Microprocessor Complex Subunit DiGeorge Syndrome Critical Region Gene 8 (Dgcr8) Is Required for Schwann Cell Myelination and Myelin Maintenance*§

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Background: Dgcr8 regulates primary miRNA processing in the nucleus.

Results: Conditionally ablating Dgcr8 in Schwann cells during development or in adulthood causes defects in myelin formation and gene expression characteristic of immature and denervated (injured) Schwann cells.

Conclusion: Dgcr8 is needed for myelin formation and maintenance.

Significance: miRNAs synchronize the translation of genes essential to myelin formation.

During the development of a multicellular organism, cells differentiate to acquire specialized functions. During the process of differentiation, precedent gene programs are turned off, and new cell-specific gene expression programs are activated. Elucidating the mechanisms underpinning the precise execution of developmental transitions is critical for understanding developmental disorders as well as plasticity of cell types.

Schwann cells (SCs) are a good model to study cellular differentiation because SC differentiation states can be readily identified by morphology and molecular markers (1, 2). During development, SCs go through multiple stages of differentiation to myelinate axons of the peripheral nervous system. SC development begins with the transition of migrating neural crest cells into SC precursors, which then generate committed immature SCs. Committed immature SCs ensheath large or small diameter axons and differentiate into myelinating or non-myelinating SCs, respectively. Non-myelinating SCs wrap around multiple small diameter axons to collectively form Remak bundles. Myelinating SCs go through a process termed radial sorting to establish 1:1 relationships with single axons, characteristic of the promyelinating stage, before producing multilamellar myelin sheaths (1). Each of these stages of SC differentiation is characterized by distinctive morphology. Proper myelination and myelin maintenance is essential for the saltatory conduction of nerve impulses as well as trophic support of axons (3). Patients with demyelinating neuropathies, in

* This work was supported, in whole or in part, by National Institutes of Health Grants 1RO1NS071081-01 from National Institute of Neurological Disorders and Stroke (to R.A.). The authors declare that they have no conflicts of interest with the contents of this article.

† This article contains supplemental File 1 and Video 1.

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2 The abbreviations used are: SC, Schwann cell; miRNA, microRNA; cKO, conditional knock-out; TAM, tamoxifen; SN, sciatic nerve; EdU, 5-ethynyl-2’-deoxyuridine; qRT, quantitative real-time; Egr, early growth response; Tcf, transcription factor; Pmp, peripheral myelin protein; Nfkb1, nuclear factor of kappa light polypeptide gene enhancer in B cells 1; Hes1, hairy and enhancer of split 1; Id2, inhibitor of DNA binding 2; Cnp, 2’,3’-cyclic nucleotide 3’-phosphodiesterase; Pou3f1, POU domain, class 3, transcription factor 1; Plp, proteolipid protein; Rara, retinoic acid receptor α; Gpr126, G protein-coupled receptor 126; Ncam1, neural cell adhesion molecule 1; Nrcam, neuron-glia-CAM-related cell adhesion molecule; Cdh2, cadherin 2; Tnc, tenascin C; Smoc1, SPARC-related modular calcium binding 1; MUT, mutant; iKO, induced knock-out; IQR, interquartile range; P, postnatal day.
which myelin is poorly developed or fails to be maintained, have accompanying dysfunction of the peripheral nervous system (4, 5).

Each stage of SC differentiation, in addition to being characterized by distinctive morphology, is also characterized by the expression of distinct sets of molecular markers and transcription factors (1, 2, 6, 7). Positive regulators of differentiation, such as Egr2, are up-regulated during development and act on cell-intrinsic signaling pathways to drive myelination (8–13); complementary negative regulators of differentiation and genes characteristic of the immature stage, such as Sox2 and Jun proto-oncogene (JUN), are down-regulated during development and up-regulated upon injury, potentially working to prevent myelin formation or maintenance (6). The master positive regulator of SC myelination, Egr2, is necessary and sufficient to drive myelination (14–17). It directly activates mature SC genes encoding key myelin proteins (8–13) and transcriptionally represses the expression of genes characteristic of the immature stage such as Sox2 and Jun (14, 18, 19). Egr2 is antagonized by Sox2 and JUN in a cross-antagonistic circuit that modulates the proper timing of myelination and governs active demyelination after nerve trauma (14, 18, 20–22). Loss of Egr2 results in the arrest of SC differentiation at the transition between the promyelinating and myelinating stages and leads to drastically reduced myelin formation in peripheral nerves (congenital hypomyelination) (14, 18, 19).

MicroRNA profiling of SCs has been reported (28–36). MicroRNAs (miRNAs) are small non-coding RNAs that regulate global gene expression in various developmental, physiological, stress, aging, and disease scenarios (37–39). They are transcribed as long primary miRNAs processed by the Microprocessor complex (formed by drosha ribonuclease type III, DROSHA, and DGCR8) into precursor miRNAs, exported into the cytoplasm, and cleaved by dicer1 ribonuclease type III (Dicer1) to mature ~22-nucleotide-long miRNAs (40). miRNAs can silence hundreds of target genes by base pairing with target mRNA, resulting in mRNA destabilization and translational repression. Although the expression of target genes is subtly regulated by miRNAs, the additive effect of miRNAs can silence hundreds of target genes by base pairing and feed-forward and feedback loops can result in strong phenotypic outputs (41–43).

Recently our group and others have found that Dicer1 is crucial for SC myelination (28–31, 44). Mice lacking SC Dicer1 during development (P0::Cre+/−, Dicer1flox/flox, or Dicer1 cKOs) display severe neurological impairment. The majority of Dicer1 cKO SCs achieve 1:1 relationships with axons but stall at the transition between the promyelinating and myelinating stages of differentiation, partially mimicking the phenotype of Egr2 mutants. On the other hand, mice with tamoxifen (TAM)-induced late deletion of Dicer1 (Pip::CreERt2+/−, Dicer1flox/flox, or Dicer1 iKOs) in SCs that have completed myelination show no apparent pathology up to 14 weeks after deletion but demonstrate delayed transitioning from proliferation to remyelination after nerve injury (31). Together, these studies suggest that miRNAs modulate the transition between differentiation states in SCs.

Ablating Dicer1 in adult SCs does not affect myelin maintenance but hinders the process of myelination and remyelination. Puzzled in part by the lack of a phenotype in Dicer1 iKO SCs (31), we sought to examine the role of miRNAs in SC development using a different conditional mutant. In this study we conditionally knocked out Dgcr8 in developing and adult SCs to examine its role in SC myelination and myelin maintenance. The novel findings of this study are that (i) Dgcr8 cKOs SCs are stalled in differentiation, like Dicer1 cKOs (28), but the phenotype of Dgcr8 mutants appears to be more severe, (ii) a de novo injury-specific program is elevated in Dgcr8 mutants at a very early age, and (iii) ablation of Dgcr8 in adult SCs results in profound gene expression changes, including activation of the de novo injury-specific gene expression program as well as SC degeneration, the latter of which is not observed in equivalent Dicer1 mutants (28). These results suggest that Dgcr8 plays an important role in myelination and myelin maintenance and that Dgcr8 and Dicer1, in addition to processing sequential steps in miRNA biogenesis, may also have some distinct functions.

Experimental Procedures

For developmental studies, P0::Cre+/− (45) and Dgcr8flox/wt (46) mice were bred to Dgcr8flox/flox mice on a mixed FVB/B6 background. Sciatric nerves (SNs) were harvested at different time points and processed for the appropriate assay. When necessary, 50 mg/kg 5-ethynyl-2′-deoxuryridine (EdU) (Molecular Probes) was administered through intraperitoneal injection 2 h before dissection. Based on Institutional Animal Care and Use Committee (IACUC) guidelines, we did not carry Dgcr8 cKO mice older than 4 weeks for humane reasons. For myelin maintenance and nerve injury studies, Plp::CreERt2+/− (47), Dgcr8flox/wt, and Ai9+/− (48) mice were bred to Dgcr8flox/flox mice on a B6 background. TAM injection and SN surgery were performed as described previously (31). Dgcr8 iKO mutants developed a severe CNS-related phenotype and were kept no longer than 12 weeks after TAM administration. For the Shh lineage study, Shh::Cre+/− mice (49), with Cre knocked into an endogenous Shh locus, were bred to R26RZsg+/+/+ mice (50) on a mixed FVB/B6 background. To obtain Egr2 null mice (Egr2−/− or Egr2Cre/Cre), Egr2Cre/wt mice were crossed on a mixed 129Sv/B6/DAB background (51).

Semithin Analyses and Electron Microscopy (EM)—SN samples were immersed in fixative (2% paraformaldehyde + 2.5% glutaraldehyde + 0.1 M sodium cacodylate); semithin analysis with EM was performed either at the Center for Advanced Microscopy of Northwestern University or in the laboratory of Dr. Wrabetz as described previously (52).

RNA Extraction and qRT-PCR—RNA extraction was performed using mirVana miRNA isolation kit (Life Technologies, Inc.) as described previously (28). cDNAs were synthesized from DNase-treated (DNA-free kit; Life Technologies) RNA samples using SuperScript VILO Master Mix (Life Technologies). qRT-PCR was performed using TaqMan Universal Master Mix II, no UNG, and Taqman Gene Assays or Taqman MicroRNA Assay on 7500HT Real-time PCR System (Life Technologies). Several internal controls were used including glyceraldehyde-3-phosphate dehydrogenase (Gapdh), hypox-
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anthine guanine phosphoribosyltransferase (Hprt), β actin (Actb), and small nuclear RNA-202 for miRNAs.

The Taqman Gene Assays used include: nerve growth factor receptor (Ngrf) (Mm01309638_m1); vimentin (Vim) (Mm01333430_m1); early growth response 1 (Egr1) (Mm00656724_m1); early growth response 3 (Egr3) (Mm00516979_m1); transcription factor 4 (Tcf4) (Mm00443210_m1); peripheral myelin protein 22 (Pmp22) (Mm00476979_m1); glial cell line-derived neurotrophic factor (Gdnf) (Mm00599849_m1); Shh (Mm00436528_m1); myelin basic protein (Mb) (Mm01266402_m1); myelin protein zero (Mpz) (Mm01290519_m1); Egr2 (Mm00456650_m1); desert hedgehog (Dhh) (Mm01310203_m1); patched homolog 2 (Ptch2) (Mm00436047_m1); hedgehog-interacting protein (Hhip) (Mm00469580_m1); SRY (sex determining region Y)-box 10 (Sox10) (Mm01300162_m1); smoothened homolog (Smo) (Mm01162710_m1); gap junction protein, α1 (Gja1) (Mm00439105_m1); delta-like 1 (Dll1) (Mm01279269_m1); paired box 3 (Pax3) (Mm00435491_m1); a disintegrin and metallopeptidase domain 17 (Adam17) (Mm00446288_m1); Jun (Mm00495662_s1); Hprt (hypoxanthine guanine phosphoribosyl transferase; Mm00446968_m1); cyclin D1 (Ccnd1) (Mm00432359_m1); Notch1 (Mm00435249_m1); Sox2 (Mm00495662_m1); SPARC-related modular calcium binding 1 (Smoc1) (Mm00491564_m1); Dgcr8 in Schwann Cells

Real-time PCR miRNA Arrays—Real-time PCR miRNA arrays were performed on TaqMan Rodent MicroRNA A+B Cards Set v3.0 (Life Technologies) according to the manufacturer’s instructions.

Immunofluorescence—Immunohistochemistry was performed as described previously (18, 28). A Click-iT EdU Alexa Fluor imaging kit (Molecular Probes) was used for labeling EdU. For adult SN labeling of F4/80 and CCND1, immunohistochemistry was performed by the Mouse Histology and Pheotyping Laboratory of Northwestern University. Slides were incubated with 3% hydrogen peroxide in double distilled H2O to block endogenous peroxidase and with avidin/biotin blocking solution to block endogenous biotin. Amplification was performed using Vectastain ABC kit (Vector Laboratories) followed by Biotinyl Tyramide Working Solution and Streptavidin-Alexa 488.

Quantification and Statistics—If not stated otherwise, p values were determined by unpaired, two-tailed, equal variance, or unequal variance Student’s t tests using Microsoft Excel. The equality of the variances was determined with F tests. Results are given as the means ± S.E. of the mean. Benjamini-Hochberg corrections were used to adjust p values for false discovery rate with miRNA arrays. χ^2 tests and one-factor analysis of variance tests were used to test significance in cases of categorical comparisons and multiple comparisons. For quantification of measures in Dgcr8 cKO and control nerves, counts were obtained manually on three representative fields (immunofluorescence 20×, semithin 100×, EM 440×) of at least three control and three mutant animals. For quantification of F4/80 staining in Dgcr8 iKO and control nerves, measurements were obtained of F4/80 + and the nerve area in 10 random, non-overlapping fields (20×) using ImageJ. For quantification of axons lacking myelin sheaths and severely degenerating SCs in Dgcr8 iKO and control nerves, counts were obtained from 10 random, non-overlapping fields (890×). Wilcoxon-Mann-Whitney tests were performed using Microsoft Excel with Analyze-it, Standard Edition (Analyze-it Software, Ltd). Semi-automated computer-based morphometry was performed on semithin sections of sciatic nerves to determine the G ratio and the distribution of fiber diameters for myelinated axons using Leica Q-Win/Pro software (Leica Microsystems). The G ratio was determined by dividing the mean diameter of an axon (without myelin) by the mean diameter of the same axon including myelin.

Results

Knocking Out Dgcr8 in SCs During Development Results in Reduced Peripheral Nerve Myelination—To obtain Dgcr8 deletions in SCs, we crossed Dgcr8^floxed/flox mice to P0::Cre mice to generate P0::Cre^+/−, Dgcr8^floxed/flox (Dgcr8 cKO) mice (28, 45). The P0::Cre strain displays activity starting at about E14 (45, 52) in most, if not all, SCs in peripheral nerves. P0::Cre-mediated recombination is specific to SCs and is not observed in DRG neurons (52). Upon exposure to Cre recombinase at midgestation, exon 3 of Dgcr8 was deleted from the Dgcr8^floxed/flox allele in SCs, generating a frameshift mutation that resulted in loss of the WW protein-protein interaction and RNA binding domains of Dgcr8. This conditional deletion was designed to disrupt Drosha/Dgcr8-dependent cleavage of precursor miRNAs into mature miRNAs and thus inhibit canonical Dgcr8-dependent miRNA biogenesis in SCs (46).

To verify successful disruption of miRNA biogenesis in SCs, we analyzed miRNA levels in SNs at postnatal day (P) 7 in control and Dgcr8 cKO mice using TaqMan Array Rodent MicroRNA Cards (supplemental File 1). Similarly to Dicer1 cKOs, the expression of only a small subset of miRNAs was drastically reduced in Dgcr8 cKOs. 12 of 641 unique mouse
miRNAs showed a >10-fold reduction in expression and 50 of 641 showed a >3-fold reduction in expression, whereas 339 miRNAs showed a <2-fold reduction or no reduction in expression (Fig. 1A and supplemental File 1). SCs are the most abundant cell type in sciatic nerve samples, and most, if not all, SCs were expected to have been recombined by the robust P0::Cre-deleted strain, making the large number of unperturbed miRNAs surprising. This result, obtained independently in both Dicer1 and Dgcr8 mutants, also diminishes the likelihood that these remnant miRNAs are a result of hypomorphic alleles. Many of the unperturbed miRNAs likely represent miRNAs that are produced in other cellular components of the nerve sample such as axons or the endoneurium or miRNAs with greater stability. Dgcr8-independent miRtrons such as miR-320 and miR-484, for example, were reduced in Dicer1 cKOs but not Dgcr8 cKOs (Fig. 1B) (28, 53).

Behaviorally, Dgcr8 cKO mice developed normally during the first postnatal week but displayed limb weakness by P14. By P21, Dgcr8 cKO mice displayed severe forelimb and hind limb paresis (supplemental Video 1). This forelimb deficit appeared to be more severe in Dgcr8 cKOs than Dicer1 cKOs (28). Gross examination of the SN at P14 revealed that whereas control nerves were opaque, Dgcr8 cKO nerves were transparent, most likely representative of defects in myelination (Fig. 1C). Semi-thin analysis revealed that Dgcr8 cKO nerves had reduced numbers of myelin sheaths at P14 (p < 0.001) (Fig. 1D). The occasional myelin sheaths that were observed in mutants could represent cells that have escaped early recombination or outliers that escaped the hypomyelination phenotype despite successful recombination.

Ultrastructural analyses of control and Dgcr8 cKO and nerves with EM confirmed an arrest in SC differentiation in mutant nerves. Although myelinating cells and Remak bundles were observed in controls at P14, few SCs in Dgcr8 cKOs had formed myelin sheaths (Fig. 1E). Most SCs were arrested at the 1:1 promyelinating stage of differentiation (p), whereas some are arrested earlier at the immature stage, with unsorted axon bundles (u). F, quantification of different SC types in control and Dgcr8 cKO SNs at P14 (n = 3). Dgcr8 cKOs have an overall different composition of SC types (χ² test, p < 0.001). Cell counts of myelinating SCs, promyelinating SCs, nonmyelinating SCs, and unsorted SCs per field are significantly different between control and mutant nerves (pairwise χ² tests, all p < 0.001).

FIGURE 1. Knocking out Dgcr8 in SCs during development results in reduced peripheral nerve myelination. A, quantification of miRNA expression in control and Dgcr8 cKO SNs at P7 using Taqman Array MicroRNA Cards (n = 3). Shown are 12 miRNAs reduced in expression by >10-fold in mutants. Black bar = controls; gray bar = mutants. Two-tailed Student’s t test, * = p < 0.05, ** = p < 0.01. B, qRT-PCR analysis of miR-320, miR-484, and miR-70 expression in Dicer1 and Dgcr8 cKO SNs at P7 (n = 3). C, gross examination of the SN. Dgcr8 cKO SNs at P14 are transparent, whereas control nerves are opaque. D, semithin analysis of control and Dgcr8 cKO SN at P14 stained with toluidine blue, cross-sections (n = 3). Mutant nerves have reduced numbers of myelin sheaths; two-tailed Student’s t test, WT = 592 ± 20, MUT = 13 ± 4, p = 0.0008. E, ultrastructural analysis of control and Dgcr8 cKO SNs at P14 with EM. Myelinating cells (m) and Remak bundles (r). Most Dgcr8 cKO SCs arrested at the 1:1 promyelinating stage of differentiation (p), whereas some are arrested earlier at the immature stage, with unsorted axon bundles (u). F, quantification of different SC types in control and Dgcr8 cKO SNs at P14 (n = 3). Dgcr8 cKOs have an overall different composition of SC types (χ² test, p < 0.001).
sciatic nerves with immunofluorescence labeling (18, 28). Like Dicer1 cKOs, Dgcr8 cKO nerves had increased numbers of cells expressing SOX2 and decreased numbers of cells expressing Egr2 and MBP. (Fig. 2, A–C) (28). Dgcr8 cKOs also appeared to have increased numbers of cells expressing JUN (not shown). In both Dicer1 cKOs and Dgcr8 cKOs, derepression of SOX2 and JUN and other negative regulators of myelination due to a deficiency in miRNAs could in part underpin their drastic hypomyelination phenotype.

Similarly to Dicer1 cKOs, immunolabeling analyses performed with Ki67 and EdU staining on control and Dgcr8 cKO nerves showed a marked increase in the number of proliferating cells in Dgcr8 cKO SNs at P14 (Fig. 2, A and B) (28). Increased proliferation in Dgcr8 cKOs is consistent with the inability of Dgcr8-deficient SCs to terminally differentiate. Several canonical miRNAs that are highly reduced in Dgcr8 cKOs (e.g. miR-138, miR-34a, miR-20b) are predicted to down-regulate cell cycle genes such as Cend1. Ceasing proliferation during devel-

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FIGURE 2. Dgcr8 cKO SCs continue to proliferate, fail to down-regulate SOX2 and up-regulate Egr2 and display an increase in proliferation. A, immunolabeling analyses of control and Dgcr8 cKO SNs at P14, longitudinal sections. Increased Ki67 and EdU staining in Dgcr8 cKO nerves indicate increased SC proliferation in mutant nerves. Decreased MBP- and Egr2-positive cells and increased SOX2-positive cells were observed in mutants. B, quantification of immunolabeling analyses in control and Dgcr8 cKOs at P14 (WT, n = 5; MUT, n = 3). There is a significantly greater number of EdU-, Ki67-, and SOX2-positive and significantly fewer number of Egr2-positive SCs in mutant nerves. Two-tailed Student’s t test, * = p < 0.05, *** = p < 0.001. C, Western blot analysis of MBP and p-ERK expression in control and Dgcr8 cKO SNs at P14 (n = 3). Levels of MBP were strongly reduced in mutant nerves, whereas p-ERK levels were similar to levels in controls. Tubulin was used as a loading control.
opment is thought to be coupled with myelin formation (28, 30, 31, 54–56), meaning that a failure to down-regulate \textbf{Ccn}d1 and other cell cycle genes may be tied to a failure of SCs to terminally differentiate. We also examined apoptosis in control and \textbf{Dgcr}8 cKOs at P14. There was no significant difference in the number of cleaved caspase 3-immunopositive cells in mutant and control nerves (WT 0.0017, MUT 0.0014, \(p = 0.4178\); not shown).

The ERK signaling transduction pathway has been implicated in the regulation of SC differentiation and proliferation (57–61). Levels of phospho-ERK were found to be increased in \textbf{Dicer}1-ablated SCs (30). To test whether ERK signaling was altered in \textbf{Dgcr}8 cKOs, we probed for phospho-ERK expression by Western blot. Although MBP levels were extremely low in \textbf{Dgcr}8 cKOs, levels of phospho-ERK did not appear to be significantly increased in \textbf{Dgcr}8 cKOs, as they are in \textbf{Dicer}1 cKOs (Fig. 2C) (30).

\textbf{Dgcr}8 cKO SCs Show Altered Gene Expression Profiles and Express Denervation-associated Genes—To further analyze gene expression in \textbf{Dgcr}8 cKOs, we performed qRT-PCR analysis for control and \textbf{Dgcr}8 cKO SNs at P14. As expected, the results were similar to those of \textbf{Dicer}1 cKO and \textbf{Egr}2 null mutants. Genes encoding many different components of myelin (including \textbf{Mpz}, \textbf{Mbp}, \textbf{Pmp}22, \textbf{Plp}1, and \textbf{Cnp}), most positive regulators of myelination (including \textbf{Egr}2, \textbf{Sox}10, \textbf{Pou}3f1, and \textbf{Pou}3f2), and neuregulin receptors (including \textbf{Erbb}2 and \textbf{Erbb}3) were down-regulated in \textbf{Dgcr}8 cKO nerves but down-regulated in \textbf{Egr}2 null nerves. C, X-gal staining of SN cross sections harvested from adult transected or uninjured \textbf{Shh}:\textbf{Cre}, \textbf{R26}G2G mice \((n = 3)\). Only SCs on the transected side are labeled with X-gal, suggesting that, at least by this assay, expression of \textbf{Shh} is not detectable in SCs during development but is only activated after transection.
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retinoic acid, which were also relatively increased. Among the putative negative regulators of myelination, levels of *Jun*, *Egr3*, *Pax3*, *Dll1*, and *Nras* (6) were not significantly higher in the mutants (Fig. 3A). In the case of *Jun*, our data suggest a possible uncoupling between mRNA and protein expression in *Dgcr8* cKOs, suggesting that miRNAs may play a key role in post-transcriptional suppression of JUN levels without affecting mRNA abundance.

We also examined the expression of genes that are expressed by SCs in nerve injury models (hereafter interchangeably called denervated SCs). qRT-PCR analysis for selected denervation-associated genes in control and *Dgcr8* cKOs at P5 revealed the up-regulation of *Shh* and *Gdnf* (denervation-associated genes), *Crabp1* (a cellular binding protein of retinoic acid), *Tnc* (an extracellular matrix glycoprotein), and *Smoc1* (a SPARC-related modular calcium ECM protein). The distinctive up-regulation of *Shh* and *Gdnf* occurs surprisingly early in *Dgcr8* cKOs; the first postnatal week is a time at which many SCs in controls have not yet even begun myelination. We next wanted to determine whether early activation of denervation-specific genes is a common feature of other models of hypomyelination. We performed qRT-PCR analysis for denervation-associated genes in control and *Egr2* null mutant SNs at P5. There was no change in *Shh* and *Gdnf* levels in *Egr2* mutants, and *Crabp1* levels were in fact lower in *Egr2* mutants than in controls (Fig. 3B). Together, these results suggest that even though *Dgcr8* mutants are similar to *Egr2* mutants in terms of hypomyelination, *Dgcr8* mutants demonstrate gene expression changes that are different from those found in *Egr2* null mice. Either through direct or indirect mechanisms, *Dgcr8* cKO SCs appear to fail to repress an injury-associated gene expression program, which another model of hypomyelination, the *Egr2* null mutant, does not.

**Shh Is Expressed de Novo in Denervated SCs**—The early expression of *Shh* in *Dgcr8* cKO nerves prompted us to ask whether this represented a failure to down-regulate *Shh* from a previous stage of development or whether it represented the expression of a *de novo* denervation-associated gene expression program. Expression of *Shh* has previously been reported in situations involving denervation but not development (69–73). However, in zebrafish, *Shh* is truly a denervation-specific gene, the expression of which is limited to the peripheral nervous system (74). To definitively confirm whether *Shh* is only a denervation-specific gene in SCs and not expressed at any point during SC development, we performed sham and transection surgery on SNs of adult *Shh* lineage tracking mice (*Shh::Cre^{+/–}, R26^{N2G+/–}*). Cells that expressed *Shh* at any point were labeled with nuclear lacZ. Multiple SCs were labeled with LacZ in transected nerves, whereas very few cells were labeled in sham nerves (Fig. 3C). These results suggest that *Shh::Cre* activity is largely undetectable in SCs during development but increases following transection, confirming that *Shh* is part of a gene expression program that is activated de novo in denervated SCs.

**Dgcr8 Is Required for Myelin Maintenance**—To test whether *Dgcr8* is required for myelin maintenance, we crossed *Dgcr8^{lox/lox}* mice to *Plp::CreER^{T2}, Dgcr8^{lox/lox}* mice to generate *Plp::CreER^{T2+/–}, Dgcr8^{lox/lox} (Dgcr8 iKO) mice. *Plp::CreER^{T2} mice display recombination in SCs after treatment with TAM, albeit in a mosaic fashion (47). *Plp::CreER^{T2}–mediated recombination is specific to SCs and is not observed in DRG neurons (47, 75). Upon exposure to TAM, CreERT was expected to be translocated into the nucleus, where exon 3 of the *Dgcr8^{lox/lox}* allele was expected to be deleted via Cre-mediated recombination in a spatially and temporally restricted fashion. We aged *Dgcr8* iKOs and their littermate controls to 4 weeks of age and then injected them daily for 10 days with either 100 μg/g of TAM or a vehicle (corn oil), the same regimen used for *Dicer1* iKOs (31). To visualize the extent of recombination after injection, we crossed *Plp::CreER^{T2+/–}, Dgcr8^{lox/lox} (Dgcr8 iKO) mice to *Ai9* mice. *Ai9* is a sensitive reporter of Cre-mediated recombination (48). Four weeks after TAM injection, widespread tdTomato expression was seen in *Plp::CreER^{T2+/–}, Dgcr8^{lox/lox} (Dgcr8 iKO) SNs, confirming successful recombination, although the percentage of recombination was difficult to determine due to the cytoplasmic expression of the tdTomato (Fig. 4A).

To assess the effectiveness of the *Dgcr8* deletion, we performed gene expression analysis using qRT-PCR on control and *Dgcr8* iKOs 4 weeks after TAM exposure. There was a significant reduction in the levels of *Dgcr8* in mutants. Levels of miR-138 and miR-338–3p were also reduced (Fig. 4B). Remnant levels of *Dgcr8* and the miRNAs likely reflect the mosaic nature of the *Dgcr8* deletion using the *Plp::CreER^{T2} strain. Gene expression was analyzed in *Dgcr8* iKO SNs 4 weeks after TAM exposure using qRT-PCR. The expression of most tested genes did not differ from their expression in controls except for the expression of genes characteristic of the immature stage *Ngfr* and *Crabp1*, which were up-regulated in *Dgcr8* iKOs, and *Dhh* and *Smo*, members of the Dhh pathway, which were down-regulated (Fig. 4C).

To evaluate gene expression in *Dgcr8* iKOs at a later time point, qRT-PCR was performed on SNs 11 weeks after TAM injection. There was significant reduction in levels of miR-138 and miR-338–3p (Fig. 4D). Gene dysregulation was pronounced at this point. Expression of compact myelin proteins (such as *Mbp* and *Mpz*), *Gja1*, and *Smo* was significantly reduced in *Dgcr8* iKOs whereas the expression of *Pmp22*, Mag and *Egr2* remained unchanged (data not shown). Many genes characteristic of immature (including *Ngfr*, *Sox2*, *Ccn1*, *Notch1*, *Tnc*, and *Crabp1*) and denervation-specific (including *Olig1*, *Shh*, and *Gdnf*) states were significantly up-regulated (Fig. 4E). Overall, the gene expression changes in *Dgcr8* iKOs at 11 weeks post ablation were more pronounced than those found at 4 weeks post ablation and were similar to those found in *Dgcr8* cKOs.

To investigate whether the observed gene expression changes could also be seen at the protein level, we performed immunolabeling analyses. We observed sparse but increased CCND1 expression in *Dgcr8* iKO SNs 11 weeks after TAM exposure (Fig. 4F). Changes in MBP, MPZ, phospho-ERK, and phospho-AKT protein levels, however, were not detectable by Western blot analysis (Fig. 4G).

To determine whether the changes in gene and protein expression in *Dgcr8* iKOs led to structural changes in the SN, we performed semithin and EM analyses on SNs 10–11 weeks post TAM exposure. Surprisingly, semithin sections revealed fasciculation defects of the nerve trunk in *Dgcr8* iKOs with the
nerve trunk in some animals containing four to six fascicles in a mid-thigh SN cross section (data not shown). Although myelination seemed largely normal as analyzed by axon size distribution and g-ratio (myelin thickness), a significant number of axons lacking myelin sheaths as well as demyelinating SCs were observed in Dgcr8 iKOs (Fig. 5A–F). Although the numbers of pathological SCs were few compared with the number of normal-appearing SCs, our results are likely to be an underestimate of the full phenotype of Dgcr8 iKOs due to the mosaic nature of tamoxifen-inducible Cre deletions. Additionally, our experiments were terminated at 11 weeks due to significant CNS related impairments (due to Plp::CreERT<sup>+/−</sup> activity in oligodendrocytes) (47, 76); it is likely that a PNS-specific inducible Dgcr8 iKO experiment, harvested after a longer period of time, would have shown a greater proportion of defective SCs.

The presence of macrophages was analyzed with EM. There was increased presence of macrophages in Dgcr8 iKOs (Fig. 5B). Immunolabeling analysis revealed increased F4/80 staining in contained regions throughout Dgcr8 iKO SNs (Fig. 4F) whereas F4/80 staining was largely absent in areas outside of these
regions (data not shown). Quantification of F4/80 positive area/unit area revealed an overall increase in F4/80 staining in Dgcr8 iKO mutant nerves ($p < 0.05$). Together these results suggest that ablation of Dgcr8 in adult SCs leads to SC degeneration and increased macrophage infiltration, confirming that Dgcr8 is required for myelin maintenance.

To examine whether loss of Dgcr8 affects the response of SCs to nerve injury, we performed sham and crush surgery on Dgcr8

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**Figure A**

Control vs Dgcr8 iKO

**Figure B**

Immunohistochemistry images showing control and Dgcr8 iKO

**Figure C**

Bar graph showing axon size distribution for control and mutant SCs

**Figure D**

Scatter plot and linear regression for G ratio against axon diameter

**Figure E**

G-ratio distribution for wild-type (wt) and mutant SCs

**Figure F**

Histogram showing percentage of axon diameter distributions for control and mutant SCs
iKO SNs 8 weeks after TAM exposure. Surprisingly, miRNA levels in Dgcr8 iKOs in crushed nerves 2 weeks after surgery were indistinguishable from levels in controls (Fig. 4D). The replenishment of miRNAs in Dgcr8 iKOs following crush surgery is most likely due to repopulation of the nerve by incompletely recombined, and therefore wild-type, SCs. Repopulation of this kind has been observed in mosaic ablations of Dicer1 in oligodendrocytes (77). Because of this significant confounding effect, we were unable to assess the role of Dgcr8 in remyelination.

Discussion

To investigate whether DGCR8 plays a role in the regulation of SC myelination and myelin maintenance, we conditionally knocked out Dgcr8 in developing and adult SCs. In both scenarios, derepression of immature and denervation (injury)-associated genes was observed, and myelin formation and myelin maintenance were respectively disrupted. Without Dgcr8, SCs cannot properly advance through the stages of differentiation or maintain their differentiated state in adulthood.

Loss of Dgcr8 Dysregulates the Balance between Positive and Negative Regulators of Myelination and Derepresses a Denervation-associated Gene Expression Program—SC differentiation is subject to the control of both negative and positive regulators of myelination (1, 6, 7, 62). The master positive regulator of myelination, Egr2, associates with coactivators and corepressors to directly or indirectly activate myelin-related genes as well as repress negative regulators of myelination (12, 15, 20, 78, 79). Egr2-deficient SCs have increased expression of negative regulators and decreased expression of positive regulators of myelination and myelin proteins and arrest at the promyelinating stage of differentiation (15, 18).

Expression of Egr2 is strongly reduced in Dgcr8 cKO SCs. Consistently with this, Dgcr8 cKO SCs display gene expression and phenotype changes similar to those found in Egr2 null and Dicer1 cKO SCs. Because overexpression of certain negative regulators of myelination, such as Notch1 and Sox2, can actively suppress the activity of Egr2 (18, 22, 80), derepression of negative regulators of myelination due to a deficiency in canonical miRNAs could in part underlie the developmental arrest observed in Dgcr8 cKOs. Several miRNAs that are reduced in Dgcr8 mutants, such as miR-138, miR146b, and miR-34a, are predicted to suppress negative regulators of myelination (28, 30, 81). An alternative explanation of the increase in expression of genes characteristic of the immature stage may be defective axon-SC communication (see below).

We found denervation-associated gene expression in Dgcr8 mutants but not in Egr2 null mutants as early as P5, a time point at which many SCs have not begun myelination in controls. The up-regulation of denervation-associated genes may occur due to autonomous mechanisms or secondarily due to the loss of axons or axonal signals. Several denervation-specific genes, such as Shh, Gdnf, and Olig1, are direct targets of JUN (82). Jun appears to be up-regulated in Dgcr8 cKOs; it is likely that the induction of denervation-specific genes in Dgcr8 mutants requires JUN at the transcriptional level. However, Jun is also up-regulated in Egr2 null mutants, suggesting that other factors must also be involved in modulating the levels of denervation-specific genes in Dgcr8 mutants.

Because Jun is not sufficient to explain the elevation in expression of denervation-associated genes, what may be the possible mechanisms by which denervated genes are induced in Dgcr8 cKOs and iKOs? First, several miRNAs that are highly reduced in expression in Dgcr8 cKOs are predicted to directly target denervation-specific genes. Shh is bioinformatically predicted to be targeted by miR-340–5p, Gdnf is predicted to be targeted by miR-9, miR-146b, miR-204, and miR-340–5p, and Olig1 is predicted to be targeted by miR-138 and miR-146b (Targetscan and MiRanda). Deficiency in these canonical miRNAs in part likely underlies the derepression of denervation-specific genes such as Shh, Gdnf, and Olig1 directly in Dgcr8 mutants. Indeed, a well recognized role of miRNAs is to function as a fail-safe mechanism to eliminate the effect of baseline levels of transcription (81, 83, 84). Second, it is also possible that DGCR8 itself directly destabilizes certain miRNAs (85), and such targets would be derepressed in Dgcr8 cKO mutants. Third, denervation-associated genes may be up-regulated indirectly due to the loss of axons or the receipt of axonal signals. In this regard, Erbb2 and ErbB3 are down-regulated in Dgcr8 cKO nerves. Although we do not see overt axonal pathology at P5, degeneration of axons and axon-SC units have been reported in Dicer1 cKO SNs at P24 through SM132 staining (44). Interestingly, Pereira et al. (44) also found using serial EM that the same axon could be bundled, engaged by a promyelinating SC, myelinating SC, or demyelinating SC in Dicer1 cKO SNs at P24 depending on the level that was imaged, indicating that the autonomous mechanisms explanation is more probable.

Comparison of Dicer1 Versus Dgcr8 Mutant Phenotypes in SC—Effects of miRNA deficiency in various systems have been studied using Dgcr8 and Dicer1 conditional knockouts (46, 86, 87). Although Dgcr8 and Dicer1 are both required for the biogenesis of most miRNAs, their conditional knockouts can, interestingly, exhibit similar or distinct phenotypes. For example, defects in Dicer1- and Dgcr8-null skin cells are indistinguishable in mice (53). Because most of the miRNAs that are

FIGURE 5. Some Dgcr8 iKOs fail to maintain myelin. A, semithin analysis of control and Dgcr8 iKO SNs 11 weeks after TAM exposure stained with toluidine blue, cross-sections (WT, n = 5; MUT, n = 4). Myelination is grossly normal in mutants. B, ultrastructural analysis of Dgcr8 iKO SNs 11 weeks after TAM exposure with EM, cross-sections (WT, n = 5; MUT, n = 4; WT not shown). Some macrophages (m) and demyelinating SCs (sc) are observed in mutants as well as axons without myelin sheaths (*); macrophages are characterized by filopodia and dark cytoplasmics containing phagocytized myelin, and demyelinating SCs are characterized by multilamellar sheaths, cytoplasms rich in ribosomes, and phagocytized myelin. There is an increased number of demyelinating SCs in Dgcr8 iKOs (Wilcoxon-Mann-Whitney test, WT, med = 0.0, interquartile range (IQR) = 0.7; MUT, med = 12.5; IQR = 58.3; p = 0.0159). C-F, control and Dgcr8 iKO SNs 10 weeks after TAM exposure (WT, n = 7; MUT, n = 3). C, percentage (%) of axons in axon diameter ranges (μm). There is no significant difference in axon diameter distribution in Dgcr8 iKO nerves. D, scatter plot of g-ratio (axon diameter/fiber diameter) versus axon diameter. G-ratio is widely used as a functional and structural index of optimal axonal myelination. There is no significant difference in the relationship between axon diameter and myelin thickness in Dgcr8 iKO nerves. E, average g-ratio. Each data point denotes one animal. There is no significant difference in average myelin thickness in Dgcr8 iKO nerves. F, percentage (%) of axons in g-ratios ranges. There is no significant difference in myelin thickness distribution in Dgcr8 iKO nerves. Similar results were obtained in control and Dgcr8 iKO SNs 11 weeks after TAM exposure (n = 3, data not shown).
abundantly expressed in skin cells are dependent on both DICER1 and DGCR8 function, the similar phenotypes of *Dicer1* and *Dgcr8*-null mutants are most likely due to deficiency in these canonical miRNAs (53). By contrast, ablation of *Dicer1* and *Dgcr8* during forebrain neurogenesis results in distinct phenotypes. In the early stages of mammalian forebrain neurogenesis, the Microprocessor complex directly binds to and destabilizes the Neurog2 mRNA. Ablating DGCR8 in this system stabilizes the expression of such proneural genes and leads to precocious differentiation that is not seen in *Dicer1* knockouts (85). Indeed, DICER1-dependent and DGCR8-independent non-canonical miRNAs, such as endogenous shRNA, siRNA, mirtrons, and H/ACA small nucleolar RNA-derived small RNA, have also been shown to underpin phenotypes that are more severe in *Dicer1* knockouts than in *Dgcr8* knockouts (88–90).

Ablating *Dgcr8* and *Dicer1* in developing SCs results in mostly similar defects, although *Dgcr8* cKOs have more severe SC sorting defects. By contrast, ablating *Dgcr8* in mature SCs results in dysregulated gene expression and evidence of SC pathology. Although gene expression was not analyzed in *Dicer1* iKOs, the presence of equivalent defective SCs was not observed in *Dicer1* iKOs even 14 weeks after TAM exposure (31), suggesting that the breakdown of myelin maintenance after ablation is specific to *Dgcr8* cKOs. Together, these studies introduce the possibility that *Dgcr8* and *Dicer1*, in addition to processing canonical miRNAs as a primary function, may have disparate interactions with other unique small RNA and mRNA targets that result in their distinct phenotypes in myelin maintenance.

**Denervated SCs Activate a de Novo Gene Expression Program**—Traditionally, differentiation during development and dedifferentiation upon injury are considered to be mirror images of the same process (6, 7); denervated SCs in injured nerves are considered to be similar to immature SCs in developing nerves. However, denervated SCs also process additional functions of axon guidance, myelin breakdown, and macrophage recruitment (6, 91). Recently, it has been demonstrated that down-regulating negative regulators of myelination such as *Jun* may be dispensable during development but necessary for the activation of denervation-associated genes (72, 92). Unclear in this data set was whether denervation-specific genes were expressed in the SC lineage during development. Our *Shh::Cre* lineage analysis indicates that denervation-associated genes such as *Shh* are not detectable during SC development and truly represent a *de novo* gene expression program activated only upon nerve injury. Our results also demonstrate that the *Shh::Cre* strain may be a useful tool for inducibly ablating genes specifically after denervation.

DICER1 and DGCR8 are both important components of the miRNA biogenesis pathway. It is logical to assume that knocking out *Dicer1* and *Dgcr8* will result in the same phenotype, and this has been demonstrated in some studies (53). However, other studies to date have demonstrated otherwise in various systems (85, 88–90). Our data, in comparison with published literature on *Dicer1* mutants (28), point to the fact that *Dgcr8* and *Dicer1*, in addition to their role in miRNA biogenesis, may also have some distinct functions. It is through such comparative models that the presence of new species of non-coding RNAs as well as other important functions of DICER1 and DGCR8 can be further elucidated. Determining the substrates of *Dgcr8* and *Dicer1* in SCs will shed light not only on SC biology and SC-axon communication but also the machinery of RNA biology and repertoire of regulatory RNA functions in modulating gene expression during cell differentiation and maintenance.

**Author Contributions**—R. A. conceived and coordinated the study. H.-P. L. wrote the paper. H.-P. L. and I. O. designed, performed, and analyzed the experiments shown in Figs. 1–4. L. W. and E. H. designed, performed, and analyzed the experiments shown in Fig. 5. All authors reviewed the results and approved the final version of the manuscript.

**Acknowledgments**—Part of this work was performed at the Center for Advanced Microscopy and the Mouse Histology and Phenotyping Laboratory of Northwestern University generously supported by National Cancer Institute, National Institutes of Health Cancer Center Support Grant P30 CA060553 (to the Robert H. Lurie Comprehensive Cancer Center). We thank Courtney Williamson, Dr. Lin Li, Mr. Lennell Reynolds Jr., and Dr. Wensheng Liu for technical assistance. We also thank statistical analyst Amy Yang from Northwestern Biostatistics Collaboration Center and Yannick Poitelon for help with our statistical analyses.

**References**

1. Jessen, K. R., and Mirsky, R. (2005) The origin and development of glial cells in peripheral nerves. *Nat. Rev. Neurosci.* 6, 671–682
2. Monk, K. R., Feltri, M. L., and Taveggia, C. (2015) New insights on schwann cell development. *Glia* 63, 1376–1393
3. Nave, K. A. (2010) Myelination and the trophic support of long axons. *Nat. Rev. Neurosci.* 11, 275–283
4. Scherer, S. S., and Wrabetz, L. (2008) Molecular mechanisms of inherited demyelinating neuropathies. *Glia* 56, 1578–1589
5. Nave, K. A., Sereda, M. W., and Ehrenreich, H. (2007) Mechanisms of disease: inherited demyelinating neuropathies, from basic to clinical research. *Nat. Clin. Pract. Neurol.* 3, 453–464
6. Jessen, K. R., and Mirsky, R. (2008) Negative regulation of myelination: relevance for development, injury, and demyelinating disease. *Glia* 56, 1552–1565
7. Mirsky, R., Woodhoo, A., Parkinson, D. B., Arthur-Farraj, P., Bhaskaran, A., and Jessen, K. R. (2008) Novel signals controlling embryonic Schwann cell development, myelination, and dedifferentiation. *J. Peripher. Nerv. Sys.* 13, 122–135
8. Bondurand, N., Girard, M., Pingault, V., Lemort, N., Dubourg, O., and Goossens, M. (2001) Human connexin 32, a gap junction protein altered in the X-linked form of Charcot-Marie-Tooth disease, is directly regulated by the transcription factor SOX10. *Hum. Mol. Genet.* 10, 2783–2795
9. Musso, M., Balestra, P., Bellone, E., Cassandrini, D., Di Maria, E., Doria, L. L., Grandis, M., Mancardi, G. L., Schenone, A., Levi, G., Ajmar, F., and Mandich, P. (2001) The D355V mutation decreases Egr2 binding to an element within the Cx32 promoter. *Neurobiol. Dis.* 8, 700–706
10. Parkinson, D. B., Dickinson, S., Bhaskaran, A., Kinsella, M. T., Brophy, P. J., Sherman, D. L., Sharghi-Namini, S., Duran Alonso, M. B., Mirsky, R., and Jessen, K. R. (2003) Regulation of the myelin gene periaxin provides evidence for Krox-20-independent myelin-related signalling in Schwann cells. *Mol. Cell. Neurosci.* 23, 13–27
11. Taveggia, C., Pizzagalli, A., Fagiani, E., Messing, A., Feltri, M. L., and Wrabetz, L. (2004) Characterization of a Schwann cell enhancer in the myelin basic protein gene. *J. Neurochem.* 91, 813–824
12. LeBlanc, S. E., Jang, S. W., Ward, R. M., Wrabetz, L., and Svaren, J. (2006) Direct regulation of myelin protein zero expression by the Egr2 transacti-
12. Developmental regulation of microRNA expression in Schwann cells. Mol. Cell. Biol. 32, 558–568
13. Yu, B., Zhou, S., Wang, Y., Ding, G., Ding, F., and Gu, X. (2011) Profile of microRNAs following rat sciatic nerve injury by deep sequencing: implication for mechanisms of nerve regeneration. Plos ONE 6, e24612
14. Li, S., Yu, B., Wang, Y., Yao, D., Zhang, Z., and Gu, X. (2011) Identification and functional annotation of novel microRNAs in the proximal sciatic nerve after sciatic nerve transection. Sci. China Life Sci. 54, 806–812
15. Yu, B., Qian, T., Wang, Y., Zhou, S., Ding, G., Ding, F., and Gu, X. (2012) miR-182 inhibits Schwann cell proliferation and migration by targeting FGFR9 and NTM, respectively, at an early stage following sciatic nerve injury. Nucleic Acids Res. 40, 10356–10365
16. Chang, L. W., Viader, A., Varghese, N., Payton, J. E., Milbrandt, J., and Nagarajan, R. (2013) An integrated approach to characterize transcription factor and microRNA regulatory networks involved in Schwann cell response to peripheral nerve injury. BMC Genomics 14, 84
17. Emde, A., and Hornstein, E. (2014) miRNAs at the interface of cellular stress and disease. EMBO J. 33, 1428–1437
18. Bartel, D. P. (2004) MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 116, 281–297
19. Bartel, D. P. (2009) MicroRNAs: target recognition and regulatory functions. Cell 136, 215–223
20. Carthew, R. W., and Sontheimer, E. J. (2009) Origins and Mechanisms of miRNAs and siRNAs. Cell 136, 642–655
21. He, L., and Hannon, G. J. (2004) MicroRNAs: small RNAs with a big role in gene regulation. Nat. Rev. Genet. 5, 522–531
22. Mendell, J. T., and Olson, E. N. (2012) MicroRNAs in stress signaling and human disease. Cell 148, 1172–1187
23. Ebert, M. S., and Sharp, P. A. (2012) Roles for microRNAs in conferring robustness to biological processes. Cell 149, 515–524
24. Pereira, J. A., Baumann, R., Normén, C., Somandin, C., Miehe, M., Jacob, C., Lühmann, T., Hall-Bozic, H., Mantei, N., Meijer, D., and Suter, U. (2010) Dicer in Schwann cells is required for myelination and axonal integrity. J. Neurosci. 30, 6763–6775
25. Feltri, M. L., D’Antonio, M., Previtali, S., Fasolini, M., Messing, A., and Milbrandt, J. (1999) P0-Cre transgenic mice for inactivation of adhesion molecules in Schwann cells. Ann. N.Y. Acad. Sci. 883, 116–123
26. Wang, Y., Medvid, R., Melton, C., Jaisiche, R., and Belloch, R. (2007) DGC8 is essential for microRNA biogenesis and silencing of embryonic stem cell renewal. Nat. Genet. 39, 380–385
27. Doerflinger, N. H., Macklin, W. B., and Popko, B. (2003) Inducible site-specific recombination in myelinating cells. Genesis 35, 63–72
28. Madisen, L., Zwingman, T. A., Sunkin, S. M., Oh, S. W., Zariwala, H. A., Gu, H., Ng, L. L., Palmiter, R. D., Hawrylycz, M. J., Jones, A. R., Lein, E. S., Gu, H., Ng, L. L., Palmiter, R. D., Hawrylycz, M. J., Jones, A. R., Lein, E. S., and Zeng, H. (2010) A robust and high-throughput Cre reporting and characterization system for the whole mouse brain. Nat. Neurosci. 13, 133–140
29. Harfe, B. D., Scherz, P. J., Nissim, S., Tian, H., McMahon, A. P., and Tabin, C. J. (2004) Evidence for an expansion-based temporal Shh gradient in specifying vertebrate digit identities. Cell 118, 517–528
30. Yamamoto, M., Shook, N. A., Kanisicak, O., Yamamoto, S., Wosczyna, N. M., Camp, J. R., and Goldhammer, D. J. (2009) A multifunctional reporter mouse line for Cre- and Flp-dependent lineage analysis. Genesis 47, 107–114
31. Voiculescu, O., Charnay, P., and Schneider-Maunoury, S. (2000) Expression pattern of a Krox-20 Cre knock-in allele in the developing hindbrain, bones, and peripheral nervous system. Genesis 26, 123–129
32. Feltri, M. L., Graus Porta, D., Previtali, S. C., Dodign, A., Migliavacca, B., Cassetti, A., Littlewood-Evans, A., Reichardt, L. F., Messi, A., Quattrini, A., Mueller, U., and Wabretz, L. (2002) Conditional disruption of β1 integrin in Schwann cells impedes interactions with axons. J. Cell. Biol. 156, 199–209
33. Yi, R., Pasolli, H. A., Landthaler, M., Hafner, M., Ojo, T., Sheridan, R., Sander, C., O’Carroll, D., Stoffel, M., Tuschl, T., and Fuchs, E. (2009) DGC8-dependent microRNA biogenesis is essential for skin development. Proc. Natl. Acad. Sci. U.S.A. 106, 498–502
34. Brown, M. J., and Asbury, A. K. (1981) Schwann cell proliferation in the postnatal mouse: timing and topography. Exp. Neurol. 74, 170–186
H. E., Provis, J. M., Madigan, M. C., Milam, A. H., Justice, N. L., Albuquerque, R. J., Blandford, A. D., Bogdanovich, S., Hirano, Y., Witta, J., Fuchs, E., Littman, D. R., Ambati, B. K., Rudin, C. M., Chong, M. M., Provost, P., Kugel, J. F., Goodrich, J. A., Dunaief, J. L., Baffi, J. Z., and Ambati, J. (2011) DICER1 deficit induces Alu RNA toxicity in age-related macular degeneration. *Nature* **471**, 325–330

91. Martini, R., Fischer, S., López-Vales, R., and David, S. (2008) Interactions between Schwann cells and macrophages in injury and inherited demyelinating disease. *Glia* **56**, 1566–1577

92. Fontana, X., Hristova, M., Da Costa, C., Patodia, S., Thei, L., Makwana, M., Spencer-Dene, B., Latouche, M., Mirsky, R., Jessen, K. R., Klein, R., Raivich, G., and Behrens, A. (2012) c-Jun in Schwann cells promotes axonal regeneration and motoneuron survival via paracrine signaling. *J. Cell Biol.* **198**, 127–141