechanisms after injury for SC regeneration and remyelination.

Deficient radial axonal sorting and myelination caused by Zeb2 loss could severely disrupt nerve conduction and, consequently, motor function in Zeb2 mutant mice, paralleling motor disabilities and delayed walking in humans with MOWS. The phenotypic variation between humans with haplo-insufficient mutations could be attributed to a differential sensitivity to gene dosage across species or dominant-negative effects of human mutant proteins. Strikingly, we find that the ZEB2^{R22G} detected in a MOWS patient failed to associate with HDAC1/2–NuRD and blocked SC differentiation, suggesting that developmental defects caused by ZEB2 mutations affecting a functional domain may contribute to neuropathies and motor dysfunction seen in these patients. Given that ZEB2-mediated functions are vital for SC development and myelination, it may serve as a pivotal hub of the myelination regulatory network. Augmenting HDAC1/2–NuRD-dependent ZEB2 activity and inhibiting Notch signaling could potentially provide a novel therapeutic means of reversing adverse neuropathies seen in people with MOWS.

METHODS

Methods and any associated references are available in the [online version of the paper](#).

Accession codes GEO: [GSE74381](#).

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

L.M.N.W. and Q.R.L. designed the experiments. L.M.N.W. carried out the studies. J.W., A.C. and L.Z. assisted with coimmunoprecipitation biochemical experiments for HDAC–NuRD and BMP–Smads. C. Zhao assisted with ChIP experiments. H.W. assisted with Notch-TP1 reporter assays. C. Zweier provided ZEB2 variant identification data. Z.F. assisted with heat hypersensitivity experiments. M.P.J. assisted with ChIP recordings. B.G.A., P.M., A.Z. and J.R.C. provided input and data interpretation. D.H. provided mice with the loxP-flanked Zeb2 allele and input on the study. L.M.N.W. and Q.R.L. wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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- Pereira, J.A., Lebrun-Julien, F. & Suter, U. Molecular mechanisms regulating myelination in the peripheral nervous system. *Trends Neurosci.* **35**, 123–134 (2012).
- Nave, K.A. & Werner, H.B. Myelination of the nervous system: mechanisms and functions. *Annu. Rev. Cell Dev. Biol.* **30**, 503–533 (2014).
- Salzer, J.L. Schwann cell myelination. *Cold Spring Harb. Perspect. Biol.* **7**, a020529 (2015).
- Dastot-Le Moal, F. *et al.* ZFH1B mutations in patients with Mowat-Wilson syndrome. *Hum. Mutat.* **28**, 313–321 (2007).
- Mowat, D.R., Wilson, M.J. & Goossens, M. Mowat-Wilson syndrome. *J. Med. Genet.* **40**, 305–310 (2003).
- Nagaya, M., Kato, J., Niimi, N., Tanaka, S. & Wakamatsu, N. Clinical features of a form of Hirschsprung's disease caused by a novel genetic abnormality. *J. Pediatr. Surg.* **37**, 1117–1122 (2002).
- McKinsey, G.L. *et al.* Dlx1&2-dependent expression of Zfh1b (Sip1, Zeb2) regulates the fate switch between cortical and striatal interneurons. *Neuron* **77**, 83–98 (2013).
- Weng, Q. *et al.* Dual-mode modulation of Smad signaling by Smad-interacting protein Sip1 is required for myelination in the central nervous system. *Neuron* **73**, 713–728 (2012).
- van den Berghe, V. *et al.* Directed migration of cortical interneurons depends on the cell-autonomous action of Sip1. *Neuron* **77**, 70–82 (2013).
- Verstappen, G. *et al.* Atypical Mowat-Wilson patient confirms the importance of the novel association between ZFH1B/SIP1 and NuRD corepressor complex. *Hum. Mol. Genet.* **17**, 1175–1183 (2008).
- Michailov, G.V. *et al.* Axonal neuregulin-1 regulates myelin sheath thickness. *Science* **304**, 700–703 (2004).
- Taveggia, C. *et al.* Neuregulin-1 type III determines the ensheathment fate of axons. *Neuron* **47**, 681–694 (2005).
- Feltri, M.L. & Wrabetz, L. Laminins and their receptors in Schwann cells and hereditary neuropathies. *J. Peripher. Nerv. Syst.* **10**, 128–143 (2005).
- Nodari, A. *et al.* Alpha6beta4 integrin and dystroglycan cooperate to stabilize the myelin sheath. *J. Neurosci.* **28**, 6714–6719 (2008).
- Monk, K.R. *et al.* A G protein-coupled receptor is essential for Schwann cells to initiate myelination. *Science* **325**, 1402–1405 (2009).
- Woodhoo, A. *et al.* Notch controls embryonic Schwann cell differentiation, postnatal myelination and adult plasticity. *Nat. Neurosci.* **12**, 839–847 (2009).
- Jaegle, M. *et al.* The POU proteins Brn-2 and Oct-6 share important functions in Schwann cell development. *Genes Dev.* **17**, 1380–1391 (2003).
- Topilko, P. *et al.* Krox-20 controls myelination in the peripheral nervous system. *Nature* **371**, 796–799 (1994).
- He, Y. *et al.* Yy1 as a molecular link between neuregulin and transcriptional modulation of peripheral myelination. *Nat. Neurosci.* **13**, 1472–1480 (2010).
- Svaren, J. & Meijer, D. The molecular machinery of myelin gene transcription in Schwann cells. *Glia* **56**, 1541–1551 (2008).
- Higashi, Y. *et al.* Generation of the floxed allele of the SIP1 (Smad-interacting protein 1) gene for Cre-mediated conditional knockout in the mouse. *Genesis* **32**, 82–84 (2002).
- Douglas, W.W. & Ritchie, J.M. Mammalian nonmyelinated nerve fibers. *Physiol. Rev.* **42**, 297–334 (1962).
- Doerflinger, N.H., Macklin, W.B. & Popko, B. Inducible site-specific recombination in myelinating cells. *Genesis* **35**, 63–72 (2003).
- Verschueren, K. *et al.* SIP1, a novel zinc finger/homeodomain repressor, interacts with Smad proteins and binds to 5'-CACCT sequences in candidate target genes. *J. Biol. Chem.* **274**, 20489–20498 (1999).
- Jessen, K.R., Mirsky, R. & Lloyd, A.C. Schwann cells: development and role in nerve repair. *Cold Spring Harb. Perspect. Biol.* **7**, a020487 (2015).
- Parrinello, S. *et al.* EphB signaling directs peripheral nerve regeneration through Sox2-dependent Schwann cell sorting. *Cell* **143**, 145–155 (2010).
- Jessen, K.R. & Mirsky, R. The origin and development of glial cells in peripheral nerves. *Nat. Rev. Neurosci.* **6**, 671–682 (2005).
- Parkinson, D.B. *et al.* c-Jun is a negative regulator of myelination. *J. Cell Biol.* **181**, 625–637 (2008).
- Feltri, M.L., Poitelson, Y. & Previtali, S.C. How Schwann cells sort axons: new concepts. *Neuroscientist* **22**, 252–265 (2016).
- Fischer, A., Schumacher, N., Maier, M., Sendtner, M. & Gessler, M. The Notch target genes Hey1 and Hey2 are required for embryonic vascular development. *Genes Dev.* **18**, 901–911 (2004).
- Xin, M. *et al.* Essential roles of the bHLH transcription factor Hrt2 in repression of atrial gene expression and maintenance of postnatal cardiac function. *Proc. Natl. Acad. Sci. USA* **104**, 7975–7980 (2007).
- Le, N. *et al.* Analysis of congenital hypomyelinating Egr2Lo/Lo nerves identifies Sox2 as an inhibitor of Schwann cell differentiation and myelination. *Proc. Natl. Acad. Sci. USA* **102**, 2596–2601 (2005).
- Mizutani, K., Yoon, K., Dang, L., Tokunaga, A. & Gaiano, N. Differential Notch signalling distinguishes neural stem cells from intermediate progenitors. *Nature* **449**, 351–355 (2007).

34. Souilhol, C. *et al.* Nas transgenic mouse line allows visualization of Notch pathway activity in vivo. *Genesis* **44**, 277–286 (2006).
35. De Strooper, B. *et al.* A presenilin-1-dependent gamma-secretase-like protease mediates release of Notch intracellular domain. *Nature* **398**, 518–522 (1999).
36. Pinnix, C.C. *et al.* Active Notch1 confers a transformed phenotype to primary human melanocytes. *Cancer Res.* **69**, 5312–5320 (2009).
37. Lauberth, S.M. & Rauchman, M. A conserved 12-amino acid motif in Sall1 recruits the nucleosome remodeling and deacetylase corepressor complex. *J. Biol. Chem.* **281**, 23922–23931 (2006).
38. Chen, Y. *et al.* HDAC-mediated deacetylation of NF- κ B is critical for Schwann cell myelination. *Nat. Neurosci.* **14**, 437–441 (2011).
39. Jacob, C. *et al.* HDAC1 and HDAC2 control the transcriptional program of myelination and the survival of Schwann cells. *Nat. Neurosci.* **14**, 429–436 (2011).
40. van Grunsven, L.A. *et al.* XSi1 neutralizing activity involves the co-repressor CtBP and occurs through BMP dependent and independent mechanisms. *Dev. Biol.* **306**, 34–49 (2007).
41. Quintes, S. *et al.* Zeb2 is essential for Schwann cell differentiation, myelination and nerve repair. *Nat. Neurosci.* <http://dx.doi.org/10.1038/nn.4321> (2016).
42. Conidi, A. *et al.* Few Smad proteins and many Smad-interacting proteins yield multiple functions and action modes in TGF β /BMP signaling in vivo. *Cytokine Growth Factor Rev.* **22**, 287–300 (2011).
43. Hung, H., Kohnken, R. & Svaren, J. The nucleosome remodeling and deacetylase chromatin remodeling (NuRD) complex is required for peripheral nerve myelination. *J. Neurosci.* **32**, 1517–1527 (2012).
44. Desmazières, A., Decker, L., Vallat, J.M., Charnay, P. & Gilardi-Hebenstreit, P. Disruption of Krox20-Nab interaction in the mouse leads to peripheral neuropathy with biphasic evolution. *J. Neurosci.* **28**, 5891–5900 (2008).
45. Reynolds, N., O'Shaughnessy, A. & Hendrich, B. Transcriptional repressors: multifaceted regulators of gene expression. *Development* **140**, 505–512 (2013).
46. Fischer, A. *et al.* Hey basic helix-loop-helix transcription factors are repressors of GATA4 and GATA6 and restrict expression of the GATA target gene ANF in fetal hearts. *Mol. Cell. Biol.* **25**, 8960–8970 (2005).
47. Doetzlhofer, A. *et al.* Hey2 regulation by FGF provides a Notch-independent mechanism for maintaining pillar cell fate in the organ of Corti. *Dev. Cell* **16**, 58–69 (2009).
48. Benito-Gonzalez, A. & Doetzlhofer, A. Hey1 and Hey2 control the spatial and temporal pattern of mammalian auditory hair cell differentiation downstream of Hedgehog signaling. *J. Neurosci.* **34**, 12865–12876 (2014).
49. Liu, Z., Owen, T., Fang, J., Srinivasan, R.S. & Zuo, J. In vivo Notch reactivation in differentiating cochlear hair cells induces Sox2 and Prox1 expression but does not disrupt hair cell maturation. *Dev. Dyn.* **241**, 684–696 (2012).
50. Stanchina, L., Van de Putte, T., Goossens, M., Huylebroeck, D. & Bondurand, N. Genetic interaction between Sox10 and Zfhx1b during enteric nervous system development. *Dev. Biol.* **341**, 416–428 (2010).

ONLINE METHODS

Animals. *Zeb2^{loxP/loxP}* mice²¹ were crossed with *Dhh-cre^{+/−}* mice to obtain *Zeb2^{loxP/+};Dhh-cre^{+/−}* mice, which were then bred with *Zeb2^{loxP/loxP}* mice to produce control (*Zeb2^{loxP/+};Dhh-cre^{+/−}*) and *Zeb2* cKO offspring (*Zeb2^{loxP/loxP};Dhh-cre^{+/−}*). Littermates *Zeb2^{loxP/loxP}* or *Zeb2^{loxP/+};Dhh-cre^{+/−}* mice were used as controls. Transgenic Notch reporter (TNR) mice were obtained from the Jackson Laboratory (Stock 018322) and bred with *Zeb2^{loxP/+};Dhh-cre^{+/−}* mice to generate *Zeb2^{loxP/loxP};Dhh-cre^{+/−};Notch-GFP* mice. Recombination, perinatally or after sciatic nerve transection, was achieved by crossing *Zeb2^{loxP/loxP}* mice with the inducible Cre recombinase Cre-ERT2 under the control of the *Plp1* promoter (*Plp1-cre^{ERT}*) followed by tamoxifen injection²³. *Zeb2^{loxP/loxP};Plp1-cre^{ERT}* mice were then bred to mice containing a Cre-responsive *Rosa26 loxP*-flanked stop tdTomato reporter allele. Animals of either sex were used in the study and littermates were used as controls unless otherwise indicated. The mouse strains used in this study were generated and maintained on a mixed C57Bl/6;129Sv background and housed in a vivarium with a 12-h light/dark cycle. No more than 4 adult mice were housed in the same cage per IACUC regulations at CCHMC. All animal experiments were conducted in mice of both genders. All animal use and studies were approved by the Institutional Animal Care and Use Committee at Cincinnati Children's Hospital, USA.

Isolation, growth and differentiation of primary rat SCs. Rat SCs from sciatic nerves of newborn rats (1–2 d old) were isolated as described previously⁵¹. SCs were grown routinely in DMEM/10% FBS (Life Technologies), supplemented with 10 ng/ml Neuregulin 1 (Nrg1; R&D Systems) and 5 μ M forskolin (Sigma) plus L-glutamine and penicillin/streptomycin, hereafter termed SC proliferation medium. Cells between passages 2 and 6 were used in all experiments. >95% SC purity was achieved, assessed by Sox10 and S100 β staining. To initiate differentiation, SCs were washed 4 times with DMEM and then cultured in differentiation medium containing DMEM/0.5% FBS and 1 mM dibutyryl cyclic AMP (Sigma) with L-glutamine and penicillin/streptomycin, for the length of time indicated in the text, depending on the assays used. All tissue culture containers and coverslips were coated with 50 μ g/ml poly-L-lysine (Sigma) in PBS for at least 30 min at room temperature and then rinsed in distilled water. Purified rat SCs seeded on coverslips were fixed in 4% paraformaldehyde (PFA) for 20 min and washed in 1 $\times$ PBS 4 times before immunofluorescence staining.

Immunostaining, *In situ* hybridization and electron microscopy. The sciatic nerves of mice at defined ages were dissected and fixed for 45 min in 4% PFA, embedded in OCT, cryoprotected in 25% sucrose and sectioned at 9 μ m as longitudinal sections. For BrdU pulse labeling, animals at P1 were injected subcutaneously with 100 mg BrdU/kg body weight 2 h before sciatic nerve collection. For immunostaining, we used antibodies to Zeb2 (Santa Cruz Biotechnology, sc-48789), Sox10 (goat; Santa Cruz Biotechnology, sc-17342; rabbit; Millipore; AB5727), Oct6 (goat; Santa Cruz Biotechnology, sc-11661), Krox20 (rabbit; Covance, PRB-236P), MBP (goat; Santa Cruz Biotechnology, sc-13914), Sox2 (goat; Santa Cruz Biotechnology, sc-17320), c-Jun (rabbit; Epitomics, 1254-1), Hey2 (rabbit; Proteintech, 10597-1-AP), Ki67 (rabbit; Thermo Scientific, RM-9106), BrdU (rat; Abcam, ab6326), cleaved caspase 3 (rabbit; Cell Signaling, #9661), S100 β (mouse; Sigma, SAB1402349), GFP (rabbit; Life Technologies, A11122); c-MYC (mouse; Santa Cruz Biotechnology, sc-40), Flag (mouse; Cell Signaling, #8146) and HA-tag (Upstate, 50-171-890). Secondary antibodies conjugated to Cy2, Cy3 or Cy5 were from Jackson ImmunoResearch Laboratories catalog numbers 705-165-147, 705-225-147, 711-225-152, 711-165-152, 711-175-152, 715-165-150 and 712-165-150. All images were acquired using a Zeiss LSM 510 or Nikon C2+ confocal microscope. RNA *in situ* hybridization was performed using digoxigenin-labeled riboprobes as described previously⁵². The probes used were murine Mbp and Mpz. Detailed protocols are available upon request. For electron microscopy, mice were perfused with 4% PFA, 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2. Sciatic nerves were dissected and fixed in the same fixative solution overnight. Nerves were rinsed in PBS, postfixed in 1% OsO₄ in PBS for 1 h, dehydrated in graded ethanol, infiltrated with propylene oxide and embedded in Epon. Semi-thin sections were stained with toluidine blue, and thin sections were stained with lead citrate.

Transient transfections and luciferase assays. For plasmid transfections, rat SCs were transfected with expression vectors using Lipofectamine 3000 (Life

Technologies) per the manufacturer's protocol and assayed for immunocytochemistry or qPCR analysis. pHA-Sox2 was purchased from Addgene.

For siRNA knockdown in SCs, we used Lipofectamine RNAiMAX (Life Technologies) per the manufacturer's instructions. SCs were induced to differentiate after 48 h of transfection for 24 h before harvesting for immunocytochemistry or qPCR analysis. For sequential siRNA knockdown, SCs in proliferation medium was transfected with *Zeb2*-specific siRNA for 24 h before *Sox2* or *Hey2* siRNA transfection. Triple knockdown was performed in the order of *Zeb2*, *Hey2* and *Sox2* siRNA, each separated by a 24-h interval. *Zeb2* siRNA: SASI_Rn01_00044097 (GCAAGAAAUGAUUGGUUU); SASI_Rn01_00044098 (GUAUGCAUGUGACUUA UGU); SASI_Rn02_00203739 (CAGAUGAACCCUGAAUUU). *Sox2* siRNA: CCACCUACAGCAUGUC CUA[dT][dT]; *Hey2* siRNA: GGAUCGAAUAAUAAACAGU[dT][dT] and CCAUGCAGAUUCUGCUCUU[dT][dT].

For DAPT (Sigma) treatment, 48 h after transfection with scramble or *Zeb2* siRNA, SCs on coverslips or in 12-well culture dishes were induced to differentiate with 1 mM dbcAMP and treated with DMSO or 5 μ M DAPT for 4 d and then assayed for immunocytochemistry or qRT-PCR analysis. Proliferation was assessed by immunostaining for Ki67 in Sox10⁺ SCs seeded on PLL-coated coverslips for 2 d. Differentiation was assessed by immunostaining for Oct6 or Krox20. Multiple images were taken from each coverslip to obtain representative images from all areas of the coverslip, and at least 400 cells/coverslip were counted using ImageJ (NIH). For quantitation, GFP⁺ or fusion tag-expressing cells were counted, and the percentage of GFP⁺/fusion-tag⁺ cells that expressed the protein of interest was determined. At least 100 GFP⁺ or fusion-tag⁺ cells/coverslip were counted.

For reporter assays, purified rat SCs were transfected with pGL3-luciferase reporters containing inserts from 2 kb upstream of *Sox2* TSS or 3.9 kb upstream of *Hey2* TSS and with expression vectors using Lipofectamine 3000 (Life Technologies) per the manufacturer's protocol, and were assayed 48 h post-transfection for luciferase activity using a Promega luciferase assay kit according to the manufacturer's instructions. The pSV- β -galactosidase control vector was included to control for variable transfection efficiencies between independent experiments ($n \geq 3$). Mutant Sox2 promoter luciferase reporter was generated by mutating a *Zeb2* consensus binding site (P1; −1.1 kb from TSS; Fig. 5i) into an EcoRI site, and mutant Hey2 promoter luciferase reporter was generated by mutating a *Zeb2* binding site into an EcoRI site.

Western blotting and coimmunoprecipitation. For western blotting, the perineurium and epineurium were removed from sciatic nerves before snap-freezing and storage at −80 °C. Sciatic nerves and rat SCs were lysed in RIPA buffer, containing protease and phosphatase inhibitors. Western blot analysis was performed as described previously³⁸. GAPDH was used as an input control. For immunoprecipitation, HEK 293T cells cultured in 10% FBS/DMEM were transfected with expression vectors using PolyJet (Signagen) for 48 h. Cells were lysed in NP-40 buffer (170 mM NaCl, 10 mM EDTA, 50 mM Tris pH 7.4, 50 mM NaF and 0.5% NP-40) supplemented with protease inhibitor cocktail and phosphatase inhibitors (Roche Diagnostics Inc.). A total of 300 μ g of cell lysate proteins were incubated with 2 μ g IgG or appropriate antibodies and immunoprecipitated using Protein A/G beads (Santa Cruz Biotechnology, sc-2003). Western blotting was performed using chemiluminescence with the ECL kit (Pierce). The antibodies used were anti-Zeb2 (Millipore, ABT-332), anti-Flag (rabbit #14793 or mouse #8146, Cell Signaling), anti-Myc (Santa Cruz, sc-40). For coimmunoprecipitation of endogenous NuRD subunits, extracts of HEK 293T cells transiently expressing Flag-tagged ZEB2^{WT} or Flag-tagged ZEB2^{R22G} were precipitated in a Flag-dependent manner and eluted using 3XFlag peptides according to Verstaappen *et al.*¹⁰. Co-immunoprecipitation experiments showing endogenous interaction between Zeb2 and HDAC1/2 were performed by incubating protein lysates from purified rat SCs with anti-Zeb2 antibody followed by immunoprecipitation using Protein A/G beads and detection of HDAC1/2 using HDAC1/2-specific antibodies. Antibodies used were HDAC1 (Santa Cruz, sc-7872), HDAC2 (Abcam #7029), Mi2 (Santa Cruz, sc-11378), MTA-1 (Santa Cruz, sc-9446), MTA-2 (Santa Cruz, sc-28731), RbAp48 (Abcam, 38185) and CtBP (Sigma, C8741). Secondary antibodies conjugated to HRP were from Jackson ImmunoResearch Laboratories. catalog numbers 111-035-144 and 111-035-003.

RNA-seq and data analysis. RNA from P7 control and *Zeb2* cKO sciatic nerves were extracted using TRIzol (Life Technologies) followed by purification using an RNeasy Mini Kit (Qiagen). RNA-seq libraries were prepared using Illumina RNA-Seq Preparation Kit and sequenced by HiSeq 2000 sequencer. RNA-seq reads were mapped using TopHat with default settings (<http://ccb.jhu.edu/software/tophat/index.shtml>). TopHat output data were then analyzed by Cufflinks to (1) calculate FPKM values for known transcripts in mouse genome reference and (2) test the changes of gene expression between *Zeb2* cKO and control. Gene ontology (GO) analysis was performed using The Database for Annotation, Visualization and Integrated Discovery (DAVID; <http://david.abcc.ncifcrf.gov>). The heat map was generated based on $\log_2$ [FPKM] by AltAnalyze^{53,54} with normalization of rows relative to row mean.

ChIP assays. ChIP assays were performed as described with minor modifications^{38,55}. Briefly, purified rat SCs grown in proliferation and differentiation (9 h in 1 mM cAMP-containing medium conditions; ~20 million cells) were fixed for 10 min at room temperature with 1% formaldehyde-containing medium. Nuclei were pelleted and sonicated in sonication buffer (10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 0.5 mM EGTA and protease inhibitor cocktail). Sonicated chromatin (~300 µg) was used for immunoprecipitation by incubation with IgG or rabbit anti-*Zeb2* antibody (H-260; Santa Cruz Biotechnology sc-48789; 4 µg) overnight at 4 °C. We kept 10% of the chromatin used for each ChIP reaction as input DNA. Pre-rinsed protein A/G plus agarose beads (50 µl) were added to each ChIP reaction and incubated for 1 h at 4 °C. The beads were then incubated in 200 µl elution buffer at 65 °C for 20 min to elute immunoprecipitated materials. For ChIP, SCs at 70–90% confluency were transfected with control or *Zeb2* siRNA for 48 h using Lipofectamine RNAiMAX per the manufacturer's instructions and induced to differentiate for 9 h with 1 mM cAMP. ChIP was performed using anti-HDAC1 (Active Motif #40967) and HDAC2 antibody (Abcam #7029). Real-time PCR was performed using quantitative SYBR green PCR mix (Bio-Rad). The relative fold-enrichments were determined by $2^{-\Delta CT}$ methods as described³⁸. Samples were normalized to input chromatin. Primers used for ChIP-qPCR analysis:

Sox2 proximal promoter (P1): forward ATTGGTGGAGGAGGGAGTTA; reverse TATGGCTGACTGTAGAGCTAGG;

Sox2 proximal promoter (P2): forward CTGCACCTGCACCTCTG; reverse CAGCGTTCCCAGATGG;

Sox2 proximal promoter (P3): forward CTCCACCCGTGTTTCCTAAAT; reverse CTTCTGCCCTCTAAGTCACAAA;

Hey2 proximal promoter (−3.9 kb from TSS): forward: GCTCACAATA GCCTGTAGTT; reverse: AAGTCAGTCTCTGTCCTGGT.

Oct6 proximal promoter (−0.8 kb from TSS): forward: TCCTAGAGCTCG CATCTCC; reverse: ATCGGCTTGGGCTTCAC.

Oct6 proximal promoter (−3.9 kb from TSS): forward: GTGTCCGT ATGTCTTCGAGATG; reverse: ACACGGTGCTATTACACAAA.

Jun proximal promoter (−6 kb from TSS): forward: CCCAAGAC CTGTGTGAGAAAT; reverse: CTCACAGTTTGATTGGCTGAAA.

Distal control region ChIP-PCR: *Sox2* (+25 kb TSS): forward GAACAGATG AGTGTTCCTTTAG; reverse ACTCCTGGACCAACATAC;

Hey2 (+20 kb TSS): forward GAAACTGTATACGGCCTCTG; reverse GATCTGCATCGCCCTTTCT;

Oct6 (+18 kb TSS): forward GTAGAGGAAAGCAGAGGAAAGG; reverse GAATCCCATAGCCAGACATGAT.

RNA isolation and quantitative real-time PCR. RNA from purified rat SCs or control and *Zeb2* cKO mouse sciatic nerves was extracted using TRIzol (Life Technologies). cDNA was synthesized from 1 µg RNA using iScript Reverse Transcription Supermix (BioRad) according to the manufacturer's instructions. qPCR was performed using quantitative SYBR green PCR mix (BioRad) as previously described⁵⁶. PCR primer sequences are available upon request.

RNA-seq and data analysis. RNA-seq libraries were prepared using Illumina RNA-Seq Preparation Kit and sequenced by a HiSeq 2000 sequencer. All RNA-Seq data were aligned to mm9 using TopHat⁵⁷ with default settings. We used Cuff-diff⁵⁸ to (1) estimate FPKM values for known transcripts and (2) analyze differentially expressed transcripts. In all differential expression tests, a difference was considered significant if the *q* value was less than 0.05 (Cuff-diff default).

Heat map of gene expression was generated using R language (<http://www.r-project.org>). GO-analysis of gene expression changes was performed using Gene Set Enrichment (GSEA, <http://www.broadinstitute.org/gsea/index.jsp>). We used ToppCluster (<https://toppcluster.cchmc.org/>) to construct the network of genes belonging to over-represented GO-term categories.

Tamoxifen injections. We used Tamoxifen (Sigma) dissolved to a stock concentration of 20 mg/ml in a vehicle of ethanol and sunflower seed oil (1:9 v/v). For perinatal tamoxifen injections, tamoxifen stock was injected i.p. at 2 mg/100 µl for 5 consecutive d to lactating mothers, thus administering tamoxifen to pups, beginning at P0. Sciatic nerves of pups were analyzed on P14 for electron microscopy. For adult tamoxifen treatment, 100 µl (2 mg/100 µl) was administered by i.p. injection once daily for 5 consecutive d to 4–6 week-old mice. Mice were treated again for 5 d after a 2-d rest period. Control mice were treated identically.

In vivo drug treatment. Control and *Zeb2* cKO were injected on P2, P3, P5 and P7 with the Notch signaling (γ -secretase) inhibitor LY411575 (Selleckchem) at 0.30 mg kg^{−1} per day. Sciatic nerves were fixed in 4% PFA on P8 for immunostaining. LY411575 was diluted in a mixture of corn oil and EtOH (95:5 v/v). Vehicle was corn oil and EtOH (95:5 v/v).

Generation and transduction of lentivirus. Lentivirus was produced by transfection of DN-MAML lentivirus construct (MAML1 1-305 subcloned into BamHI and XhoI sites of PLU-ires-GFP mod vector, a kind gift from A.J. Capobianco) into HEK 293T cells with the packaging vectors psPAX2 and pMD2.G using Polyjet *in vitro* DNA transfection reagent (Sigma) according to the instructions of the manufacturer. The supernatant was collected 72 h after transfection and frozen in liquid nitrogen. A titer of 10⁷/µl was achieved. SCs previously transfected with control or *Zeb2* siRNA for 24 h were infected with control GFP lentivirus or DN-MAML-GFP lentivirus in the presence of 4 µg/ml Polybrene for 48 h before harvesting for RNA.

ZEB2 variant identification. The novel ZEB2 variant c. 64A>G [p.(Arg22Gly)] was identified by targeted diagnostic sequencing of the coding exons of ZEB2 in a patient with a facial gestalt reminiscent of MOWS (OMIM 235730). The patient had mild manifestation of MOWS phenotype. This mutation was not detected in any other patients and is not contained in the ExAC database. This variant is atypical since missense mutations in ZEB2 are very rare in MOWS; usually truncating mutations are found. Due to the young age (4 years old) of the patient, peripheral nerve-related phenotyping was clinically impossible to test for, and therefore we could not assess the peripheral nerve related phenotype.

Sciatic nerve transection surgeries. Sciatic nerve transection was performed 10 d after the last tamoxifen injection in 6–8 week-old mice. Under general anesthesia after injection of a mixture of ketamine (90 mg/kg) and xylazine (10 mg/kg), right sciatic nerves were exposed and transected at the midhigh level. Exposed left sciatic nerves were used as uncut controls. Mice were treated with tamoxifen for an additional 8 d following injury. Nerves were collected 14 d after surgery and processed for immunohistochemistry.

Compound muscle action potential recording. To analyze nerve conduction and motor unit function, single CMAPs were recorded *in vivo* from the lateral gastrocnemius muscles of wild-type littermate controls or *Zeb2* cKO mice during electrical stimulation of the sciatic nerve under sodium pentobarbital anesthesia (50 mg/kg, i.p.). The lateral gastrocnemius muscle was first exposed from the knee to approximately 6 mm above the ankle. A small opening was then made in the overlying fascia to facilitate implantation of the recording wires into the muscles. Next, the sciatic nerve was exposed near the biceps femoris muscles. Polymide-coated steel recording wires (California Fine Wire Inc. stainless steel 304; size = 0.0092 and insulated with polyimide; material #100192) were then implanted into the lateral gastrocnemius muscles and reference wires were inserted under the nearby skin. To induce electrical activation of the nerve *in vivo*, a concentric bipolar stimulating electrode was placed on the sciatic nerve. CMAPs were amplified and obtained using a Micro 1401 data acquisition unit and analyzed using Spike2 software (Cambridge Electronic Design, Cambridge, UK). Three successive electrical stimulations of the sciatic nerve at 2 mA (0.25–0.5 Hz, 0.1 ms duration) were initiated immediately proximal to the tibial, sural and common

peroneal branches via a stimulus isolator (WPI Inc.) connected to the Micro 1401. After recording, the sciatic nerve was axotomized distal to the site of concentric electrode placement and the proximal end of the sciatic nerve was stimulated to ensure CMAPs were generated from direct nerve stimulation. Conduction velocity was calculated after determining the latency of CMAP onset relative to the stimulus artifact induced by electrical stimulation of the sciatic nerve and the distance between recording and stimulating electrodes measured directly on the nerve. Peak CMAP amplitude and CMAP duration were calculated from each stimulation paradigm. The average of the stimulations of the sciatic nerve for each paradigm were obtained and then averaged across animals.

Heat hypersensitivity assessments. Mice at 3–4 weeks old were acclimatized individually in a box of at least $15 \times 12 \times 10 \text{ cm}^3$ in dimensions for 15 min. Mice were then gently held and both hindpaws were immersed into a 50°C hot water bath. Latency (in s) to withdrawal from the heat stimulus was determined. Three trials were performed with a 5-min interval in between each trial. The average of these three trials was then determined to obtain the baseline withdrawal latency. Next, mice were anesthetized under 3% isoflurane in order to induce cutaneous inflammation. Using a syringe with a 30 G needle, approximately $10 \mu\text{L}$ of 3% carrageenan (in 0.9% NaCl) was injected into the right hairy hindpaw skin. 24 h later, thermal withdrawal latency using the methods described above were measured again to determine heat hypersensitivity (withdrawal latency) post inflammation^{59,60}.

Morphometric analysis. The morphometric measurements in the early postnatal *Zeb2* iKO experiment and the axon regeneration/remyelination assays were performed in Toluidine blue-stained semi-thin sections ($0.5 \mu\text{m}$). An entire sciatic nerve cross section per animal was reconstructed by merging several high magnification photographs taken by bright field microscopy at $100\times$ magnification. The quantifications of the 1:1 promyelinating cells, myelinated fibers and numbers of axon were performed by manually counting all of the figures present in the entire sciatic nerves cross section using the cell counter plugin from ImageJ software (National Institutes of Health).

The number of myelinated axons per nerve, the G-ratio and the number of unmyelinated axons in Remak bundles were analyzed in ultrathin sections using a JEOL 1200 EXII or Hitachi H7650 electron microscope. Quantification was done by manually counting myelinated or unmyelinated axons present in the sciatic nerves using the cell counter plugin from ImageJ software. For G-ratio analysis, images were calibrated based on the scale bar corresponding to the magnification used for each micrograph. The axons (without myelin) and fiber areas (including myelin) of randomly chosen fibers were measured, except for those surrounded by a Schmidt Lanterman incisure. The axon and fiber diameters were estimated as

$$2\sqrt{\frac{\text{area}}{\pi}}$$

and the G-ratio was calculated as axon diameter/fiber diameter. At least 100 nerve fibers per animal ($n = 3$ for each group) were analyzed.

For quantification of labeled cells in transfection studies, since the ZEB2, ZEB2^{R22G} and ZEB2^{ΔCTBP} expression plasmids carry a GFP-reporter, GFP⁺ cells represent ZEB2 or ZEB2-mutant transfected cells. The quantification was performed by manually counting antibody co-labeled cells based on the total number of transfected cells indicated by GFP expression.

Statistical analysis. All analyses were done using Microsoft Excel or GraphPad Prism 6.00 (San Diego California, <http://www.graphpad.com>). Data are shown in dot plots as mean $\pm$ s.e.m. or as box-and-whisker plots; $P < 0.05$ is deemed statistically significant. Data distribution was assumed to be normal, but this was not formally tested. Count data was assumed to be non-parametric, and appropriate statistical tests were used. Statistical analysis was performed by two-tailed unpaired Student's *t* tests, Mann-Whitney test, one-way ANOVA with post-hoc analysis by Tukey's multiple comparison test, two-way repeated measures ANOVA with Sidak's multiple comparisons test, or as indicated. Quantifications were performed from at least three experimental groups in a blinded fashion. No statistical methods were used to predetermine sample sizes, but our sample sizes are similar to those generally employed in the field. No randomization was used to collect all the data; they were quantified blindly. No animals or data points were excluded from analyses.

A **Supplementary Methods Checklist** is available.

Data availability. All the RNA-seq data have been deposited in the NCBI Gene Expression Omnibus (GEO) under accession number [GSE74381](https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE74381). The data that support the findings of this study are available from the corresponding author upon reasonable request.

51. Brockes, J.P., Fields, K.L. & Raff, M.C. Studies on cultured rat Schwann cells. I. Establishment of purified populations from cultures of peripheral nerve. *Brain Res.* **165**, 105–118 (1979).
52. Lu, Q.R. *et al.* Common developmental requirement for Olig function indicates a motor neuron/oligodendrocyte connection. *Cell* **109**, 75–86 (2002).
53. Emig, D. *et al.* AltAnalyze and DomainGraph: analyzing and visualizing exon expression data. *Nucleic Acids Res.* **38**, W755–62 (2010).
54. Salomonis, N. *et al.* Alternative splicing regulates mouse embryonic stem cell pluripotency and differentiation. *Proc. Natl. Acad. Sci. USA* **107**, 10514–10519 (2010).
55. Yu, Y. *et al.* Olig2 targets chromatin remodelers to enhancers to initiate oligodendrocyte differentiation. *Cell* **152**, 248–261 (2013).
56. Becker, W. Recent insights into the function of DYRK1A. *FEBS J.* **278**, 222 (2011).
57. Trapnell, C., Pachter, L. & Salzberg, S.L. TopHat: discovering splice junctions with RNA-Seq. *Bioinformatics* **25**, 1105–1111 (2009).
58. Trapnell, C. *et al.* Transcript assembly and quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell differentiation. *Nat. Biotechnol.* **28**, 511–515 (2010).
59. Ririe, D.G., Bremner, L.R. & Fitzgerald, M. Comparison of the immediate effects of surgical incision on dorsal horn neuronal receptive field size and responses during postnatal development. *Anesthesiology* **109**, 698–706 (2008).
60. Marsh, D., Dickenson, A., Hatch, D. & Fitzgerald, M. Epidural opioid analgesia in infant rats I: mechanical and heat responses. *Pain* **82**, 23–32 (1999).

Corrigendum: Zeb2 recruits HDAC–NuRD to inhibit Notch and controls Schwann cell differentiation and remyelination

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In the version of this article initially published online, Danny Huylebroeck was listed under affiliation 2 (Institute of Pharmacology and Toxicology, College of Pharmaceutical Sciences, Zhejiang University, Hangzhou, China) instead of affiliation 3 (Department of Cell Biology, Erasmus University Medical Center, Rotterdam, the Netherlands). The error has been corrected for the print, PDF and HTML versions of this article.